Reiner Westermeier, Tom Naven,
Hans-Rudolf Höpker

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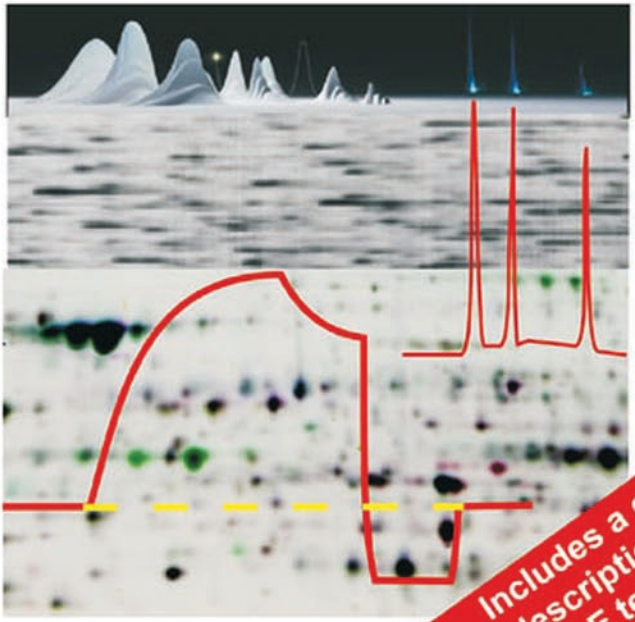
Proteomics in Practice

A Guide to Successful Experimental Design

Second, Completely Revised Edition



Includes a detailed description of the DIGE technology

Reiner Westermeier, Tom Naven, and
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Contents

Preface

More than a decade after the beginning of the "Proteomics Rush" the methodological and systematic approaches for the analysis of proteomes have evolved from being holistic and non-hypothesis driven to phenomenon-based, dedicated protein studies. Importantly and correctly so, it has been widely reported that it is very difficult to obtain all necessary information from one single workflow, e.g. 2-D gel-mass spectrometry; and that different workflows deliver complementary information rather than similar, overlapping results. Therefore it is necessary to make additions to the manuscript for the second edition of Proteomics in Practice and include a comprehensive description of chromatography methods, written mainly by Hans-Rudolf Höpker.

The objective of the second edition of Proteomics in Practice is to provide the reader with a comprehensive reference and practical guide for the successful analysis of proteins by 2-D electrophoresis, chromatography and mass spectrometry. The book includes a theoretical introduction into the most-applied methodologies, a practical section complete with worked examples, a unique troubleshooting section and a thorough reference list to guide the interested reader to further details.

The theoretical section introduces the fundamentals behind the techniques applied in proteomics and describes how the techniques are used for proteome analysis. However, the practical aspects of the book focus on 2D-DIGE technology and mass spectrometry. 2-D DIGE is increasingly cited for studying differential protein expression and, as such, a considerable section of the text is dedicated to this technique. The core components of 2D-DIGE, sample preparation and labeling, 2-D electrophoresis and image analysis are addressed in considerable detail. Further, the importance of mass spectrometry, sequence databases and search engines for successful protein identification are discussed.

The practical section of the book is, in principle, a course manual, which has been optimized over a number of years. The experimental

section describes how to achieve consistent, reliable and reproducible results using a single instrumental setup, instead of presenting a wide choice of techniques and instruments.

Fundamentally, the book celebrates the attention to detail that is necessary to perform proteome analysis routinely, with confidence.

As the technical developments in this field are proceeding quickly, the contents of the book will need to be updated every few months. The reader can have access to a web-site at http://www.wiley-vch.de/books/info/3-527-31941-7/index.html/, which will contain the updated chapters and recipes.

The authors would like to thank Professor Richard Simpson, LICR in Melbourne, for writing the foreword, and Dr. Axel Parbel, GE Healthcare in Munich, for critical reading of the manuscript and delivering valuable contributions to the LC MS sections.

Reiner Westermeier
Tom Naven
Hans-Rudolf Höpker January 2008

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Foreword

Biological macromolecules are the main actors in the makeup of life. To understand biology and medicine at a molecular level, we need to visualize the activity and interplay of large macromolecules such as proteins. To study protein molecules, the principles of their separation, quantitation, and determination of their individual characteristics had to be developed. One of the most important separation techniques used today for the characterization and analysis of proteins is electrophoresis: a separation technique involving the movement of charged species through a matrix under the influence of an applied electric field. In 1948, the Nobel Prize in Chemistry was awarded to Arne Tiselius "for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins". This acknowledgement followed his seminal work in 1937, which led to the development of an apparatus purposely designed for the separation of serum proteins – the Tiselius moving-boundary apparatus. Explosive developments in electrophoresis occurred in the 1940s and 1950s when, in addition to zone electrophoresis, two other electrophoretic techniques emerged: isolectric focusing and isotachophoresis. Concomitant with these discoveries was the development of the matrices employed for these techniques (e.g., paper, polymer gels, such as agar or starch, and in 1959, polyacrylamide gels), each yielding distinct advantages for different samples. Of these, acrylamide gel support media emerged as the most widely used in the separation of proteins, in particular SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis, independently discovered in 1975 by Joachim Klose and Patrick O'Farrell. Today, electrophoresis still remains the seminal technique in the armory of methods that biologists apply to protein separation and characterization problems.

More and more, as students and experienced researchers from different disciplines delve into intricate biological questions that require protein chemistry input, they are confronted with the pressing need to learn fundamental protein separation methods and techniques.

Often, finding suitable resources to accomplish this task may present as big a challenge as mastering the subject field itself. In 2002, Reiner Westermeier and Tom Naven accomplished this formidable task by condensing background information, electrophoretic theory, didactic protocols, complete source lists for the tested materials, practical tips, and information resources into a single volume: Proteomics in Practice – A Laboratory Manual of Proteome Analysis. Of immense value are the sections that cover sample preparation (considered the "Achilles' heel" of proteomics) and the development of purification strategies. Given the ever-broadening landscape of proteomic technique development, Reiner Westermeier and his coauthors Tom Naven and Hans-Rudolf Höpker have now completely rewritten most parts of the First Edition according to the new developments which have happened since 2002.

Proteomics in Practice – A Guide to Successful Experimental Design (Second, completely revised edition) by Reiner Westermeier, Tom Naven and Hans-Rudolf Höpker is an invaluable information resource both for the experienced protein chemist venturing into cutting-edge electrophoretic separation methodologies tailored for a mass spectrometric protein identification endpoint and for researchers from diverse biological fields who are novices to analytical protein chemistry. This volume represents an essential tool for every laboratory involved in contemporary proteomics research.

Richard Simpson
Member, Ludwig Institute for Cancer Research, Melbourne
Professor, University of Melbourne

Abbreviations, Symbols, Units

1D electrophoresis	One-dimensional electrophoresis
2D electrophoresis	Two-dimensional electrophoresis
1D-LC	One-Dimensional liquid chromatography
2D-LC	Two-Dimensional liquid chromatography
^{12}C	Monoisotopic peak in a peptide isotopic envelope
A	Ampere
AC	Affinity chromatography
A,C,G,T	Adenine, cytosine, guanine, thymine
AEBSF	Aminoethyl benzylsulfonyl fluoride
AIEX	Anion exchange
Å	Ångström
ANOVA	Analysis of variance
API	Atmospheric pressure ionization
APS	Ammonium persulfate
Asn-Xxx-Ser/Thr	N-linked glycosylation sequon, where Asn = Asparagine, Ser = Serine, and Thr = Threonine
AU	Absorbance units
16-BAC	Benzyldimethyl-*n*-hexadecylammonium chloride
BAC	Bisacryloylcystamine
Bis	N, N′-methylenebisacrylamide
BLAST	Basic local alignment search tool
BN Page	Blue native polyacrylamide gel electrophoresis
bp	Base pair
BPB	Bromophenol blue
BSA	Bovine serum albumin
C	Crosslinking factor (%)
CAF	Chemically assisted fragmentation

CAM	Co-analytical modification
CAPS	3-(cyclohexylamino)-propanesulfonic acid
CBB	Coomassie brilliant blue
CCD	Charge-coupled device
CF	Chromatofocusing
CHAPS	3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate
CIEX	Cation exchange
CE	Capillary electrophoresis
CID	Collision induced dissociation
conc	Concentrated
CM	Carboxylmethyl
CMOS	Complementary metal oxide semiconductor
CMW	Collagen molecular weight marker
const.	Constant
CSF	Cerebrospinal fluid
CTAB	Cetyltrimethylammonium bromide
CV	Column volume
Da	Dalton
DALPC	Direct analysis of protein complexes
DB	Database
DBM	Diazobenzyloxymethyl
DDRT	Differential display reverse transcription
DEA	Diethanolamine
DEAE	Diethylaminoethyl
DGGE	Denaturing gradient gel electrophoresis
2,5-DHB	2,5-dihydroxybenzoic acid
DIGE	Difference gel electrophoresis
Disc	Discontinuous
DMF	Dimethyl formamide
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dpi	dots per inch
DTE	Dithioerythreitol
DTT	Dithiothreitol
E	Field strength in V/cm
ECD	Electron capture dissociation
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
EST	Expressed sequence tag
FAB	Fast atom bombardment
FDR	False discovery rate

FT-ICR	Fourier transform – Ion cyclotron resonance
GF	Gel filtration
GLP	Good laboratory practice
GMP	Good manufacturing practice
h	Hour
H_3PO_4	Phosphoric acid
HCCA	α-cyano-4-hydroxycinnamic acid
HED	Hydroxyethyldisulfide
HeNe	Helium neon
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanane-sulfonic acid
HFBA	Heptafluorobutyric acid
HMW	High Molecular Weight
HPLC	High Performance Liquid Chromatography
HUPO	Human Proteome Organization
I	Current (A, mA)
ICAT	Isotope coded affinity tags
i.d.	Internal diameter
ID	Identification
IEF	Isoelectric focusing
IEP	Isoelectric point
IEX	Ion exchange
IgG	Immunoglobulin G
IMAC	Immobilized metal affinity chromatography
IP	Immunoprecipitation
IPAS	Intact protein analysis system
IPG	Immobilized pH gradients
IRMPD	Infra red multiphoton dissociation
ITP	Isotachophoresis
kB	Kilobase
kDa	Kilodalton
L	Liter
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LMW	Low molecular weight
LOD	Limit of detection
LWS	Laboratory workflow system

M	mass
mA	Milliampere
MALDI	Matrix assisted laser desorption ionization
MDLC	Multidimensional liquid chromatography
min	Minute
mol/L	Molecular mass per liter
m_r	Relative electrophoretic mobility
mRNA	messenger RNA
MS	Mass spectrometry
MS^n	Multistage tandem mass spectrometry where *n* is greater than 2
MS/MS	Tandem mass spectrometry
M_r	Relative molecular mass
MudPIT	Multi dimensional protein identification technology
m/z	mass/charge ratio (*x*-axis in a mass spectrum)
Nonidet	Non-ionic detergent
NEPHGE	Non equilibrium pH gradient electrophoresis
NHS	*N*-hydroxy succinimide
NPS	Non-porous silica
NR	Non-redundant
NSE	Neuron-specific enolase
NTA	Nitrilotriacetic acid
O.D.	Optical density
P	Power (W)
PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PAGIEF	Polyacrylamide gel isoelectric focusing
PBS	Phosphate-buffered saline
PC	Peak capacity
PEG	Polyethylene glycol
PEEK	Polyetherether ketone
PFPA	Pentafluoroproprionic acid
pI	Isoelectric point
p*K* value	Dissociation constant
PMF	Peptide mass fingerprint
PMSF	Phenylmethyl-sulfonyl fluoride
PPA	Piperidinopropionamide
PPF	Protein pre-fractionation
ppm	Parts per million (measure of mass accuracy)
PSA	Prostate-specific antigen
PSD	Post-source decay

PTM	Post-translational modification
PVC	Polyvinylchloride
PVDF	Polyvinylidene difluoride
QTOF	quadrupole time-of-flight
r	Molecular radius
Rf value	Relative distance of migration
R_m	Relative electrophoretic mobility
RNA	Ribonucleic acid
RP	Reversed Phase
RPC	Reversed phase chromatography
rpm	Revolutions per minute
RuBPS	Ruthenium II tris (bathophenanthroline disulfonate)
s	Second
SAX	Strong anion exchange
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SILAC	Stable isotope labeling of amino acids in cell culture
S/N	Signal/noise ratio
SOP	Standard operation procedure
SP	Sulfopropyl
T	Total acrylamide concentration (%)
t	Time (h, min, s)
TAP	Tandem affinity purification
TBP	Tributylphosphine
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl) phosphine
TEMED	N,N,N′,N′-tetramethylethylenediamine
TFA	Trifluoroacetic acid
THPP	Tris(hydroxypropyl)phosphine
TiO_2	Titanium dioxide
TNF	Tumor necrose factor
TOF	Time of flight
Tricine	N,tris(hydroxymethyl) methyl glycine
Tris	Tris(hydroxymethyl) aminoethane
U	Volt
UV	Ultraviolet

V	Volume (L)
v	Speed of migation (m/s)
v/v	Volume per volume
W	Watt
w/v	Weight per volume (mass concentration)
ZiO_2	Zirconium dioxide

Introduction

In a living cell, most activities are performed by proteins. Therefore proteins are the subject of intense research in life science. "Proteomics" is the study of quantitative changes of protein expression levels and their application to drug discovery, diagnostics and therapy. Thereby it is important to apply the correct strategy to discover induced biological changes against the background of inherent biological variations of the sample sources.

Proteomics research has many different application areas: Pharmaceutical companies search for faster identification of new drug targets in transformed cell lines or diseased tissues. Also the validation of the detected targets, *in vitro* and *in vivo* toxicology studies, and checks for side effects can be performed with this approach. Clinical researchers want to compare normal versus disease samples, diseased versus treated samples, find molecular markers in body fluids for diagnosis and prognosis, monitor diseases and their treatments, determine and characterize post-translational modifications. In clinical chemistry it would be interesting to subtype individuals to predict response to therapy. Biologists study basic cell functions and molecular organizations. Another big field is microbiology for various research areas. Proteomics is also applied for plant research for many different purposes, for instance for breeding plants of higher bacterial, heat, cold, drought, and other resistances, increasing the yield of crop and many more.

1 History

The original definition of the "Proteome" analysis means "The analysis of the entire PROTEin complement expressed by a genOME, or by a cell or tissue type" (Wasinger *et al.* 1995). Originally the technologies behind proteome analysis were two-dimensional electrophoresis and identification of proteins by subsequent MALDI mass spectrome-

Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I. Electrophoresis 16 (1995) 1090–1094.

try with peptide mass fingerprinting. Therefore the proteins spots of interest were picked from the gel and digested with trypsin. In case of failure of identification the peptide mixtures were submitted to sequencing by tandem mass spectrometry. Although the concept of Proteome analysis is older than the phrase, it only began to become widely employed, because several prerequisites came real at the same time:

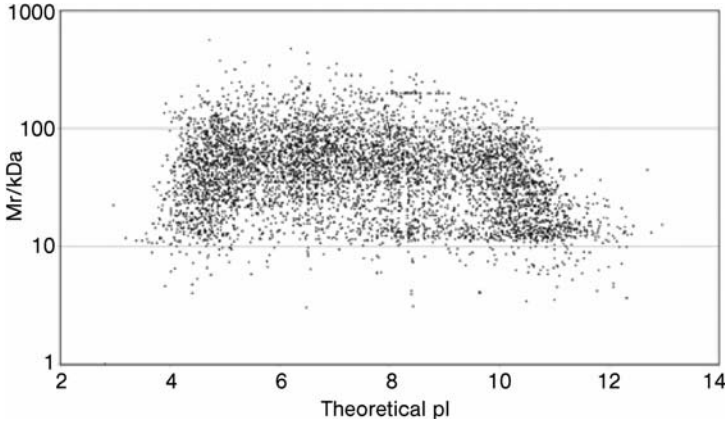
There are more definitions to find. Often they are linked to the application area.

- Availability of genomic sequence information
- Development of novel techniques of mass spectrometry.
- Availability of computing power, memory, and database accessibility.
- Improvement of separation technologies.

Furthermore it became obvious that the genomic sequence and protein function cannot be directly correlated: Co- and post-translational protein modifications cannot be predicted from the genome sequence. And it is known, they play a very important role in causing diseases. However, the DNA sequence can be "in silico" translated into the protein sequence, and therefore genome databases can be used for identification.

The theoretical 2-D maps of other organisms look in principle similar; they differ mainly in the complexity.

As an example a plot of the molecular masses versus the isoelectric points of the theoretically expressed proteins of the yeast genome is shown in Figure 1. There are many reasons, why this picture looks very different from the result of a 2-D electrophoresis of a yeast cell extract (see Figure 2):



Wildgruber R, Harder A, Obermaier C, Boguth G, Weiss W, Fey SJ, Larsen PM, Görg A. Electrophoresis 21 (2000)

Fig. 1: Theoretical two-dimensional map of masses and isoelectric points calculated from the protein sequences which have been "in silico" translated from the open reading frames of the yeast genome (from Wildgruber *et al.* 2000).

- A proteome reflects the actual metabolic state of a cell. It is a highly dynamic object and strongly dependent on many parameters.
- The plot cannot reflect the protein expression levels.
- Not all possible proteins are expressed.
- Many proteins are expressed in low copy numbers, often they are below the detection limit. Particularly proteins in the basic area, like regulatory proteins, transcription factors, and other DNA-binding proteins are mostly missed.
- A number of proteins have become modified in different ways during or after translation.
- A number of proteins are outside the working range of 2-D electrophoresis.

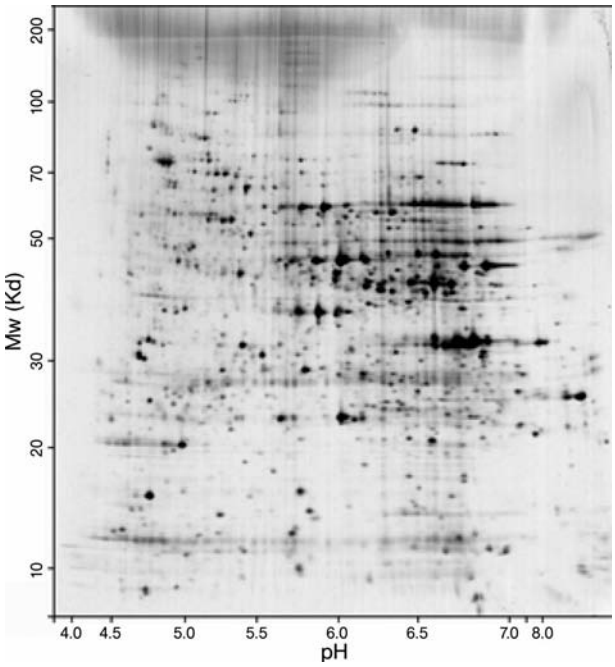


Fig. 2: Two-dimensional electrophoresis of yeast proteins as shown on the SWISS-2DPAGE database on the free accessible Expasy server (from Sanchez *et al.* 1996).

Sanchez JC, Golaz O, Frutiger S, Schaller D, Appel RD, Bairoch A, Hughes GJ, Hochstrasser DF. Electrophoresis 17 (1996) 556–565.

A large 2-D electrophoresis gel of 20 × 20 cm has a theoretical separation space of about 10,000 proteins.

A view on the working range of 2-D electrophoresis – as displayed in Figure 3 – can explain, why 2-D electrophoresis had been selected as the first choice of separation methods for the analysis of proteomes. Still the separation according to two completely independent physicochemical parameters of proteins, isoelectric point and size, offers the highest resolution. Several thousands of proteins can be separated, displayed and stored in one gel. Proteins in the size range from 10 kDa to 200 kDa and with isoelectric points between 3 and 11 can be analyzed. Because the separation takes place under completely denaturing conditions, also quite hydrophobic proteins are included in the work range. It seems like two-dimensional electrophoresis will remain the major separation technique, because its resolution and the advantage of storing the isolated proteins in the gel matrix until further analysis is unrivalled by any of the alternative techniques.

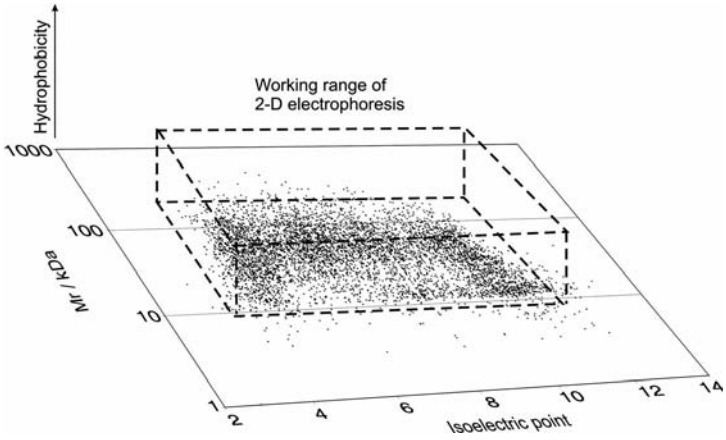


Fig. 3: Estimated working range of 2-D electrophoresis for separating highly complex protein mixtures.

However, there also some shortcomings of 2-D electrophoresis:

- Small, very large, very basic, and very hydrophobic proteins are widely excluded.
- 2-D electrophoresis is rather complex, not automated, labor-intensive, and therefore dependent on the skills of the operator.
- Even optimal separations show gel-to-gel variations. This results in difficult image analysis procedures.
- The peptide yield after in-gel digestion of proteins is considerably lower than in liquid phase. This leads to limited sensitivity in the subsequent mass spectrometry analysis.

Therefore proteomics researchers started to look for alternatives to either replace or – at least – to complement the results acquired with the 2D gel-based workflow. The most successful approach employs tryptic digestion of the entire protein mixture and analysis of the peptides with the combination of nanoscale liquid chromatography and electrospray mass spectrometry. This procedure was either called DALPC (Direct analysis of protein complexes, see Link *et al.* 1999) or MudPIT (Multidimensional protein identification technology, see Washburn *et al.* 2001). The major advantages of the LC-based workflows are the superior sensitivity and the possibility of automation by an LC-ESI MS via on-line connection. Several orthogonal separation techniques are combined to MDLC (Multi Dimensional Liquid Chromatography).

Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR III. Nature Biotech 17 (1999) 676–682.

Washburn MP, Wolters D, Yates JR III. Nature Biotech 19 (2001) 242–247.

At the present time, most multi-dimensional LC applications in proteomics deal with the separation of tryptic peptides. A variety of semi-automated off-line and fully automated on-line, as well as high-throughput configurations are available as commercial systems or can be customized according to the individual needs and preferences of the operators. Although this type of advanced tryptic peptide separation is often referred as multi-dimensional, actually it only utilizes two dimensions, namely ion exchange chromatography – cation exchange chromatography preferred – in combination with reversed phase chromatography.

Still in its infancy, multi-dimensional chromatography is enjoying more and more acceptance as a sample preparation tool for the pre-fractionation of intact proteins further upstream the proteomics workflow. The techniques and methods applied in protein pre-fractionation have been derived and adapted from protein purification, which are in use since decades with great success and reliability.

Finally, the orthogonal, high resolution separation at both protein and peptide level would deserve the term multidimensional liquid chromatography (MDLC).

Practice has shown that these different workflows develop different subsets of the same proteome with surprisingly little overlaps. A typical example can be found in the paper by Vanrobaeys *et al.* (2005). Thus none of them can be replaced by the other one. But it has been recognized that several complementary workflows need to be employed in order to keep the number of missed proteins as low as possible.

Vanrobaeys F, Van Coster R, Dhondt G, Devreese B, Van Beeumen J. J Proteome Res 4 (2005) 2283 – 2293. There are even differences within the same workflows, caused by different design of equipment.

Furthermore, another important aspect is stated in a paper by Chamrad and Meyer (2005): Today … "there are no basic rules on how to perform a proteomic study and manuscripts can frequently be found that publish results from single … experiments without any repetition, which can become problematic for further independent validation steps. Thus, search strategies and data evaluation methods

Chamrad D, Meyer HE. Nat Methods 2 (2005) 647–648.

Elias JE, Haas W, Faherty BK, Gygi SP. Nat Methods 2 (2005) 667–675.

in ... proteome studies must be improved, and the manuscript by Gygi and colleagues gives some very useful directions ...".

Other combinations than 2-D gel-MS and LC-MS have been introduced, which deliver highly satisfying results for special samples and experiments. For instance, very frequently one-dimensional SDS PAGE followed by tryptic digestion of proteins with subsequent LC-MS is employed. Also for separations on the peptide level electrophoretic alternatives have been developed to complement liquid chromatography, at least in the first stage. Furthermore, it became obvious that pre-fractionation of the highly complex protein mixtures leads to more successful protein identifications than direct analysis of crude samples. Figure 4 shows an overview of analysis modules applied in proteomics, which can be assembled to various workflows.

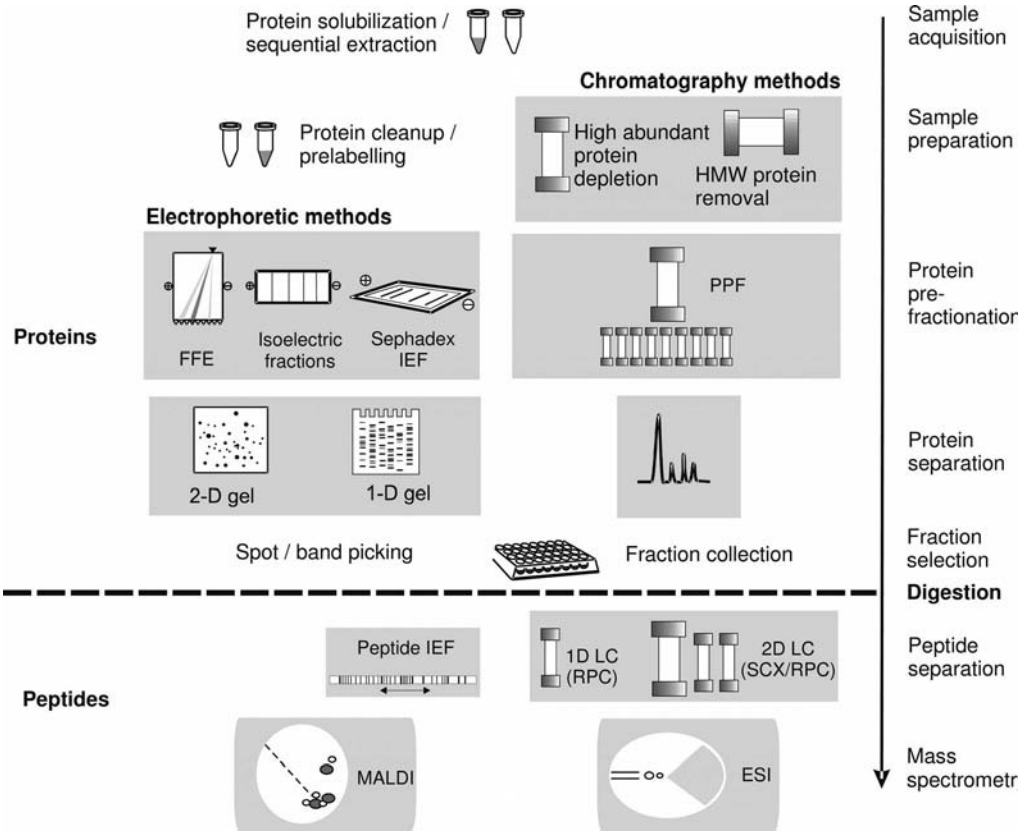


Fig. 4: Toolbox for proteome analysis. The modules can be combined to various workflows in different ways. The functions and features of the techniques displayed here will be described in more detail in the following book chapters. On the right hand side the chronological order of the analysis is indicated. Note the important division between protein and peptide level.

These technologies and their combinations will be described in the first part of the book.

Since the start of the "Proteomics Era" huge progress has been made in the instrumental development for improved nanoscale liquid chromatography, higher resolution and more sensitive mass spectrometers, evaluation software, and peripherical technologies.

A great step forward is the concept of difference gel electrophoresis (DIGE). With this method, introduced by Ünlü *et al.* (1997), protein samples are pre-labeled with modified cyanine dyes (CyeDye™), mixed, and separated together in the same gel. The co-migrated protein spots of the different samples are detected by scanning at different wavelengths; their abundance ratios are determined with dedicated software, which employs a spot co-detection algorithm. This approach makes it now possible to use an internal standard in 2-D gel electrophoresis (Alban *et al.* 2003). In this way gel-to-gel variations are compensated, which leads to highly confident quantitative and qualitative results. The technique has been applied on almost all different sample types, and during the last couple of years the number of papers on the DIGE method has increased exponentially (see Figure 5).

Ünlü M, Morgan EM, Minden JS. Electrophoresis 19 (1997) 2071–2077.

Alban A, David S, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I. Proteomics 3 (2003) 36–44.

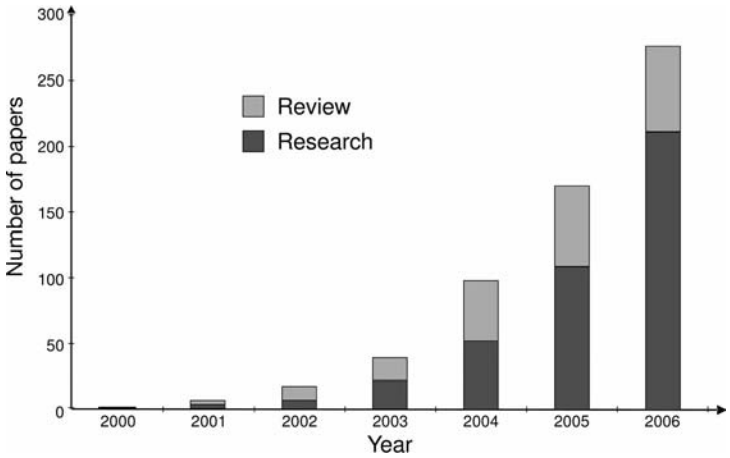


Fig. 5: Graphical representation of the number of published papers in DIGE until end of the year 2006.

At present, some major projects and developments are pursued, which raise high expectations for proteomics. Here are a few examples:

- The systematic exploration of the human proteome with Affinity (Antibody) Proteomics to generate quality assured antibodies to all non-redundant human proteins (Uhlén and Ponten, 2005).

Uhlén M, Ponten F. Mol Cell Proteomics 4 (2005) 384–393.

Misek DE, Kuick R, Wang H, Galchev V, Deng B, Zhao R, Tra J, Pisano MR, Amunugama R, Allen D, Walker AK, Strahler JR, Andrews P, Omenn GS, Hanash SM. Proteomics 5 (2005) 3343–3352.

- The combination of DIGE labeling, liquid chromatography of proteins, SDS PAGE and LC-MS for finding biomarkers in samples with very wide dynamic ranges of protein expression levels (Misek *et al.* 2005).

The generation of small subsets of intact proteins is still a challenge.

- The further developments for the top-down approach with FT-ICR mass spectrometry.
- The development of protein arrays.

Many of these critical points will be described in the following sub-chapter.

During the first few years of the proteomics era holistic approaches, mostly not hypothesis driven, were preferred in order to study complete proteomes at once by high-throughput methods. It was assumed that a proteome could be analyzed in a similar way like a genome, just with a higher effort. Unfortunately it turned out that these protein samples have more challenges in store than expected. Thus it can be observed that Proteomics is now evolving from a high-throughput industrial-scale concept ("shotgun proteomics") to carefully planned experiments and hypothesis driven analyses in order to answer certain biological questions.

2 Critical Points

2.1 Challenges of the Protein Samples

As many steps as necessary, but as little as possible!

Example: the human genome contains about 22,000 genes. With PTMs a few hundred thousand human proteins can be expected.

Usually the complexity of the protein and/or peptide mixture lies beyond the theoretical separation space of any separation method. This issue can only be solved by intelligent pre-fractionation of the sample and analyzing smaller protein subsets. But it should be noted that the more separation steps are involved, the more proteins can get lost due to technical reasons. Furthermore, the analysis of one complex sample can take quite a long time.

Five steps with 80% recovery each – which is not too bad – gives less than 40% overall recovery (see Figure 6). It becomes obvious, if not choosing a proper strategy, that there is a high risk of losing the entire sample.

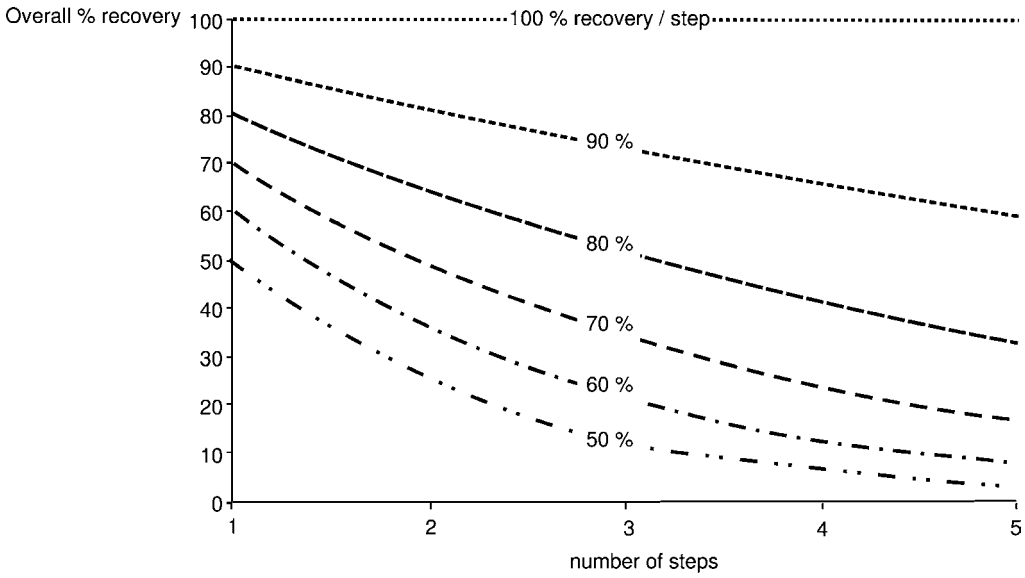


Fig. 6: Graph showing the dependence of overall recovery versus number of steps.

Note: **The way of sample acquisition and sample preparation prior to the separation needs to be optimized for each sample type. This optimization work can take up to several months.**

In tissue samples protein concentrations span a dynamic range of six orders of magnitudes.

Kislauskis EH, Zhu X, Singer RH. Cell Biol 136 (1997) 1263–1270.

Anderson NL, Anderson NG. Mol Cell Proteomics 1 (2002) 845–867.

The concentrations of proteins are not evenly distributed within a sample. It is very difficult for all separation methods and for mass spectrometry to detect and analyze very low concentrated proteins in the presence of highly abundant proteins. While regulatory proteins and transcription factors are expressed in very low copy numbers (a few proteins per cell), house keeping proteins exist in some million copies per cell: e.g. β-actin in 15 million molecules per cell (Kislauskis *et al*. 1997). The protein concentrations in human plasma even span over ten orders of magnitude (Anderson and Anderson 2002).

Enzyme inhibitors can only partly stabilize the sample.

Often the complex protein samples have limited stability. Because many proteins exhibit biological activities, some of them start to modify other proteins in the sample mixture immediately during and after taking the sample. Some enzymes, like proteases are even active under "denaturing" conditions. The measures for sample stabilization need to be optimized for different sample types. Only when the sample acquisition and sample preparation is under control, it is worth to invest time and money in a major experiment.

Statisticians would demand at least five replicates, in many cases three replicates can already deliver highly confident results. In clinical proteomics the numbers of required patients are much higher.

Hunt SMN, Thomas MR, Sebastian LT, Pedersen SK, Harcourt RL, Sloane AJ, Wilkins MR. J Proteome Res. 4 (2005) 809–819.

Moumen A, Masterson P, O'Connor MJ, Jackson SP. Cell 123 (2005) 1065–1078.

It is often necessary to apply special methods, which are suited for membrane protein analysis, like for instance blue native electrophoresis.

. . .not to mention the many different possibilities of glycosylation types.

Alternative identification strategies like, for instance, western blotting need to be further developed.

Many parameters influence the composition of a proteome. In a proteomics experiment it is critical to distinguish between induced biological changes and inherent biological variations. Those are, for instance, genetic differences, gender and age of patients, slightly different cell or plant growth conditions. Therefore it is important to always analyze several biological replicates. The required number of replicates can be different for different sample types. Some guidelines can be found in the paper by Hunt *et al.* 2005.

The expression level differences of target proteins and biomarkers associated with induced biological changes are mostly subtle, as shown for instance in the paper by Moumen *et al.* (2005). On and off effects are very rare. Therefore it is very important to be able to detect and prove the significance of the experimental effect over the background of bio-variability of the sample source and the "noise" of the analytical tool with high statistical confidence. Also this fact greatly influences the required sample size (number of replicates).

Membrane proteins are very difficult to solubilize, highly hydrophobic, and they can get easily lost during sample preparation and separation by sticking to a surface or by aggregation.

Post-translational modifications like phosphorylation and glycosylation require sophisticated analysis tools like MS^n (MS/MS/MS), where the peptide ions get several times fragmented. At present about 350 different ways of post-translational modifications are published (see Delta mass database: http://www.abrf.org/index.cfm/dm.home). It is very difficult to keep the overview and have all tools available for their identification and characterization.

The presently available detection methods are not sufficiently sensitive. It is desirable to reach down to a LOD of one protein expressed in a cell. Should the protein detection method be sensitive enough, then there is still another limitation of detection in mass spectrometry for protein identification.

When the proteome analysis starts on the peptide level, as it is nowadays still the case for the MudPIT type of workflow, any type of correlation to the parent proteins such as information on post-translational modifications and different protein isoforms can easily get lost or is difficult to trace.

Automation as much as possible or available is desirable, in order to keep the results independent of differences of human skills and to increase the sample throughput.

2.2 Challenges of the Analysis Systems

Depending on their methodological focus proteomics researchers have different points of view on the analytical steps:

- Mass spectrometry experts call every technology used prior to mass spectrometry "*Sample preparation*"; this includes also electrophoresis, chromatography, and protein arrays.
- Separation technologists sometimes call mass spectrometry "*Detection*".

This should be kept in mind, when methodical issues are discussed.

There are inherent variabilities in each analysis system, from pre-fractionation and separation to detection and mass spectrometry. The major sources of variations are: gel-to-gel variations in 2-D electrophoresis, protein losses inherent with each analysis step, retention time drifts and shifts in liquid chromatography, and ionization differences in mass spectrometry. Therefore of each biological replicate several analytical replicates are required.

Statisticians would demand at least five replicates, in many cases three replicates can already deliver highly confident results.

Most of the techniques employed in proteomics are quite demanding for hands-on skills, or they are highly sophisticated to operate. Good operator skills and knowledge are at least as important as the performance of the equipment. Education programs and tutorials have become an indispensable part of the various proteomics societies, which have been established almost everywhere around the world.

Also this book is intended as a contribution.

Because of the points described above, there are standards needed for quality control of the results. Development of standards for proteomics is on the way. There is the International Proteome Standardization Initiative (PSI), and the relevant scientific journals regularly review their instructions to the authors.

For the top-down approach of analysis of intact proteins with high resolution mass spectrometry, the resolving power of the sample preparation procedure should be as high as possible. Polyacrylamide gel electrophoresis would be the preferred method of choice. However, it is very difficult to elute intact proteins from the polyacrylamide gel matrix.

Quantitative proteomics is based on relative quantifications. Absolute quantification would be desirable, but is still not possible.

Univariate statistics like Student's *t*-test and ANOVA (Analysis of variance between groups) need to be applied to analyze whether the detected biological event is significant or not.

Even with very sophisticated equipment and highly developed analysis concepts, errors like mixing up samples or wrong annotation cannot be completely avoided.

The application of multivariate statistical tools like principal component analysis (PCA), hierarchical clustering, and *K*-means cluster-

ing on large experimental data sets help to discover errors and deliver very important information:

- They can reveal experimental errors like wrong sample annotation, for instance by detecting outliers.
- They find proteins with similar expression profiles, prognostic or diagnostic markers, and classifiers without bias of the operator.

3 Proteomics Strategies

In short the following proteomics strategy is pursued: At first target proteins are identified, characterized and correlated with biochemical pathways. This part is often called "Classical Proteomics" or "Expression Proteomics". Once some structural information is known, smaller subsets of proteins are analyzed with milder separation and measuring techniques: for instance, some proteins are fished out of a cell lysate with affinity chromatography and then proteins with intact tertiary structure, or protein–protein complexes are studied. This is called "Functional Proteomics".

3.1 Proteome Mapping

See for instance Swiss-prot (now Uni-prot) and TrEMBL.

As already mentioned above, non-hypothesis-driven proteomics experiments do not deliver meaningful results. In contrast to a genome a proteome is never static. Therefore a snapshot of a protein expression status without any biological context cannot contain valuable information. However, proteome maps are necessary for establishing databases for protein identification and characterization.

3.2 Differential Analysis

And it is very helpful to have software tools available which are dedicated to differential display analysis.

Differential analysis between wild type and mutant, diseased and healthy material, treated and control, is a powerful approach to detect significant changes in protein expression levels. In practice it is very rare that a protein is either present or completely absent. In most cases only partly up- and down-regulations of certain proteins are observed. These quantitative changes should be determined with precision and confidence.

In such a case the protein detection is carried out with a multifluorescence imager at different wavelengths.

When samples are pre-labeled with spectrally distinct fluorescent dyes, mixed, and separated together under completely identical condi-

tions (like in 2-D DIGE), possible protein losses will be qualitatively and quantitatively the same for the different samples. This makes the differential analysis highly reliable.

In order to study protein functions and pathways, cells can be stimulated for instance by heat or cold, applications of chemicals or drugs, oxygen or nutrients withdrawal. This can result in up-regulation of certain proteins, which are otherwise not expressed.

This strategy is often performed in microbial proteomics.

3.3 Time Point Experiments

In pharmaceutical drug development it is very interesting to watch changes in protein expression levels over a certain time period. With the help of statistical tools for pattern analysis proteins with similar expression profiles can be detected and grouped together.

This type of analysis needs high-throughput methods.

3.4 Verification of Targets or Biomarkers

The functions of the detected targets or biomarkers must be proven by a second entirely independent analysis method.

In most cases Western Blotting is employed for this task.

3.5 Integration of Results into Biological Context

The major goal of all proteomics research is the acquisition of knowledge on the function of the identified protein. With the help of external databases and the literature it is studied in what pathways and processes the protein is involved, also depending on the location in the cell.

3.6 Systems Biology

Eventually proteomics data will be linked to genomics, transcriptomics, and metabolomics data. The goal is to develop mathematical models to describe complex biological systems (Aebersold *et al.* 2000; Souchelnytskyi, 2005).

Aebersold R, Hood LE, Watts JD. Nature BioTech 18 (2000) 359.

Souchelnytskyi S. Proteomics 5 (2005) 4123–4137.

4
Concept of Experimental Planning

4.1
Biological Replicates

The more stringent the conditions for the biological experiment can be controled, the less biological replicates are required.

For cell cultures, animal and plant experiments usually three biological experiments tend to be sufficient to detect induced biological variations on the background of inherent biological variations. However, for analyzing patients in clinical studies, the situation is more complex: it is advised to analyze at least six patients for control, diseased, and treated samples, in special cases many more.

4.2
Pooling of Samples: Yes or No?

Pooling of samples is only performed for creating an internal standard for DIGE (see page 73 ff).

It is not advised to pool samples for analysis. Each sample should be analyzed separately. If samples have been pooled, the results cannot be traced back any longer. It would be impossible to differentiate between inherent biological variations, methodological, and induced biological variation.

4.3
Pre-fractionation of Samples: Yes or No?

No single separation or detection device is capable of deciphering an entire proteome. Only the smartest combination of techniques and methods will deliver superior results. Depending on the complexity of the initial sample, in order to obtain significant and reproducible data, it might be inevitable to consider the application of pre-fractionation.

Reducing the complexity of a proteome by pre-fractionation considerably increases the number of identifications (see page 189 ff).

In general every manipulation applied on a complex protein mixture will lead to some unspecific losses of proteins. This fact would suggest that any pre-fractionation of a protein mixture for proteome analysis should be avoided. However, the practical experience collected over the last couple of years shows that there is anyhow no workflow existing, which offers a complete coverage of an entire proteome. For instance, protein detection and identification using different workflows, the protein 2-D gel-based and the peptide chromatography-based, are complementary with only small overlaps. Because each analysis method covers only a part of a proteome, the claim to acquire a complete proteome with one procedure is anyhow obsolete. Thus, also from this aspect, pre-fractionation can be seen as a valuable tool in proteomics.

4.4 Which is the Best Workflow to Start With?

2-D gels allow high-throughput analysis on the protein level. It is advisable to optimize first the biological experimental conditions with 2-D gels, and only at a second step apply the slow and cost-intensive LC-MS based workflow.

As all major workflows, if executed properly with all their modifications and adaptations lead to valuable results, it can be good advice to begin with those techniques that are already successfully established in a laboratory. Once good results have been obtained in a reproducible and repeatable fashion complementary techniques can be trained and introduced.

Part I:
Proteomics Technology

The following chapters describe selected techniques, which in the eyes of the authors form the "mainstream" in proteomics research with respect to future developments. Recently introduced methodical modifications and improvements are also included. If you are looking for a very comprehensive collection of methods with detailed description of the particular steps, it is highly recommended to look into the two books by Richard Simpson, issued in 2003.

Simpson RJ. Proteins and proteomics. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2003).

Simpson RJ, Ed. Purifying proteins for proteomics: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2003).

As already mentioned above, it was the approach of using 2-D electrophoresis in combination with mass spectrometry which triggered the introduction "proteome analysis" and "proteomics". Therefore we begin the chapter of proteomics technology with a description of the electrophoresis methods.

1
Electrophoretic Techniques

In the presently applied proteomics workflows various electrophoretic techniques are employed: for protein pre-fractionation, for high resolution separation of proteins and peptides prior to direct mass spectrometry or LC-MS, and for verification of results.

1.1
The Principle of Electrophoresis and Some Methodological Background

The principle of electrophoresis is the migration of charged particles in an electric field. Because different particles have different sizes and net charges, they migrate with different velocities and form therefore distinct zones. The higher the net charge and the smaller the molecule, the faster is its electrophoretic migration. Electrophoresis methods can be applied for the separation of the components of a mixture, but also for creating characteristic images of a substrate for differential analysis.

In proteomics mostly protein and peptide molecules are analyzed; but also intact cell organelles and native protein complexes are separated with electrophoretic methods.

Proteins and peptides are amphoteric substances: they can become positively or negatively charged, depending on the pH value of their environment. Thus they will migrate towards the cathode or the anode respectively. Electrophoretic separations are carried out in buffers with precise pH value and a constant ionic strength. The conditions are most frequently chosen in such a way that all molecules become charged in the same direction to achieve migration into the same direction.

For anionic electrophoresis very basic and for cationic electrophoresis very acidic buffers are used.

In the electric field also the buffer ions are migrating: negatively charged ions towards the anode, positively charged ions towards the cathode. To guarantee constant pH and buffer conditions the supplies of electrode buffers must be large enough. The buffer ion migration transports the electric current: the more ions the higher the current.

Yet a minimum buffering capacity is required so that the pH value of the samples analyzed does not have any influence on the system.

The ionic strength should be as low as possible to keep Joule heat development to a minimum. And, the higher the ionic strength of the buffer, the lower will be the mobilities of the sample ions.

Isoelectric focusing will be described later in detail.

In isoelectric focusing a different buffer concept is applied: either amphoteric buffering components migrate to an endpoint in the system and become decharged, or the buffering groups are fixed to the static separation medium.

In contrast to all other methods FFE is a continuous system.

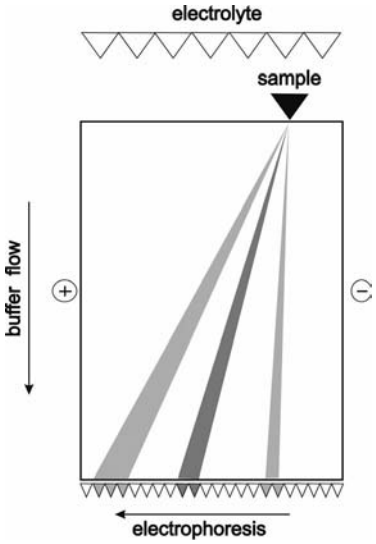
Another special case is free flow electrophoresis (FFE).

1.1.1 Free Flow Electrophoretic Methods

Hannig K. Electrophoresis. 3 (1982) 235–243.

In the free-flow approach, originally developed by Hannig (1982), a continuous stream of buffer flows in a 0.5–1.0 mm wide layer inside a cooled glass cuvette. The buffer is supplied to the cuvette over the entire width. At one end the sample is injected at a defined spot, and at the other end the fractions are collected in an array of tubes. The electrical field is applied perpendicular to the buffer flow.

The varying electrophoretic mobilities perpendicular to the flow lead to differently strong but constant deviations of the components, so that they reach the end of the separation chamber at different though stable positions (see Figure 1.1).



Wagner H, Kuhn R, Hofstetter S. In: Wagner H, Blasius E. Ed. Praxis der elektrophoretischen Trennmethoden. Springer-Verlag, Heidelberg (1989) 223–261.

Fig. 1.1: Schematic drawing of continuous free flow electrophoresis, according to Wagner *et al.* (1989).

Different separation methods can be applied: zone electrophoresis in homogeneous and discontinuous buffer systems, and isoelectric focusing (see below).

All these separations are performed in a continuous flow.

Discontinuous field electrophoresis is a specialty of free flow electrophoresis with a zone concentration effect: The sample solution is supplied in a wide zone through the central openings, the buffer solutions on the right and on the left hand side have a twenty times higher conductivity than the sample solution. The sample ions are more or less strongly deflected towards the anode or the cathode, depending on their charges. When they reach the borderline between sample and buffer stream, their electrophoretic mobility is considerably reduced, resulting in a concentration of the fraction at the borderline.

This one of the rare methods where a high buffer concentration is employed for trapping sample components in a certain zone.

Free flow electrophoresis is the method of choice for separating large particles like sub cellular components, as shown in the paper by Zischka *et al*. (2003).

Zischka H, Weber G, Weber PJA, Posch A, Braun RJ, Bühringer D, Schneider U, Nissum M, Meitinger T, Ueffing M, Eckerskorn C. Proteomics 3 (2003) 906–916.

1.1.2 Gels for Electrophoretic Techniques

Most electrophoretic separations in proteomics are performed in gel matrices. There are basically two types of gels: granulated and compact gels. Granulated gels, like dextranes, are almost exclusively used for chromatography, compact gels for electrophoretic techniques. There is just one exception: dextrane gels are well suited for preparative applications and pre-fractionation according to charge, as shown below. A detailed description for working with dextrane gels for preparative IEF can be found elsewhere (Westermeier, 2004).

Westermeier R: In Cutler P. Ed. Protein Purification Protocols. Second edition. Methods in Molecular Biology, Volume 244, Humana Press, Totowa, NJ (2004) 225–232.

The authors are not aware of any proteomics applications in agarose gels; therefore we describe only polyacrylamide gels.

1.1.3 Electroendosmosis Effects

This phenomenon occurs, when fixed charges belonging to the gel matrix or to glass surfaces are present in an electric field. Those are for instance carboxylic groups in gels and silicium oxide groups on the glass surface. These groups become ionized in basic and neutral buffers: in the electric field they will be attracted by the anode. As they are fixed in the matrix, they cannot migrate. In order to maintain the physico-chemical balance, this will be compensated by the counterflow of H_3O^+ ions towards the cathode. This effect is called electroendosmosis. In practice, this phenomenon is observed as a water flow towards the cathode, which carries proteins along with it and causes blurred zones and streaking. As described further below, it

can also cause difficulties during the transfer of proteins between IPG strips and the second-dimension gel.

1.2 Polyacrylamide Gel Electrophoresis

1.2.1 The Polyacrylamide Gel

Polyacrylamide gels are polymerized from acrylamide monomers and a cross-linking reagent – usually N,N'-methylenebisacrylamide. The reaction is started with ammonium persulfate as catalyst; TEMED provides the tertiary amino groups to release the radicals. The pore size can be exactly and reproducibly controlled by the total acrylamide concentration T and the degree of cross-linking C:

$$T = \frac{(a+b)\times 100}{V}\,[\%],\; C = \frac{b\times 100}{a+b}\,[\%].$$

a is the mass of acrylamide (g),
b the mass of methylenebisacrylamide (g),
V the volume (mL).

When C remains constant and T increases, the pore size decreases. When T remains constant and C increases, the pore size follows a parabolic function: at high and low values of C the pores are large. For T = 10% the minimum lies at C = 4%; with higher C values the gels become are brittle and relatively hydrophobic. For T = 16% the minimum lies at C = 6%; these gels are used for separation of small peptides

For protein and peptide separations T values between 4% and 16% T are used. It has been observed that higher T values can cause protein degradation. In practice the applied C values are 2.5–3.0% for zone electrophoresis and 3% for isoelectric focusing.

When the monomer concentration is very low and the pH value very high, the gel will not polymerize. For instance, it would be very difficult to polymerize a 4%T gel with a pH of 8.8.

Polyacrylamide gels must be polymerized in closed cassettes to exclude oxygen, which would interrupt the polymer chain formation. Often the monomer solutions are degassed with the help of a vacuum pump for optimal polymerization effectiveness. The polymerization efficiency is additionally influenced by the monomer concentration, the quality of the reagents, temperature, and pH value. The polymerization is exothermic. Particularly, when a highly concentrated gel solution polymerizes, a substantial amount of heat is generated. The higher the temperature, the faster and more efficient is the polymerization.

The standard catalyst system with TEMED and ammonium persulfate works only for gels containing neutral and basic buffers. Acidic gels are polymerized with alternative reagents, like ascorbic acid and ferrous sulfate together with hydrogen peroxide. Preparation of acidic gels is not trivial, because polymerization occurs very quickly and is difficult to control.

1.2.1.1 Silent Polymerization

It should be noted that after the crude gel has formed, there is a silent polymerization following, which takes several hours and completes the formation of the final matrix. Therefore the gels should not be used immediately after preparation, and they should not be placed into a refrigerator or a cold room. The gels need to be left at room temperature for a couple of hours or overnight. After this they can be used or stored in the cold. Gels containing an alkaline buffer have a limited shelf life, because at high pH values the matrix begins to hydrolyze after a few weeks.

If a gel is not perfectly polymerized it will become particularly obvious during mass spectrometry analysis of tryptic digests of cut-out protein bands or spots. Incompletely polymerized gels cause high background in the mass spectrogram.

Practical tip: ***In order to get reproducible gels, the concentrations of the catalysts have to be balanced in favor of a higher TEMED and a lower ammonium persulfate concentration. At basic pH, ammonium persulfate can react with the Tris; this effect is minimized by adding more TEMED, and by reducing the ammonium persulfate content.***

1.2.1.2 Vertical and Horizontal Gels

In a classic setup polyacrylamide gels are run in vertical chambers while they are still in the casting cassettes. The samples are applied into wells formed with a comb during polymerization (see Figure 1.2). As an alternative, the gels can be run with an open surface on a horizontal flatbed system. Here the sample wells are positioned within the stacking gel area (see Figure 1.4).

A comprehensive guide how to prepare and run horizontal polyacrylamide gels can be found in "Electrophoresis in Practice" (WILEY VCH).

Laboratory-made horizontal gels are usually run with liquid buffers, connected to the gel with paper wicks. The electrodes are then placed into the buffer tanks. The Laemmli buffers are used. Ready-made horizontal gels contain a different buffer (see below). Horizontal gels can also be run without the buffer tanks: either thick filter cardboard, soaked with a concentrated buffer, or ready-made polyacrylamide buffer strips, which contain the necessary buffer ions for one run, are placed on the edges of the gel (see also page 108). In this case electrode wires are placed on top of these electrode strips. The

Kleine B, Löffler G, Kaufmann H, Scheipers P, Schickle HP, Westermeier R, Bessler WG. Electrophoresis 13 (1992) 73–75.

buffer strip concept reduces chemical and radioactive liquid waste considerably (Kleine *et al*. 1992).

1.2.1.3 **Discontinuous Gels**

The mode of functioning of the stacking gel is in detail described in "Electrophoresis in Practice" fourth edition 2004, page 34ff.

In order to prevent aggregations of proteins or other sample molecules during entering the gel matrix, usually a stacking gel is polymerized on top of the resolving gel. The stacking gel contains a different buffer composition and has larger pore sizes than the resolving gel. Figure 1.2 shows how such discontinuous gels are prepared. The stacking gel is poured about one hour before electrophoresis.

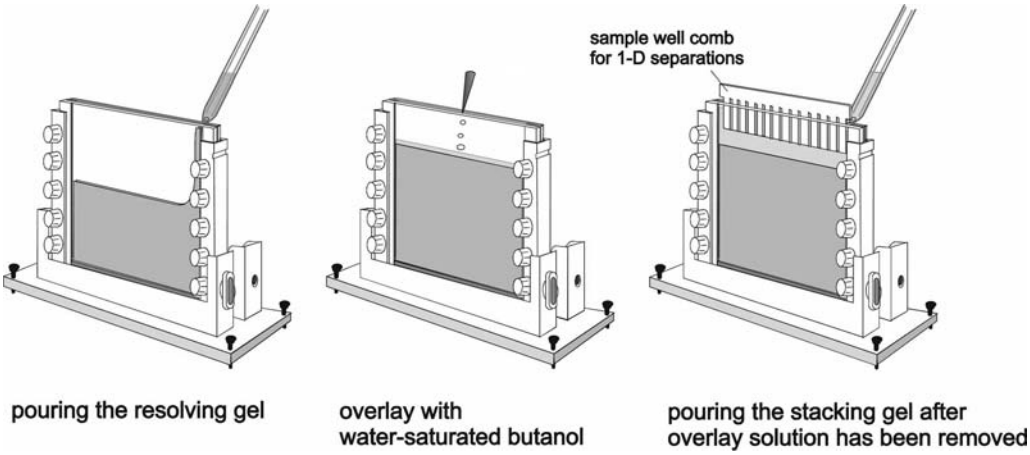


Fig. 1.2: Preparation of a discontinuous polyacrylamide gel. The resolving gel is polymerized for at least 5 hours or overnight at room temperature. The stacking gel is poured about one hour before electrophoresis.

For 2-D electrophoresis in vertical systems the stacking gel is not necessary.

Stacking gels are used for one-dimensional separation, when the applied sample molecules are in liquid phase, like in 1-D SDS electrophoresis, cationic detergent acidic electrophoresis, and Blue native electrophoresis.

1.2.1.4 **Gradient Gels**

The easiest is to use gravitation to create the liquid flow; the velocity can be controlled by placing the gradient maker on a certain level and opening the clamp only partly. In some laboratories the support of a peristaltic pump is preferred.

Porosity gradients are prepared by continuously changing the acrylamide concentration in the polymerization solution while pouring the gel, so that the concentration in the casting cassette decreases from bottom to top. The density of the highly concentrated solution is supplemented with glycerol so that the layers in the cassette will not mix. In principle a concentration gradient is poured. For casting linear gradients the law of the communicating vessels is followed: when the level in one of the tubes decreases, liquid will flow through the con-

necting channel until the level is equal. The light solution will be immediately mixed with the heavier solution in the mixing chamber. Figure 1.3 shows casting of a single gel in two different ways: on the left hand side the solution is poured from the top of the cassette. In this case the mixing chamber contains the dense solution, the reservoir the light solution. A compensation rod is placed into the reservoir for volume and density compensation. Alternatively the monomer solution can be introduced through the bottom, then the solutions are swapped, the compensation bar is not inserted (see right hand side of Figure 1.3). This option is also preferred for casting multiple gels.

Exponential gradient gels are prepared by placing a stopper into the mixing chamber, thus keeping the volume of the mixing chamber fixed. Exponential gradients are not often used.

This procedure is described in "Electrophoresis in Practice" fourth edition 2004, page 37.

In the field of proteomics gradient gels are used as immobilized pH gradients for isoelectric focusing, or as porosity gradients in Blue Native Electrophoresis, and sometimes for SDS gel electrophoresis.

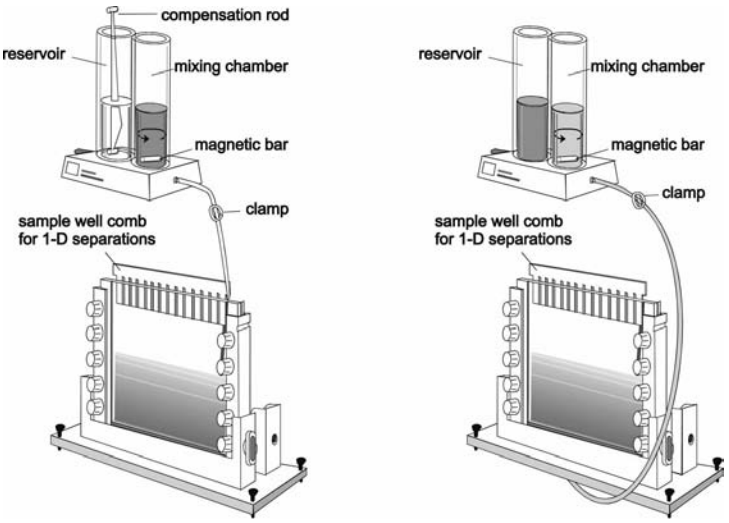


Fig. 1.3: Preparing a linear acrylamide gradient.
Left side: pouring from the top. In the beginning the mixing chamber contains only dense solution, when the liquid level decreases; light solution from the reservoir flows in and is mixed with the liquid in the mixing chamber.
Right side: pouring from the bottom. The solutions are swapped; the gel solution is introduced through a hole in the bottom of the casting stand. The magnetic bar is rotated with a magnetic stirrer (not shown).

1.2.1.5 Gel Sizes

Klose J, Kobalz U. Electrophoresis 16 (1995) 1034–1059.

Proteomics requires large gels, because highly complex protein mixtures have to be separated as efficiently as possible. Large gels also offer more space for highly abundant proteins; they have a higher dynamic range. Some expert laboratories work with gel sizes of 40×30 cm (Klose and Kobalz, 1995). But in general, the standard size for a large 2-D gel is 25×20 cm, because there are upper limits for practically handling the gels. Also, the prices for scanners increase exponentially, when a size of letter format or A4 is exceeded. For vertical systems mostly the gels are 1 mm thick. Some laboratories prefer 1.5 mm thick gels because those have a higher mechanical stability. However, staining of these thicker gels takes longer and is in some cases less sensitive. Horizontal gels are usually 0.5 mm thin.

Nevertheless many laboratories are satisfied with the resolution of 2-D separations in smaller gels.

There are two other standard gel sizes for vertical systems: 16×16 cm and 8×8 cm (minigels); these gels are much easier to handle, however for 2-D electrophoresis they suffer from limited resolution. Their major application area in proteomics is 1-D SDS electrophoresis and blotting. For 1-D separations in horizontal flatbed systems 25 cm wide and 11 cm long gels are used for running multiple samples.

1.2.1.6 Mechanically Supported Polyacrylamide Gels

Gels with improved mechanical strength and elasticity are obtained with these reagents, however on cost of resolution and pattern quality.

Although polyacrylamide gels have a much better mechanical stability than agarose gels, they can swell, shrink, and become fragile after they have been removed from the cassettes. Particularly during staining gels can easily break into many pieces. Some laboratories use therefore gel strengtheners, which are either acrylamide derivatives or supplements to acrylamide monomer solutions.

If the gel has to be stained, the reagents and dyes can diffuse into the gel only from the open side, which can lead to longer staining time and to lower sensitivity, for instance for silver staining.

As an alternative, gels can be bound to one of the cassettes glass plates, which has been pretreated with Bind-Silane. This procedure is particularly useful, when selected bands or spots have to be cut out with an automated spot picker. In this case the spot picker cuts the spots from the gel while using the information on the spot position coordinates from the image analysis results. This concept would not work, when the gel would shrink or swell between scanning and spot picking.

Gels on film supports are easier to ship, handle and store than glass plate supported gels.

Instead of binding a gel to a glass plate, it can be cast on a film support. This has become a standard for gels, which are run on a horizontal flatbed apparatus, but is also applied for vertical gels. Lately new supporting films have been developed, which do not exhibit fluorescent background anymore. Furthermore these films are treated with a novel binding chemistry, which does not increase the gel pore sizes near the film surface.

1.2.1.7 Reversible Polyacrylamide Gels

Practical experiences in mass spectrometry analysis of peptide mixtures show that better signals are obtained from samples digested in liquid phase rather than in gel plugs. Besides methylenebisacrylamide a number of other cross-linking reagents exist, listed and compared by Righetti (1983). Some of them possess a cleavage site, which allows solubilization of the gel matrix after electrophoresis (see Table 1.1).

Righetti PG.: Isoelectric focusing: theory, methodology and applications. Elsevier Biomedical Press, Amsterdam (1983).

Tab. 1.1: Alternative crosslinkers for polyacrylamide gels (examples).

Substance	Cleavage site	Cleavage agent	Comment
N,N′-methylenebisacrylamide (Bis)	None	Not possible	Standard crosslinker
N,N′-(1,2-Dihydroxyethylene)bisacrylamide (DHEBA)	1,2-diol	Periodic acid	
N,N′-Diallyl-ditartardiamine (DATD)	Ester bonds	Hydrolysis with a base	Problems with inefficient polymerization
N,N′-Bisacryloylcystamine (BAC)	Disulfide bond	Thiol reagents	DTT in the sample must be completely scavenged by iodoacetamide

Furthermore, reversible polyacrylamide gels can become very interesting as a sample preparation method for the "top-down" approach in proteomics, where intact proteins are directly analyzed with very high resolution mass spectrometry like FT-ICR and orbitraps (see page 232 f).

1.2.2 SDS Polyacrylamide Gel Electrophoresis

In general this is the mostly applied electrophoretic method for protein analysis. In proteomics SDS PAGE of proteins is employed for many different purposes:

- Second dimension in high-resolution 2-D electrophoresis;
- 1-D protein separation prior to tryptic digestion and LC-MS of peptides;
- Second dimension for "dual detergent" electrophoresis (after separation with 16-BAC or CTAB);
- Second dimension for Blue native electrophoresis;
- Third dimension in IPAS.

1.2.2.1 Theoretical Background

Ibel K, May RP, Kirschner K, Szadkowski H, Mascher E, Lundahl P. Eur J Biochem 190 (1990) 311–318.

Sodium dodecyl sulfate (SDS) is a very strong anion detergent, and solubilizes all proteins including the very hydrophobic ones. It denatures proteins by dissolving hydrogen bonds and unfolds the tertiary and secondary structures. SDS and proteins form complexes with a necklace-like structure composed of protein-decorated micelles connected by short flexible polypeptide segments (Ibel *et al.* 1990). As a result of the necklace structure large amounts of SDS are incorporated in the SDS–protein complex in a ratio of approximately 1.4 g SDS/g protein. SDS masks the charge of the proteins themselves and the formed anionic complexes have a reasonably constant net negative charge per unit mass. Usually a reducing agent such as DTT is added to the sample to cleave the disulfide bridges between cysteines. Then the polypeptides become completely unfolded, all quaternary structures are dissolved.

For complex protein mixtures mostly gels with T values of 12 to 13 are used for the optimal separation in the range between 10 kDa and 100 kDa.

With SDS PAGE (SDS–polyacrylamide gel electrophoresis) the polypeptides are separated according to their molecular weights (M_r). All proteins – also those with basic pIs – will migrate towards the anode. The electrophoretic mobility of proteins treated with SDS and DTT depends largely on the molecular weight of the protein. At a certain polyacrylamide percentage there is an approximately linear relationship between the logarithm of the molecular weight and the relative migration distance of the SDS–polypeptide complexes of a certain molecular weight range. The molecular weights of the sample proteins can be estimated with the help of co-migrated standards with known molecular weights.

Exact masses can only be determined with mass spectrometry.

Note: ***SDS PAGE cannot deliver the exact molecular mass of a protein, it allows only estimation.***

1.2.2.2 Sample Preparation

It should be noted that during electrophoresis the polypeptides become partly alkylating by acrylamide. For database searches after mass spectrometry it is very important that either none or all cysteines are alkylated.

In the standard sample preparation procedure for 1-D SDS electrophoresis the proteins are boiled for 3 minutes in the sample buffer containing 2% SDS, 50 mmol/L Tris-Cl pH 8.8, 0.01% Bromophenol blue, and 1% (w/v) DTT. For vertical gels the sample buffer must contain 25% glycerol to prevent mixing of the sample with the upper buffer. When the sample has cooled down, back folding and aggregating of subunits must be prevented: either by adding the same amount of reductant again or by alkylating the cysteines with 2.5% (w/v) iodoacetamide. Alkylation has several advantages: higher stability of the samples, prevention of artifactual lines across the gel and for down-

stream analysis with mass spectrometry ensuring alkylation of all cysteines.

For some clinical applications, or for the detection of antibodies by blotting, sometimes non-reduced samples are applied, in order to maintain the quaternary structure of the immunoglobulins. In this case the molecular weights cannot be determined, because the disulfide bridges are still intact, many of the polypeptides are still folded. Non-reduced samples must not be boiled; this would cause fragmentation of some polypeptides.

Some people call this "native" SDS electrophoresis.

1.2.2.3 Buffers and Gels

Tris-chloride / Tris-glycine The standard buffer system for SDS PAGE is based on the discontinuous Tris-chloride / Tris-glycine system described by Laemmli (1970). With this buffer system, reproducible and well resolved patterns are obtained even with high protein loads. It should be noted that different laboratories prepare the buffers in slightly different ways, this can cause differences of the running conditions. When the gel buffer is titrated with a pH meter, the measuring electrode should be well calibrated. Some laboratories measure the added hydrochloric acid by the volume.

Lämmli UK. Nature 227 (1970) 680–685.

The running buffer and the gels contain 0.1% SDS. Sometimes the gels are cast without SDS, because the SDS migrating into the gel from the cathodal buffer is sufficient. During electrophoresis the negatively charged chloride, SDS and glycine ions migrate towards the anode, the positively charged Tris ions migrate towards the cathode. The buffer reservoirs of the electrophoresis chamber must be large enough to prevent depletion of the buffer ions.

Note: ***Use only Tris base for making the buffers, not "Tris buffer". The gel buffers are made with Tris and HCl, they contain only chloride. The cathodal running buffer must not contain any chloride; only SDS, Tris and glycine. Do not titrate the upper running buffer to adjust the pH!***

Adding HCl to the upper running buffer eliminates the stacking effect and causes extended running time.

Because of the very alkaline pH 8.8 in the gel of the Laemmli system, the polyacrylamide matrix becomes hydrolyzed during storage. After two months the sieving property of the matrix is almost completely gone. In the standard laboratory practice this is not a big issue, because the gels are consumed within one week after preparation. The situation is different, when ready-made gels are used.

The problem is not only the limitation in shelf life, but also the lack of reproducibility because of the ongoing hydrolysis of the matrix.

Long shelf life SDS gels For long shelf life, the pH value of the gel buffer has to be reduced to or below pH 7. This requires a modification of the buffer composition.

Because of the tricine in the cathodal buffer, these gels show a very good separation of small peptides, when a gradient gel or a 15% T gel is used.

Tris-acetate / Tris-tricine Tris-acetate buffer with a pH of 6.7 has proven to have a good storage stability and separation capacity for flatbed gels. Glycine has to be replaced by tricine as the terminating ion. Since tricine is more expensive, it is only used in the cathodal buffer. The anodal buffer contains Tris-acetate. The running buffer can be applied as polyacrylamide electrode strips, instead of connecting the gels to liquid buffers in tanks, see also page 108. The molecular weight distribution of proteins obtained with this buffer system is slightly different from that achieved with the Laemmli system. This buffer system is also applied by rehydratization of pre-polymerized, washed film-supported gels for SDS in horizontal flatbed chambers (CleanGel). Figure 1.4 shows a SDS electrophoresis result in a 10% *T* gel.

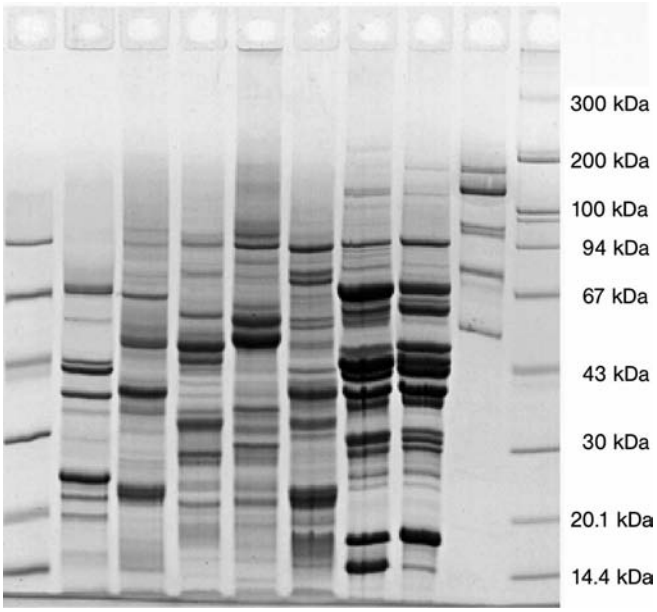


Fig. 1.4: SDS electrophoresis in a CleanGel 10% *T*. Samples: plant seed proteins and *M*, standards. Hot Coomassie brilliant blue staining.

Wiltfang J, Arold N, Neuhoff V. Electrophoresis 12 (1991) 352–366.

Bis-Tris / Bis-Tris-tricine This is similar to the above system. Tris is replaced by Bis-Tris to obtain improved buffer capacity at neutral pH (Wiltfang *et al.* 1991). Also with this buffer system the spot patterns are different from Laemmli buffer patterns.

PPA-chloride / Tris-glycine Here the Tris in the gel is replaced by PPA (piperidino propionamide) and titrated with hydrochloric acid to pH 7. This gel buffer can be combined with the standard running buffer Tris-glycine in the cathodal buffer. For the anodal buffer PPA would be required instead of Tris. Because this compound is rather expensive, the large volume anodal buffer usually contains a cheaper compound: diethanolamine-acetate (DEA).

The M_r distribution is very similar to Laemmli gels.

Tris-borate for improved separation of glycoproteins Glycoproteins migrate slower than pure polypeptides, since the sugar moiety does not bind SDS. When a Tris-borate-EDTA buffer is used, borate sticks to the sugars, the sugar moieties become also negatively charged: therefore the speed of migration will increase (Poduslo, 1981). The glycine in the running buffer is simply replaced by boric acid. The use of gradient gels is also beneficial for better MW estimations. See also cationic detergent electrophoresis on page 34 f.

Poduslo JF. Anal Biochem 114 (1981) 131–139.

Homogeneous gels Usually a homogeneous gel with 12.5% *T* and 3% *C* is used; the proteins of major interest in the size range from 10 kDa to 100 kDa are optimally resolved. Only in special cases the matrix concentration is modified to increase the resolution in certain molecular size ranges.

The rule of thumb is:

- Lower *T* value: better resolution for high molecular weight proteins.
- Higher *T* value: better resolution for low molecular weight proteins.

Gradient gels Gradient gels offer a broader separation interval than homogeneous gels, also the linear relation interval between the logarithm of M_r and the migration distance is wider than for homogeneous gels. And they exhibit a zone sharpening effect.

Peptide gels The resolution of peptides below 10 kDa is not sufficient in conventional Tris-chloride / Tris-glycine systems. The peptides smaller than 10 kDa co-migrate with the SDS front. A number of modifications of the standard gel and buffer system have been proposed, for instance the addition of 8 mol/L urea to the gel by Hashimoto *et al.* (1983), and, additionally, increasing the Tris concentration in the gel to 1 mol/L by Anderson *et al.* (1983). The most efficient technique has been developed by Schägger and von Jagow (1987): The resolving gel has a composition of 16% *T* and 6% *C*; the gel buffer concentration is increased to 1 mol/L Tris-chloride; the pH is lowered to 8.4; and tricine is used as terminating ion instead of glycine. In this way the destacking of peptides and SDS is much more effi-

Hashimoto F, Horigome T, Kanbayashi M, Yoshida K, Sugano H. Anal Biochem 129 (1983) 192–199.

Anderson BL, Berry RW, Telser A. Anal Biochem 132 (1983) 365–275.

Schägger H, von Jagow G. Anal Biochem 166 (1987) 368–379.

cient than in the standard Tris-glycine system. This method yields linear resolution from 100 kDa to 1 kDa.

The Schägger system requires long running time because of the high buffer concentration in the gel, which would otherwise become overheated with high electric power. By adding 30% v/v ethyleneglycol to the monomer solution, the buffer concentration can be reduced to 0.7 mol/L (Westermeier, 2004). The resolution is still very good and cracking of glass plates and smiling effects are avoided.

Westermeier R. Electrophoresis in Practice. WILEY-VCH, Weinheim (2004) 242–245.

1.2.3 Blue Native Electrophoresis

Schägger H, von Jagow G. Anal Biochem 199 (1991) 223–231.

Blue Native polyacrylamide electrophoresis has been developed by Schägger and von Jagow (1991) for the separation of membrane proteins and hydrophobic protein complexes in enzymatically active form. Membrane proteins and complexes are extracted with the help of a mild non-ionic detergent like Triton X-100, dodecyl-β-D-maltoside, or digitonin. The gel buffer is composed of ε-amino caproic acid and Bis-Tris and titrated to the physiological pH 7.4. The anode buffer is Bis-Tris titrated to pH 7.0 with HCl, the cathodal buffer tricine, Bis-Tris and Coomassie blue dye. The anionic dye Coomassie brilliant blue G-250 is added to the sample and the cathodal buffer. The dye binds to the hydrophobic proteins and complexes without disturbing protein–protein interactions and provides negative charges to them independently of their original net charge. Thus under the mild condition of pH 7.4 all sample components migrate into the anodal direction, and they are visible during the separation. In fact the dye–protein complexes are soluble in the detergent-free buffer medium. Because all protein and complex surfaces are negatively charged, they repel each other, and they will not aggregate. In the middle of the run, the cathodal buffer is exchanged with a non-dye-containing buffer, in order to achieve a clear background. The gels do not need to be stained, because the Coomassie dye is still bound to hydrophobic proteins and the complexes. Valuable hints for performing Blue native PAGE including a trouble shooting guide can be found in a recently published review by Wittig *et al.* (2006).

Wittig I, Braun H-P, Schägger H. Nat Protocols 1 (2006) 419–428.

Blue Native electrophoresis is often run in minigels or medium gel format. In the proteomics environment large gel systems are preferred with 25×20 cm gels in the second dimension.

Porosity gradient gels from 4.5% *T* to 16% *T* are used, to allow large super-complexes to enter and to prevent smaller molecules and complexes to leave the gel on the end. Very sharp zones can be observed, because the complexes migrate until the gel network gets too tight for further migration. The working range is between 10 kDa and 10,000 kDa. A stacking gel with the same buffer, but with a slightly lower concentration than the end of the gradient of 4% *T* is used for optimum sample entry.

In order to determine the complex partners the lanes are cut out, these polyacrylamide strips are equilibrated in SDS buffer and placed on top of a SDS gel. Thereby the complexes become dissociated into the single proteins, which are separated in a second dimension. Because in SDS electrophoresis the Coomassie dye will separate from the proteins and migrate in the front, the gels need to be stained afterwards, which is mostly performed with Coomassie blue (Granvogl *et al.* 2006).

Granvogl B, Reisinger V, Eichacker LA. Proteomics 6 (2006) 3681–3695.

Figure 1.5 shows an example of two-dimensional electrophoresis employing Blue Native electrophoresis in the first dimension (from Eubel *et al.* 2003) and SDS electrophoresis in the second.

Eubel H, Jänsch L Braun H. Plant Physiol 133 (2003) 274–286.

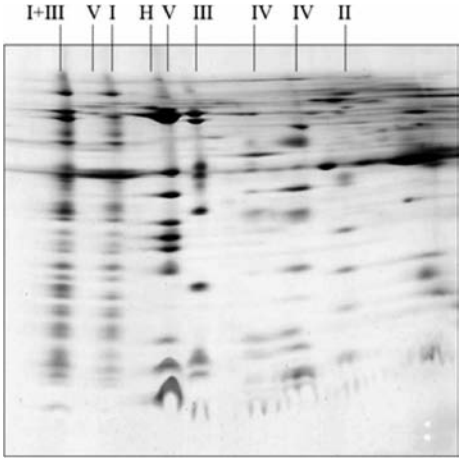


Fig. 1.5: Blue Native PAGE / SDS PAGE electrophoresis of mitochondrial complexes of *Arabidopsis thaliana* (from Eubel *et al.* 2003). The complexes were solubilized with digitonin and they are annotated with Roman numerals. I+III is a super complex composed of complexes I and III. Coomassie brilliant blue post-staining of the SDS gel.

The technique is applied in two major areas of proteomics:

- Analysis of very hydrophobic proteins, like membrane proteins.
- Functional proteomics: analysis of protein complexes and super-complexes.

A comprehensive review on applications of Blue native PAGE can be found in the paper by Krause (2006).

Krause F. Electrophoresis 27 (2006) 2759–2781.

For differential analysis of complexes it is very useful to apply the DIGE concept. The complexes can easily be labeled with the CyDyes after solubilization, prior to adding the Coomassie dye (Perales *et al.* 2005).

Perales M, Eubel H, Heinemeyer J, Colaneri A, Zabaleta E, Braun H-P. J Mol Biol 350 (2005) 263–277.

Sunderhaus S, Dudkina N, Jänsch L, Klodmann J, Heinemeyer J, Perales M, Zabaleta E, Boekema E, Braun H-P. J Biol Chem 281 (2006) 6482–6488.

When digitonin is used for extraction, even the big super-complexes are kept together and intact during the separation. After cutting out the lanes and equilibration in a less mild detergent, namely dodecyl maltoside containing buffer, the single complexes can be separated in a second-dimension Blue Native gel. In the resulting pattern the composition on the super-complexes is displayed. With activity staining, a zymogram technique, its function can be assigned to the particular spot. All this is nicely demonstrated in a paper by Sunderhaus *et al.* (2006). In this paper these complexes are also directly visualized by single particle electron microscopy.

This procedure can also be categorized into pre-fractionation with Blue native electrophoresis followed by 2-D electrophoresis.

Werhahn W, Braun H-P. Electrophoresis 23 (2002) 640–646.

Three-dimensional electrophoresis The analysis of smaller subsets of protein mixtures frequently provides improved pattern and allows to detect proteins which otherwise do not enter the gel. A possible procedure is the three-dimensional electrophoresis procedure introduced by Werhan and Braun (2002). The first dimension is the separation of protein complexes by blue native polyacrylamide electrophoresis. The visible bands are eluted electrophoretically from the gel, destained, and further analyzed by standard denaturing 2-D electrophoresis: IEF/SDS PAGE. Interestingly, this method reveals also proteins with high hydrophobicity; most probably due to the considerable reduction of complexity of the protein mixture.

Stegemann J, Ventzki R, Schrodel A, de Marco A. Proteomics 5 (2005) 2002–2009.

Stegemann *et al.* (2005) have introduced a three-dimensional gel block for performing blue native electrophoresis and SDS electrophoresis in one go.

1.2.4 Cationic Detergent Electrophoresis

Another possibility for getting poorly soluble proteins separated and analyzed is the combination of two electrophoretic separations in presence of two different ionic detergents. Usually the extraction and separation with a cationic detergent is selected as the first dimension, because cationic detergents are less denaturing than SDS. The protein–detergent micelles migrate towards the cathode. The separation pattern is different from that obtained with SDS electrophoresis. Proteins are either solubilized with cetyltrimethylammonium bromide (CTAB) or benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) and applied on an acidic polyacrylamide gel containing the respective detergent. A sodium or potassium phosphate buffer pH 2.1 with a stacking gel pH 4.1 is used. It has been observed that membrane glycoproteins are separated much better in acidic CTAB electrophoresis than in basic SDS electrophoresis (Buxbaum, 2003).

Buxbaum E. Cationic electrophoresis and electro transfer of membrane glycoproteins. Anal Biochem 314 (2003) 70–76.

For 2-D electrophoresis the first dimension is either run in individual gel rods in thin glass tubes or carried out in slab gels, which are cut into strips for the second dimension (see Figure 1.6). After the first dimension the strip is equilibrated in SDS sample buffer and applied on the second-dimension gel (MacFarlane, 1989).

Large gel formats are preferred for this technique, because the spots are crowded along the diagonal axis and require as high resolution as possible.

MacFarlane DE. Anal Biochem 132 (1983) 231–235.

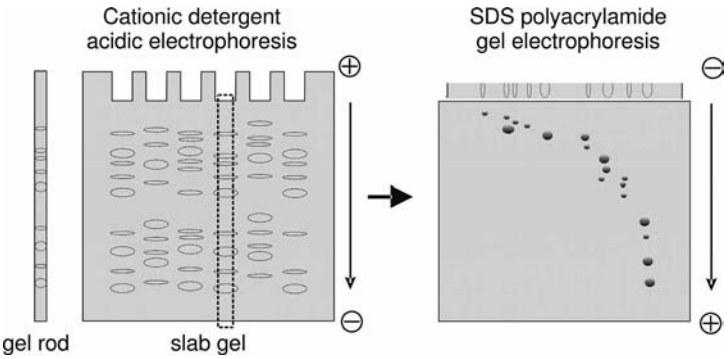


Fig. 1.6: Schematic representation of 2-D electrophoresis with cation detergent / SDS PAGE. Here the first dimension is performed in a vertical slab gel.

Hartinger *et al.* (1996) were the first to use the technique to successfully separate integral membrane proteins. Usually the patterns achieved with this technique are not easy to compare. However, when the samples are prelabeled with different CyDyes, mixed and applied together on the gel, the same proteins of the different samples will co-migrate, and the patterns can be analyzed with a spot co-detection algorithm. It was shown by Helling *et al.* (2006) that very scarce samples can be analyzed in this way by pre-labeling the cysteines of the proteins with saturation dyes. In this approach the first dimension was performed with CTAB in narrow gel rods instead of slab gels.

Hartinger J, Stenius K, Hogemann D, Jahn R. Anal Biochem 240 (1996) 126–133.

Helling S, Schmitt E, Joppich C, Schulenborg T, Müllner S, Felske-Müller S, Wiebringhaus T, Becker G, Linsenmann G, Sitek B, Lutter P, Meyer HE, Marcus K. Proteomics 6 (2006) 4506–4513.

1.3 Blotting

Towbin H, Staehlin T, Gordon J. Proc Natl Acad Sci USA. 76 (1979) 4350–4354.

Blotting is the transfer of macromolecules on immobilizing membranes for specific and sensitive detection. The electrophoretic transfer of electrophoretically separated proteins onto a membrane with subsequent immuno detection was introduced by Towbin *et al.* (1979). This method is frequently called "Western blotting". For protein blotting either nitrocellulose or PVDF is used as membrane material. PVDF membranes have a higher binding capacity for proteins than nitrocellulose, but nitrocellulose binds small proteins better.

The term Western Blotting is derived from the name of Edwin Southern, who introduced the first blotting technique: the transfer of DNA onto nitrocellulose by capillary forces for subsequent hybridization (Southern EM. J Mol Biol. 98 (1975) 503–517).

For blotting small or medium sized gels are preferred, in this way the volume of expensive antibody solutions is kept to a minimum.

Usually SDS polyacrylamide electrophoresis is employed for the separation, because all proteins have been solubilized and migrate in the same direction, and the epitopes are easier accessible due to the denaturing effect of SDS. Proteins are too big to elute quantitatively from a SDS polyacrylamide gel by diffusion or capillary forces, therefore they are transferred electrophoretically.

1.3.1 Electrophoretic Transfer

As shown in Figure 1.7 there are several ways to perform the electrophoretic transfer: tank blotting, semi-dry blotting and semi-wet blotting. All three have in common that the gel and the membrane form a sandwich with a stack of filter papers on both sides.

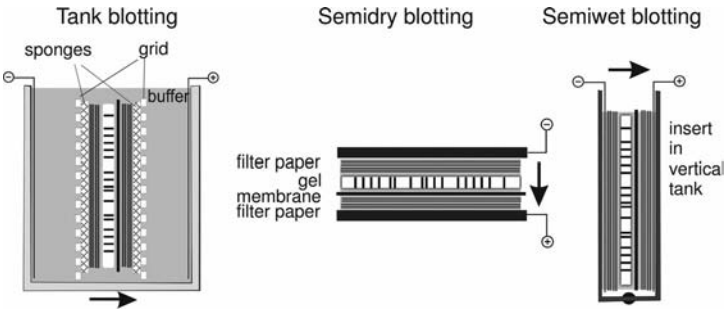


Fig. 1.7: Blotting transfer methods.

Towbin H, Özbey Ö, Zingel O. Electrophoresis. 22 (2001) 1887–1893.

If isoelectric focusing is used for the separation of the proteins, it is more efficient to transfer the proteins by diffusion with pressure blotting (Towbin *et al*. 2001).

1.3.2 Protein Detection on the Membrane

More detection techniques are described in "Electrophoresis in Practice".

After the transfer, which can take about an hour (semidry blotting) to overnight (tank blotting), the free binding sites of the membrane is blocked with a protein mixture, which will not interfere with the subsequent probing with an antibody. After some washes this "primary antibody" is detected with a secondary antibody, which recognizes this particular antibody. This secondary antibody is conjugated with a set of specific molecules, which can be easily detected with a subsequent development procedure with high sensitivity. The most sensitive detection methods are using enhanced chemiluminescence (ECL): the antibody–horseradish conjugate recognizes the primary antibody; the substrate reaction is coupled with a secondary reaction

which causes chemiluminescent light emission for a certain time period. This light signal is accumulated by exposing the membrane on an X-ray film, or by placing it into an absolutely dark cabinet where the signal is recorded with a sensitive CCD camera. With a special variant of ECL down to 1 pg of a protein band is detectable. Figure 1.8 shows an example for detection of a single protein with ECL versus a Coomassie blue-stained gel showing all proteins.

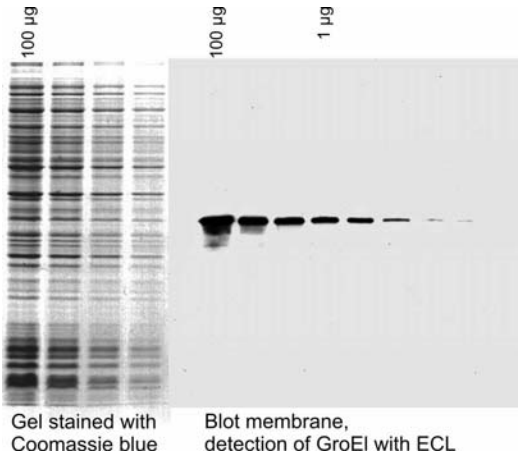


Fig. 1.8: Comparison of a gel with total proteins Coomassie blue-stained versus a blot membrane with one protein detected with ECL. Note the high sensitivity of blotting detection.

The proteins on a blotting membrane can be reprobed for several times after stripping the antibodies off and washing the membrane. For this case it is recommended to use PVDF membranes or supported nitrocellulose.

Multiplex detection on blots As an alternative to enzyme–substrate detection methods fluorescent dyes, like CyDye, can be conjugated to the secondary antibody. In this case the fluorescent signal is directly recorded with a fluorescent scanner. This procedure offers almost the same sensitivity like enhanced chemiluminescence, and with different fluorescence-conjugated secondary antibodies several different antigens can be probed in one procedure without the need for stripping antibodies off from the membrane. Besides multiplex detection this allows also a relative quantification of changes of expression levels of an antigen, when the second primary antibody is directed against a protein with stable expression level.

For this method low fluorescent membranes must be used.

Blotting from 2-D electrophoresis gels There are many papers published where immunoblotting was successfully performed after 2-D

Blotting from 2-D electrophoresis gels had been the standard for protein identification, until mass spectrometry became so sensitive that it detected too many constituents of the blotting membranes.

electrophoresis. However, it is frequently observed that an antibody does not recognize a protein anymore, when the sample had been separated with 2-D electrophoresis; but the same protein can easily be detected on a blot after 1-D SDS electrophoresis. It is still not known, what causes this effect. There is a hypothesis that focusing a protein at its isoelectric point and/or the presence of high molar urea in the first dimension causes a different and stronger denaturation for some proteins than SDS treatment alone.

Non-immunological detection procedures There are a number of general protein staining techniques for blot membranes, which are reversible or will not interfere with the subsequent specific detection methods. The most practical are the fluorescent dyes, which are introduced below in the context of staining of 2-D gels on page 119 ff.

Burgess R, Arthur TM, Pietz BC. Methods Enzymol. 328 (2000) 141–157.

Specific proteins can be detected with other ligands than antibodies. For instance, glycoproteins are detected with high specificity using lectins coupled to very sensitive avidin–biotin detection procedures. This method is the most reliable specific detection of glycosylated proteins, as the glyco-specific staining methods often produce some wrong positive signals. For the detection of protein–protein ligands there is a method called "Far Western Blotting": The probing protein is tagged with a labeled antibody for detection (Burgess *et al.* 2000).

The application of blotting for N-terminal sequencing or mass spectrometry analysis has almost been completely abandoned.

In proteomics blotting is mostly employed for the verification of a biomarker or drug target. When the new approach of antibody proteomics has succeeded with delivering antibodies against each human protein, blotting will gain increasing importance for the identification of very low abundant proteins.

1.4 Isoelectric Focusing

In proteomics isoelectric focusing is mainly applied for the following purposes:

- First dimension in high-resolution 2-D electrophoresis of complex protein mixtures.
- Pre-fractionation of complex protein mixtures according to charge.
- For the separation of very heterogeneous mixtures of tryptic peptides instead of strong cation exchange chromatography in MDLC-MS.

1.4.1 Theoretical Background

Isoelectric focusing is performed in a pH gradient. Proteins are amphoteric molecules with acidic and basic buffering groups. Those become protonated or deprotonated depending on the pH environment. In basic environment the acidic groups become negatively charged, in acidic environment the basic groups become positively charged. The net charge of a protein is the sum of all negative or positive charges of the amino acid side chains. When net charges of proteins are plotted over the pH scale, each protein has an individual net charge curve. The intersection of the net charge curve with the *x* axis is the isoelectric point – the pH value where the net charge is zero. This is shown for two model proteins in Figure 1.9.

When a protein is applied on a certain pH value of the gradient, and an electric field is applied, it will start to migrate towards the electrode of the opposite sign of its charge. Because it migrates inside a gradient, it will arrive at a pH value of its isoelectric point (see Figure 1.9) after some time. At its pI it has no net charge anymore and stops migrating. Should it diffuse away above or below its pI it will become charged again and migrate back to its pI. This is called the "focusing effect", which results in very high resolution.

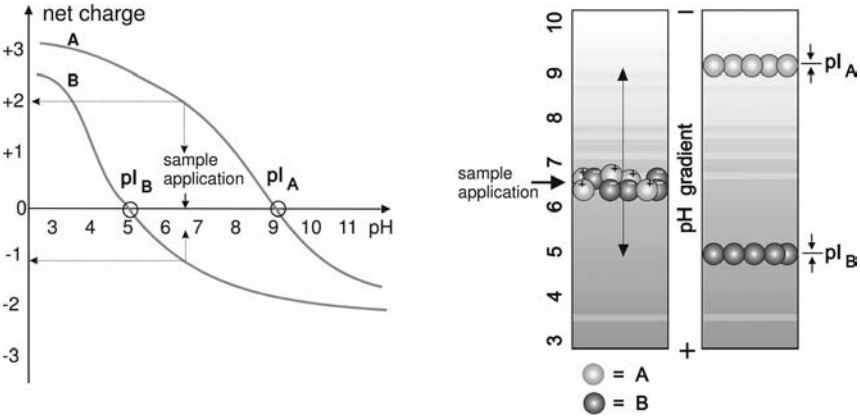


Fig. 1.9: The principle of isoelectric focusing.
Left: Net charge curves of two model proteins A and B. At the point of application, A will have two positive, B will have one negative charge(s).
Right: Migration of A and B to their pIs in the pH gradient of an isoelectric focusing gel.

In principle: ***Isoelectric focusing is a very high resolution separation method, and the pI of a protein can be measured.***

1.4.1.1 Titration Curve Analysis

Rosengren A, Bjellqvist B, Gasparic V. In: Radola BJ, Graesslin D. Ed. Electrofocusing and isotachophoresis. W. de Gruyter, Berlin (1977) 165–171.

The net charge curves are also called "titration curves". The titration curves of proteins can be displayed by a simple method (Rosengren *et al*. 1976): A square gel – containing carrier ampholytes, but no samples – is submitted to an electric field until the carrier ampholytes form a pH gradient from 3 to 10. Then the gel is rotated by 90 degrees, the sample proteins are pipetted into a groove in the gel across the pH gradient. Now an electric field is applied perpendicular to the pH gradient: the carrier ampholytes are uncharged at their pIs and will not move. But, as shown in Figure 1.10, the proteins will migrate towards the cathode or the anode according to their charge sign and mobility, and will thus form titration curves (net charge curves).

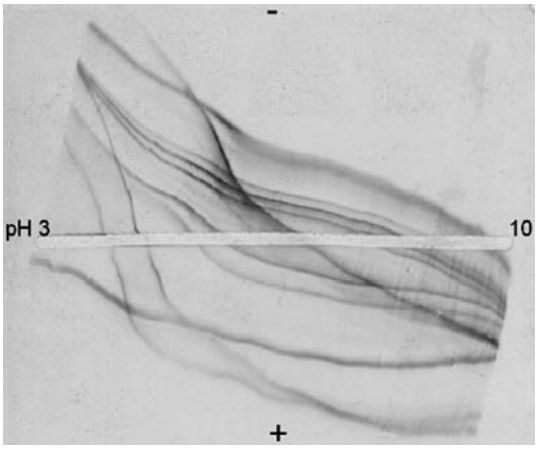


Fig. 1.10: Titration curves of a mixture of pI marker proteins under native conditions. The steeper the curve, the faster will be the migration of a protein in IEF. Note that some proteins have a flatter curve above their pI.

The proteins possess very individually shaped curves also under denaturing conditions. Practical experience has shown that more proteins have a steeper curve below their pI. This is the reason, why mostly sample application close to the anode results in better pattern.

1.4.1.2 pH Gradients

In practice there are two ways to establish a pH gradient in a gel:

- pH gradients which are formed in the electric field by amphoteric buffers, the carrier ampholytes;

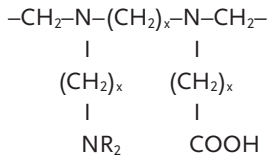
- Immobilized pH gradients in which the buffering groups are part of the gel medium.

Carrier ampholytes generated pH gradients This was the first developed technique for isoelectric focusing. Svensson (1961) has designed the theoretical basis for preparing "natural" pH gradients, while the practical realization is the work of Vesterberg (1969): the synthesis of a heterogeneous mixture of isomers of aliphatic oligoamino-oligocarboxylic acids. The result is a spectrum of low molecular weight ampholytes with closely neighboring isoelectric points, and with high buffering power at their pI.

Svensson H. Acta Chem Scand 15 (1961) 325–341.

Vesterberg, O. Acta Chem. Scand 23 (1969) 2653–2666.

The general chemical formula is the following:



R = H or $-(CH_2)_x-COOH$, x = 2 or 3

These carrier ampholytes possess the following properties:

- High buffering capacity and solubility at the pI;
- Good and regular electric conductivity at the pI;
- Absence of biological effects;
- Low molecular weight.

Naturally occurring ampholytes such as amino acids and peptides have only low or no buffering capacity at their isoelectric point. They can therefore not be used.

Alternative synthesis chemistries have been developed later. The chemical structures of other amphoteric buffers are different. Their function is the same, but the properties of the gradients can be different.

The pH gradient is established by the electric field. At the beginning, the gel with carrier ampholytes has a uniform mean pH value. Almost all the carrier ampholytes are charged: those with the higher pI positively, those with the lower pI negatively.

When an electric field is applied, the negatively charged carrier ampholytes migrate towards the anode, the positively charged ones to the cathode, with velocities depending on their net charges. The carrier ampholytes align themselves in between the two electrodes according to their pI and will determine the pH of their environment. A (relatively) stable and gradually increasing pH gradient results; for instance from 3 to 10. The carrier ampholytes lose a great part of their charge, so the conductivity of the gel decreases substantially.

By controlling the synthesis and the use of a suitable mixture the composition can be monitored for the preparation of regular and linear gradient.

The anodal end becomes acidic and the cathodal side basic.

Because these are mixtures of buffers, the gradient profile will never be perfectly linear. By adding narrow range gradient mixtures to wide gradient mixtures the final shape of the gradient can be modified according to special resolution requirements.

To maintain a gradient as stable as possible, electrode solutions are applied between the gel and the electrodes, an acid at the anode and a base at the cathode. Should, for example, an acidic carrier ampholyte reach the anode, its basic buffering group would acquire a positive charge from the medium and it would be attracted back by the electric field.

Isoelectric focusing in carrier ampholytes generated pH gradients can be performed in various media:

- In free liquid phase in capillaries, free flow electrophoresis apparatus and rotating tubing;
- In gels made from agarose with very low electroendosmosis;
- In granulated dextrane gels (Sephadex®);
- In polyacrylamide slab gels or individual gel rods in glass or plastic tubes.

Carrier ampholytes as solvents for proteins Carrier ampholytes have another very important function: they help to solubilize proteins, which stay in solution only in presence of buffering compounds. Therefore they are also needed for the immobilized pH gradient technique.

Particularly under denaturing conditions the matrix is highly viscous leading to slow migration. Additionally, unfolded polypeptides migrate slower in the gel than native proteins.

Limitations of carrier ampholyte pH gradients Isoelectric focusing is a relatively slow separation method, because proteins have only very low net charges when they come close to their isoelectric points, and therefore low mobility. High spatial resolution requires long separation distance, which causes a long migration time under a high electric field strength.

Carrier ampholyte generated gradients are also influenced by the sample load and salt contents in the sample.

Because the carrier ampholytes are in free solution, the gradient will become instable during long IEF time: the gradient drifts. The pattern becomes time-dependent and most of the basic proteins are drifting out of the gel together with the basic part of the gradient. Another problem: proteins of the sample behave like additional carrier ampholytes and modify the profile of the gradient. Thus the gradient shape becomes also sample-dependent.

Bjellqvist B, Ek K, Righetti PG, Gianazza E, Görg A, Westermeier R, Postel W. J Biochem Biophys Methods 6 (1982) 317-339.

Immobilized pH gradients These problems are solved with the application of immobilized pH gradients, generated by buffering acrylamide derivatives copolymerized with the gel matrix (Bjellqvist *et al.* 1982). The acrylamide derivatives containing the buffering groups are called Immobilines®. An Immobiline is a weak acid or weak base defined by its pK value.

The general structure of an Immobiline is the following:

$$CH_2{=}CH{-}\underset{\underset{O}{\|}}{C}{-}\underset{\underset{H}{|}}{N}{-}R$$

R contains either a carboxylic or an amino group.

The wider the pH gradient, the more different Immobiline homologues are needed.

The acidic substances have dissociation constants in the range from pK 0.8 to pK 4.6, the basic from pK 6.2 to pK 12. In order to buffer at precise pH values, at least two different Immobilines are necessary, an acid and a base. Figure 1.11 shows a diagram of a polyacrylamide gel with polymerized Immobilines, the pH value is defined by the ratio of the Immobilines in the mixture.

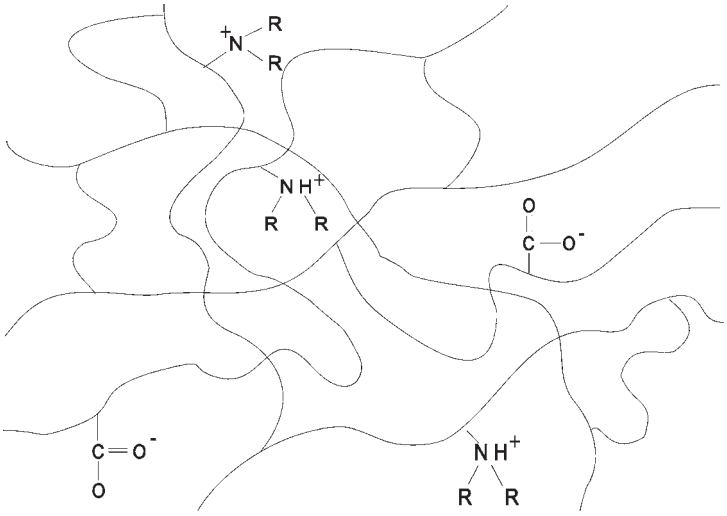


Fig. 1.11: Immobilized pH buffer. Schematic drawing of a polyacrylamide network with co-polymerized buffering groups.

This concept generates an absolutely continuous pH gradient.

A pH gradient is obtained by continuously varying the ratio of Immobilines. The principle is that of an acid/base titration, and the pH value at each stage is defined by the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{pK}_B + \log\frac{C_B - C_A}{C_A}$$

when the buffering Immobiline is a base.

C_A and C_B are the molar concentrations of the acid and basic Immobiline, respectively.

If the buffering Immobiline is an acid, the equation becomes:

$$\mathrm{pH} = \mathrm{pK}_A + \log \frac{C_B}{C_A - C_B}$$

Celentano F, Gianazza E, Dossi G, Righetti PG. Chemometr Intel Lab Systems 1 (1987) 349–358.

Altland K. Electrophoresis 11 (1990) 140–147.

Celentano *et al.* (1986) and Altland (1990) developed PC programs for the calculation and simulation of immobilized pH gradients for the optimization of buffering power and ionic strength distribution. Practice shows that a pre-calculated pH gradient recipe does not automatically result in a usable IEF gel. Every recipe has to be tested and optimized with practical experiments.

Righetti PG. In: Work TS, Ed. Burdon RH. Elsevier Biomedical Press, Amsterdam (1983).

Righetti PG. In: Burdon RH, van Knippenberg PH. Ed. Elsevier, Amsterdam (1990).

Further reading There are two books by P.G. Righetti which deal exclusively with isoelectric focusing in theory and applications.

1.4.2 Preparation of IEF Gels

Carrier ampholyte gels Casting of such homogeneous polyacrylamide gels is very simple: the monomer solution containing the carrier ampholytes is mixed with the catalysts and pipetted into a polymerization cassette, or into glass or plastic tubes. The carrier ampholyte concentration is mostly 2% (w/v), the gel concentration 4% *T* and 3% *C*. To exclude oxygen the solution is carefully overlaid with distilled water.

Westermeier R. In Cutler P. Ed. Protein Purification Protocols. Second edition. Methods in Molecular Biology, Volume 244, Humana Press, Totowa, NJ (2004) 225–232.

The preparation of granulated gels is more intricate, because it is very important to achieve the optimum liquid content (Westermeier, 2004).

Immobilized pH gradients Immobiline stock solutions with concentrations of 0.2 mol/L are used. Two solutions with acrylamide monomers, crosslinker, and catalysts are required to prepare an immobilized pH gradient: one contains the Immobiline cocktail for the acidic end, the other the cocktail for the basic end. The gel forming monomers are diluted to 4% *T* and 3% *C*. The acidic solution is made denser by adding glycerol. The gel is prepared by linear mixing of the two different monomer solutions with a gradient maker (see Figure 1.12), like for pore gradients. In principle a concentration gradient is poured. 0.5 mm thick Immobiline gels, polymerized on a support film have proved most convenient. During polymerization, the buffering carboxylic and amino groups covalently bind to the gel matrix. Because of the low electric conductivity of immobilized pH gradients, all contaminating compounds, like acrylamide monomers and the catalysts, have to be removed by washing the gels several times with deionized water.

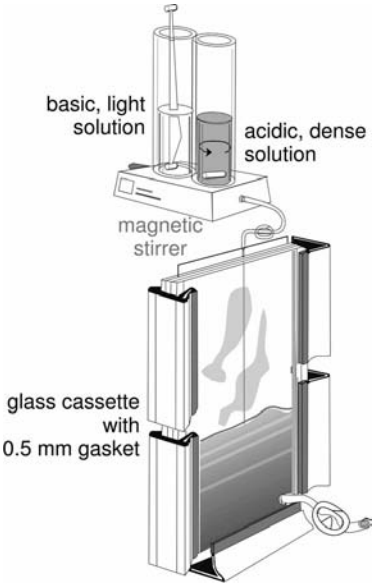


Fig. 1.12: Preparing an immobilized pH gradient. Pouring a gradient into a gel cassette.

1.4.3 Isoelectric Focusing in Proteomics

1.4.3.1 IEF Based on Carrier Ampholyte Gradients

Free flow IEF The free flow electrophoresis apparatus described above can also be used for isoelectric focusing. Either carrier ampholytes or multi-components buffers, which are mixtures of amphoteric and non-amphoteric buffers, are added. The latter mixture cannot develop linear pH gradients, but they reduce the costs. With IEF a higher number of fractions can be obtained than with the discontinuous field electrophoresis.

Free flow electrophoresis and isoelectric focusing has successfully been applied for fractionation and proteins in proteomics workflows (Hoffmann *et al.* 2001; Obermaier *et al.* 2005). Lately it has also been used for the separation of peptide mixtures as a replacement of strong cation exchange chromatography (Xie *et al.* 2005). Free flow IEF is a very reliable and reproducible technique, however good operator skills and highly sophisticated instrumentation is required.

Hoffmann P, Ji H, Moritz RL, Connolly LM, Frecklington DF, Layton MJ, Eddes JS, Simpson RJ. Proteomics 1 (2001) 807–818.

Obermaier, C, Jankowski V, Schmutzler C, Bauer J, Wildgruber R, Infanger M, Kohrle J, Krause E, Weber G, Grimm D. Electrophoresis 26 (2005) 2109–2116.

Xie H, Bandhakavi S, Griffin TJ. Anal Chem 77 (2005) 3198–3207.

Liquid phase isoelectric focusing systems Several types of IEF systems in liquid phase are on the market, which employ membranes for the separation of the fraction compartments. Temperature differ-

ences and sticking of proteins are tried to be avoided either by continuously streaming fluids or rotation. The instrumental efforts are less than with free flow apparatus, but there is always the danger that hydrophobic proteins stick to membranes.

Görg A, Boguth G, Köpf A, Reil G, Parlar H, Weiss W. Proteomics 2 (2002) 1652–1657.

IEF in a granulated gel bed Görg *et al.* (2002) have developed a very quick, reproducible and efficient pre-fractionation method: preseparation of complex protein mixtures by carrier ampholyte IEF in a granulated gel bed. The procedure is performed on standard flatbed electrophoresis equipment at an exactly adjusted temperature of 20 °C. The technique is simple: the sample is mixed with low electroendosmosis Sephadex, which has been reswollen with rehydration solution containing urea, CHAPS, DTT and carrier ampholytes. For the visualization of pH value zones after IEF a mixture of non-protein amphoteric dyes is added. The slurry is pipetted into a trough and covered with a thin layer of silicone oil. After 4 hours the proteins are focused at their isoelectric points. The pH gradient can be determined by interpolation between the colored marker zones. As shown in Figure 1.13, the pH fractions are removed with a spatula and directly applied onto rehydrated IPG strips. In the electric field the proteins migrate out from the granulated gel into the polyacrylamide gel of the IPG strip. The fractions can also be stored in a freezer at –70 °C.

This technique has several advantages:

- Standard laboratory equipment is used.
- The technique is easy to carry out.
- The separation is quick.
- Denaturing sample pre-fractionation according to pI in presence of urea, detergents, etc.
- No protein losses.
- It can be done with high and low protein amounts
- It is also desalting the sample – no microdialysis is required.

Subsequent separations of the pH fractions in the respective narrow pH interval IPG strips show a considerable increase in spot number. Many more of the lower expressed proteins can be detected in this way.

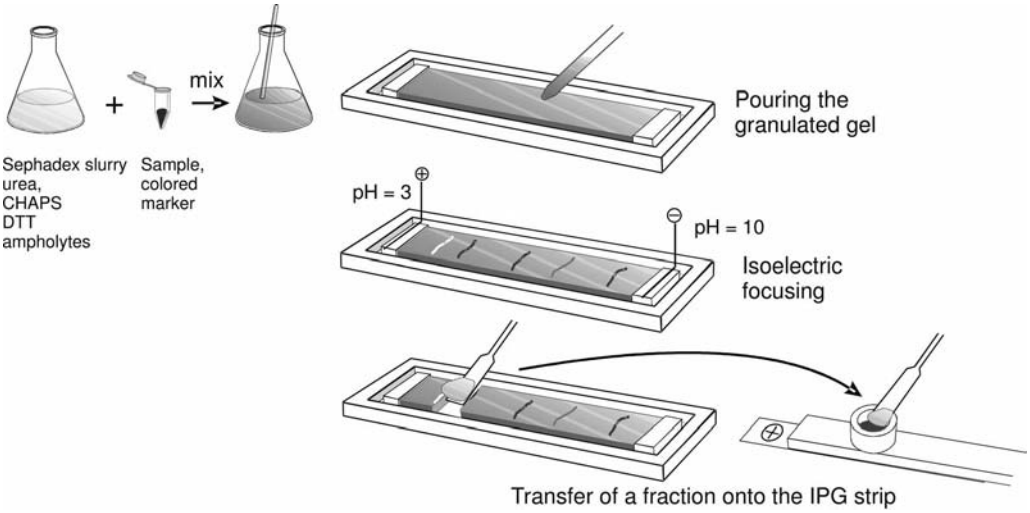


Fig. 1.13: Pre-fractionation of a complex protein mixture with isoelectric focusing in Sephadex.

Isoelectric focusing in gel rods for 2-D electrophoresis In the original high resolution 2-D electrophoresis method the first dimension is carried out in thin gel rods in glass or plastic tubes. The history of this technique goes back to a paper in German language by Stegemann (1970), combining isoelectric focusing (IEF) and SDS polyacrylamide gel electrophoresis. The resolution of 2-D electrophoresis was considerably increased by the introduction of denaturing conditions during sample preparation and isoelectric focusing by O'Farrell in the year 1975. With this modification the method gained a wide acceptance.

Stegemann H. Angew Chem 82 (1970) 640.

O'Farrell PH. J Biol Chem 250 (1975) 4007–4021.

The gel rods contain urea, detergent, reductant and carrier ampholytes to form the pH gradient in the electric field. Usually the sample is loaded onto the cathodal side of the gel rod, which becomes the basic end of the gradient in the electric field. This "O'Farrell" technique has been used for about two decades – and is still used by some laboratories – without major modifications. The potential of the method for a systematic approach to create a protein database had been recognized very soon. Anderson and Anderson (1978) have designed instruments and operation procedures to prepare and run multiple 2-D gels under the most reproducible conditions, in order to develop a "Human protein index" (Anderson and Anderson, 1982).

Anderson NG, Anderson NL. Anal Biochem 85 (1978) 331–354.

Anderson NG, Anderson NL. Clin Chem 28 (1982) 739–748.

Gels with about 20×20 cm have become standard for an adequate spatial resolution. If one assumes that up to 100 bands can be resolved in a 20 cm long one-dimensional gel, a theoretical separation space of 10,000 proteins could be reached in such a gel. In practice several thousands of proteins can be detected.

Because of the technical limitations, mainly acidic proteins could be studied in the past.

Due to the high concentration of urea the viscosity in the gel is very high. The denatured proteins are unfolded and thus highly retarded by the gel matrix. Therefore long migration times are needed, which lead to a destabilization of the gradient. The soft, thin, and long gel rods demand high experimental skill. Between pH 6.7 and 7 there is very often a lack of buffering power, leading to mechanical instability of the thin gel rods, and an empty area in the spot pattern. The handling of the technique was rather cumbersome; in many cases the patterns were not reproducible enough. Besides the influence of the sample composition also batch to batch variations of the carrier ampholyte mixtures were the reasons for differences in the profiles of the pH gradients. Gradient drift with prolonged isoelectric focusing time lead to losses of almost all basic and some of the acidic proteins.

2-D electrophoresis had sometimes been seen as a technique, which produces operator-dependent results.

O'Farrell PZ, Goodman HM, O'Farrell PH. Cell 12 (1977) 1133–1142.

A remedy had soon been found for the display of basic proteins: O'Farrell *et al.* (1977) have introduced a modification of the first dimension run: NEPHGE (non-equilibrium pH gradient electrophoresis). Here the sample is loaded onto the acidic end of the gel and the proteins are separated while the gradient drifts towards the cathode. The run is stopped after a defined time period, which is approximately one third of the regular focusing time. However, due to the time factor it is hard to achieve a good reproducibility. The proteins are not focused like in IEF; the resolution is limited by the number of different carrier ampholyte homologues.

The proteins are not focused, but stacked between the different carrier ampholyte homologues.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. Electrophoresis 21 (2000) 1037–1053.

Meanwhile most laboratories prefer to use the technique of IEF in individual IPG strips according to Görg *et al.* (2000), which will below be described in detail. In these gels all proteins are strongly focused at their isoelectric points.

This observation has been made particularly with membrane proteins.

There are indications that it is not always an advantage, when proteins are tightly focused at their isoelectric points. High molecular weight and hydrophobic proteins aggregate with themselves when they are too tightly focused, and cannot migrate out from the first dimension gel anymore. This can explain, why sometimes proteins are missing in an IPG strip 2-D gel, but they are present after a 2-D electrophoresis run with the traditional NEPHGE procedure.

Some laboratories employ isoelectric focusing in gel rods and immobilized pH gradient for 2-D electrophoresis side by side as complementary methods.

1.4.3.2 IEF Based on Immobilized pH Gradients

Görg A, Weiss W. In Rabilloud T, Ed. Proteome research: Two-dimensional gel electrophoresis and identification methods. Springer, Berlin Heidelberg New York (2000) 107–126.

Immobilized pH gradient strips Generally it is very useful to run different samples in completely separated individual gels. Immobilized pH gradient gels are the only electrophoresis slab gels, which do not show any edge effects during the run, when they are cut into strips. The gels are cut after they have been dried down on the film. Instruc-

tions and pH gradient recipes for preparing immobilized pH gradient strips in the laboratory can be found in several book chapters: Görg and Weiss (2000 and 2005) and Westermeier (2004). However, this is a multistep procedure, which needs some expertise and skill.

Görg A, Weiss W. In: Cell Biology. A Laboratory Manual, Vol. IV, Ch. 23, 3rd edition. (JE Celis, Ed.), Elsevier Science & Academic Press (2005) pp. 175–188.

Westermeier R. Electrophoresis in Practice. WILEY-VCH, Weinheim (2004).

Much more reproducible results are obtained, when ready-made IPG strips are used. Commercially produced strips are prepared according to GMP industry standards, and they are quality controlled. A wide choice of different gradients and strip lengths is available on the market. Additional gradients are being developed for achieving higher resolution.

It is very annoying, when the valuable sample is not separated well, just because of a little mistake occurring in the laboratory.

Gel sizes The commercial strips are 3 mm wide and have to be reconstituted to the original thickness of 0.5 mm with rehydration solution prior to IEF. Thicker and wider strips would have a higher protein loading capacity. This would, however, not be an advantage, because the SDS polyacrylamide gel of the second dimension would show overloading effects of proteins and a high detergent background. In theory this could be compensated by using SDS gels thicker than the conventional 1 to 1.5 mm. However, staining of such thick gel slabs would be very time consuming and not allow a high-throughput approaches.

Strips are available in several different lengths from 7 cm to 24 cm. In proteomics usually 18 and 24 cm strips are used, because the highest possible resolution is required for proteome analysis. Miniformat strips with 7 cm length are ideal for optimization of the sample preparation method.

With miniformat gels results are obtained after a few hours.

When spatial resolution does not have the highest priority, like for protein identification with western blotting, small gel formats from 7 to 13 cm are sufficient.

pH gradient types One of the advantages of immobilized pH gradients is the possibility to reproducibly produce linear gradients from pH 3 to pH 10. However, there are samples with an uneven distribution of the proteins over the pH gradient from 3 to 10. The non-linear gradient pH 3–10 is flat in the acidic range to accommodate more different and higher concentrations of acidic proteins. So far the widest gradient was designed and applied by Görg *et al.* (1999): pH 3–12. For increased resolution and higher protein loads acidic (pH 4–7, pH 3–7) and basic (pH 6–11, pH 6–9, pH 7–11) gradients are employed. Narrow intervals with only one or two pH units allow very high protein loads and excellent spatial resolution. The narrowest intervals, so called "ultra zoom gels" with 0.5 pH units, have been applied to 2-D electrophoresis by Hoving *et al.* (2000).

Görg A, Obermaier C, Boguth G, Weiss W. Electrophoresis 20 (1999) 712–717.

Hoving S, Voshol H, van Oostrum J. Electrophoresis 21 (2000) 2617–2621.

The consequence: The very basic proteins are lost, the wrong isoelectric point values are determined for basic proteins.

Basic IPG gradients As already mentioned above, polyacrylamide gels are sensitive to high pH values. The higher the pH value, the faster they become hydrolyzed. There is no problem during storage of IPG gels, because the pH is buffered with a small amount of acidic compounds, and at the freezing temperatures of storage and delivery of ready-made gels this process does not occur. But during isoelectric focusing these buffering anions will be electrophoretically removed. At 20 °C running temperature the very basic pH value will cause hydrolysis of the matrix, and the basic buffering groups will be partly cut off the gel. This results in a flattening out of the gradient at the basic end with time: what was pH 11 will come closer to a real pH value of 9. Basic IPGs should therefore be run at a minimum of time; narrow basic IPG gels were restricted to 18 cm length because of the restricted running time.

The recipe for these IPGs and the reagent are proprietary to GE Healthcare Life Sciences.

A new development allows using IPG gels with a gradient 7–11 with 24 cm length. By replacing acrylamide monomers with a more stable reagent, these IPG gels can be prevented from hydrolysis.

Advantages of immobilized pH gradient strips in 2-D electrophoresis

As already mentioned above, isoelectric focusing in pre-manufactured IPG strips is a highly reproducible method compared to gels with carrier ampholytes generated pH gradients. There are several reasons for this fact:

Readymade IPG strips are usually produced according to GMP routines.

- Industrial production reduces variations caused by human interference.

In contrast to carrier ampholytes, which are mixtures of several hundred homologues.

- The chemistry of the buffering acrylamide derivatives is better controllable.

Thin gel rods require high experimental skill.

- The film-supported gel strips are easy to handle.

Very important for reproducible results.

- The fixed gradients are not modified by the sample composition, and they do not drift with IEF time.

In contrast to NEPHGE, where the basic proteins are not focused at their pI.

- Stable basic pH gradients allow reproducible separation and display of basic proteins.

The concept of immobilized pH gradients offers a number of additional beneficial features:

This procedure prevents proteins from precipitating at the application point.

- Different ways of sample applications are feasible; the dried strips can be directly rehydrated with the sample solutions.

This allows also the detection of low expressed proteins.

- Higher protein loads are achievable.

- Various additives, like detergents and reductants, can be added to the rehydration solution.
- Less proteins are lost during equilibration in SDS buffer, because the fixed charged groups of the gradient hold the proteins back like a weak ion exchanger (Righetti and Gelfi, 1984).
- Probably the most powerful feature is the possibility to reach almost unlimited spatial resolution with very narrow pH intervals. This is needed for protein identification and characterization.

Some of them would inhibit gel polymerization.

Righetti PG, Gelfi C. J Biochem Biophys Methods. 9 (1984) 103–119.

Gradients can be engineered according to special needs; they are absolutely continuous.

Dried gel strips can be stored at –20 °C to –80 °C from months to years before reconstitution.

Fractionation between buffering isoelectric membranes The principle of this technique is based on the concept of immobilized pH gradients. The separation occurs in the liquid phase in a multi-compartment apparatus, which is divided by buffering isoelectric membranes with defined pIs (Wenger *et al*. 1987). The electrodes are located in the two outer segments. The membranes are prepared by polymerizing a polyacrylamide gel layer with basic and acidic Immobilines around a micro fiber filter or polyester grid. The amounts of Immobilines needed for a certain pH value are precalculated in the same way like for the immobilized pH gradients. Figure 1.14 shows the principle of fractionation with isoelectric membranes.

Wenger P, de Zuanni M, Javet P, Righetti PG. J Biochem Biophys Methods 14 (1987) 29–43.

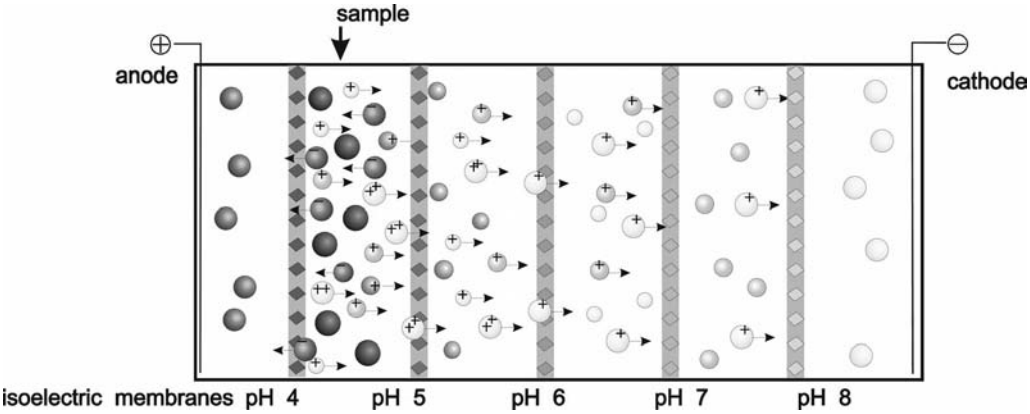


Fig. 1.14: Pre-fractionation of a protein mixture in liquid phase with isoelectric membranes.

In a mixture the sample proteins are charged. In the electric field, a protein with a high pI is protonated and migrates through the compartments towards the cathode until it reaches a membrane with a higher pI. It cannot pass through this membrane, because it will become deprotonated. If it will get a negative net charge there, it will migrate back towards the anode until it reaches the previous membrane with a lower pI. There it will become protonated again, and so on. It is thus trapped in the compartment.

Herbert B, Righetti PG. Electrophoresis 21 (2000) 3639–3648.

Speicher DW, Zuo X. Anal Biochem. 284 (2000) 266–278.

Herbert and Righetti (2000) as well as Speicher and Zuo (2000) could show that sample prefractionation *via* multicompartment electrolyzers with isoelectric membranes greatly enhances the loadability, resolution and detection sensitivity of 2-D maps in proteome analysis. In contrast, it has been reported that sometimes sticking of hydrophobic proteins to the membranes are causing electroendosmotic effects and clogging of the membrane pores.

Michel PE , Reymond F, Arnaud IL, Josserand J, Girault HH, Rossier JS. Electrophoresis 24 (2003) 3–11.

Off-Gel™ isoelectric focusing in a multicompartment device The proteins or peptides are separated according to their isoelectric point in a multiwell device, which is connected to immobilized pH gradient strips (Michel *et al*. 2003). The separation occurs in liquid phase, not in the gel. The protein fractions are directly recovered in solution for further analysis.

Giorgianni F, Desiderio DM, Beranova-Giorgianni S. Electrophoresis 24 (2003) 253–259.

Isoelectric focusing followed directly by mass spectrometry Analogous to the 1-D SDS electrophoresis LC-MS approach the proteins can be digested directly after isoelectric focusing in IPG strips with subsequent LC-MS (Giorgianni *et al*. 2003).

Essader AS, Cargile BJ, Bundy JL, Stephenson JL. Proteomics 5 (2005) 24–34.

Isoelectric focusing of peptides Essader *et al*. (2005) have found that the use of narrow range immobilized pH gradients pH 3.5–4.5 for peptide separation in the first dimension yielded 13% more protein identifications than the optimized off-line strong-cation-exchange chromatography as a first dimension in shotgun proteomics. In this work greater than 11,500 peptides and 3,700 proteins were identified with high statistical confidence. The approach has a second advantage: Accurate p*I* prediction can then be employed using currently available algorithms to very effectively filter data for peptide/protein identification, and thus lowering the false-positive rate for standard peptide identification algorithms.

1.5
Two-dimensional Electrophoresis

As already mentioned above, high-resolution two-dimensional electrophoresis is still the mainly applied separation technique in proteomics. The separation according to two completely independent physico-chemical parameters of proteins, isoelectric point and size, offers the highest resolution. Several thousand proteins can be separated, displayed and stored in one gel without degradation.

It is often tried to compare a 2-D gel result of a certain sample with a 2-D pattern displayed on a website or in a scientific paper. That does not work, because there are too many variables. There are standardization initiatives on the way, which try to design recommended procedures for sample preparation and running conditions. The conditions for these procedures must be highly stringent; otherwise pattern comparisons across different laboratories will never be possible.

However, a protein can never securely be identified by its spot position in a 2-D gel. The spot has to be excized from the gel, the protein has to be digested with a proteolytic enzyme, and the peptides have to be analyzed with mass spectrometry.

1.5.1
Sample Preparation

The sample treatment is the key to adequate results. The protein composition of the cell lysate or tissue must be reflected in the pattern of the 2-D electrophoresis gel without any losses or modifications. Of course, the sample must not be contaminated with proteins and peptides not belonging to the sample. "Co-analytical modifications" (CAM) of proteins need to be avoided. This is not trivial, because various protein–protein interactions may happen in such a complex mixture, and pre-purification of the sample can lead to uncontrolled losses of some of the proteins.

With higher protein loads sample preparation becomes even more critical, because also the contaminants concentrations are increased.

The key points for appropriate sample acquisition and preparation for 2-D electrophoresis in proteomics are:

- Avoid uncontrolled loss of proteins; — *The lost protein might be the most important ones*
- Disrupt all aggregates and complexes — *Because they are too big to enter the gel*
- Prevent enzyme activities; — *Proteases and phosphatases are also active under denaturing conditions*
- Remove disturbing compounds without removing proteins. — *Such as salts, lipids, polysaccharides, nucleic acids, polyphenols*

1.5.1.1 Sample Acquisition

Sköld K, Svensson M, Kaplan A, Björkesten L, éström J, Andren PE. Proteomics 2 (2002) 447–454.

It should be mentioned that the way, how samples are collected and stored, is very important. It is relatively easy for body fluids and lysates from cultured cells, because the proteins are evenly distributed. Strict rules have been designed and are followed for collecting clinical samples to prevent degradation. Some samples are particularly critical. It has, for instance, been reported that the protein composition of brain cells starts to change dramatically immediately after death. Sköld *et al*. (2002), for instance, keep the time between removal of a total rat brain and having it completely frozen at –80 °C below 3 minutes.

Banks R. Dunn MJ, Forbes MA, Stanly A, Pappin DJ, Naven T, Gough M, Harnden P, Selby PJ. Electrophoresis 20 (1999) 689–700.

When patient tissue material is studied, the cells to be analyzed must be well defined. A highly selective procedure for tissue analysis is needle dissection or laser capture micro dissection under the microscope (Banks *et al*. 1999), where diseased cells and control cells are separately collected to gain enough material for a 2-D electrophoresis.

Pre-labeling of cysteines with CyDyes increases the sensitivity of detection substantially, and therefore reduces the amount of required cells considerably.

As a rule of thumb about 10^7 to 10^8 cells – or about 100 µg protein – are required for a silver- or fluorescent dye-stained 2-D gel. If the sample material has to be collected with laser capture or needle micro dissection of tissue cells under the microscope, it can take several days to a week to obtain enough protein sample for a 2-D electrophoresis.

1.5.1.2 Sample Treatment

To avoid protein losses, the treatment of the sample must be kept to a minimum; to avoid protein modification, the sample should be kept as cold as possible; to avoid losses and modifications, the time should be kept as short as possible.

Too much salt in the sample disturbs isoelectric focusing and leads to streaky patterns. Amphoteric buffers in cell cultures, like HEPES, overbuffer the gradient in the areas of their pIs, which results in vertical narrow areas without protein spots. The chemicals used must be of the highest purity.

The proteins have to be extracted from cells or tissue material. Liquid samples have to be denatured to prevent the formation of polypeptide oligomers, aggregates and interactions. Some material contains proteolytic enzymes, which are still active under denaturing conditions. Phosphatases have to be inhibited to maintain the post-translational modifications to be determined.

1.5.1.3 **Denaturing Conditions**

A frequently expressed wish of proteomics researchers is the possibility to separate the protein mixtures under native conditions, in order to conserve the three-dimensional structures of the proteins. There are a number of reasons, why high molar urea, a reductant, and nonionic or zwitterionic detergents must be present in a sample for high-resolution 2-D electrophoresis:

Unfortunately it is impossible to separate complex protein mixtures under native conditions.

- Aggregates and complexes between proteins and proteins, polysaccharides, and lipids would be too big to enter a gel matrix. They are usually disintegrated by the addition of high urea concentrations and reduction of the sample.

Sometimes the sample needs to be treated by precipitation to dissolve strongly coherent complexes.

- Under native conditions, a great part of the proteins exists in several different conformations. This would lead to even more complex 2-D pattern, which could not be evaluated. The proteins have to be denatured in order to display them in single conformations.

In presence of more than 7 mol/L urea most polypeptides exist only in one single configuration.

- Different oxidation steps must be prevented by the addition of a reducing agent.
- All proteins, also the hydrophobic ones, have to be brought into solution. Because buffers and salt ions would disturb isoelectric focusing, noncharged chaotropes, like urea and thiourea, and detergents have to be used for the solubility of all proteins.

Also the addition of carrier ampholytes supports the solubility of hydrophobic proteins.

- Protein–protein interactions are avoided by applying denaturing conditions.
- For the analysis of the proteome, it should be possible to match the theoretically calculated isoelectric point of a polypeptide with pI position in the 2-D map. This is only possible, when the three-dimensional structure is cancelled and all buffering groups are exposed to the medium. Bjellqvist *et al*. have shown that this is possible with immobilized pH gradients.

In the three-dimensional configuration some buffering groups are hidden, which causes a shift of the pI.

Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez J-C, Frutiger S, Hochstrasser D. Electrophoresis 14 (1993) 1023–1031.

Composition of the standard lysis solution:
9 mol/L urea, 4% (w/v) CHAPS, 1% (w/v) DTT, 2% (v/v) carrier ampholytes, 0.002% (w/v) Bromophenol blue.

Urea is not stable in solution, repeated freeze – thawing must be avoided.

Urea The high urea concentration is needed to convert proteins into single conformations by canceling the secondary and tertiary structures, to get and keep hydrophobic proteins in solution, and to avoid protein–protein interactions. Only in special cases a second, stronger denaturing chaotrope has to be added to urea in order to increase the solubility of very hydrophobic proteins, like membrane proteins: thiourea. The purity of urea is very critical: Isocyanate impurities and heating must be avoided, because these would cause carbamylation of the proteins, resulting in artifactual spots.

Mass spectrometry is particularly sensitive to contaminations coming from detergents like Triton X-100.

Detergent CHAPS is a zwitterionic detergent, and preferred to nonionic polyol mixtures like Triton X-100 and Nonidet NP-40, because of its higher purity. It increases the solubility of hydrophobic proteins.

Righetti PG, Tudor G, Gianazza E. J Biochem Biophys Methods 6 (1982) 219–22.

Parness J, Paul-Pletzer K. Anal Biochem 289 (2001) 98–99.

Reducing agents The reductants DTT or DTE prevent different oxidation steps of the proteins. Both are interchangeable. 2-mercaptoethanol should not be used, because of its buffering effect above pH 8 (Righetti *et al.* 1982). This would cause horizontal streaks in the area between pH 8 and 9. Also, keratin contaminations, seen with mass spectrometry, have been traced back to contaminated 2-mercaptoethanol (Parness and Paul-Pletzer, 2001).

Unfortunately DTT as well as DTE can become ionized above their pK of 8 and migrate towards the anode during IEF in basic pH gradients after some time. The cysteines are no longer protected, which leads to back folding and aggregation of some of the polypeptides, resulting in modifications of their pIs and thus horizontal streaks. Additionally, non-specific reactions of cysteines with urea cause artifactual spots.

Herbert BR, Molloy MP, Gooley AA, Walsh BJ, Bryson WG, Williams KL Electrophoresis 19 (1998) 845–851.

Herbert B, Galvani M, Hamdan M, Olivieri E, MacCarthy J, Pedersen S, Righetti PG. Electrophoresis 22 (2001) 2046–2057.

Tributylphosphine (TBP), introduced into 2-D electrophoresis by Herbert *et al.* (1998), is generally not recommended as a replacement, because of its poor stability in the electric field. Also its replacement by Tris(2-carboxyethyl) phosphine (TCEP) does not improve the separation. As a remedy Herbert *et al.* (2001) have then proposed alkylation of proteins prior to IEF, particularly for separations in basic pH gradients. Also this procedure cannot be recommended, because complete alkylation of all proteins in a complex mixture is not easy to control. Therefore additional artifactual spots are observed, resulting from incomplete alkylation. Furthermore, the pIs of the basic proteins become modified, which is another disadvantage.

Olsson I, Larsson K, Palmgren R, Bjellqvist B. Proteomics 2 (2002) 1630–1632.

The phenomenon of streaking and appearance of artifactual spots in basic regions is abolished, when the first dimension IEF is run with an excess of hydroxyethyldisulfide (HED, "DeStreak") according to Olsson *et al.* (2002). For rehydration of the IPG strip the reductant is replaced by 100 mmol/L HED ("DeStreak™"). The proteins are

extracted in the conventional way with a reductant like DTT or DTE; also alternative reductants like 2-mercaptoethanol, TBP, or TCEP can be used. The lysis solution must not contain DeStreak. When the reduced proteins electrophoretically enter the IPG strip, the HED immediately oxidizes the protein cysteinyl groups to mixed disulfides. In this way all thiol groups are converted into one defined form, which will not react anymore. Back folding and aggregation of subunits are thus prevented. Non-specific reactions of cysteines with urea cannot happen anymore. The resulting pattern shows round spots, the number of spots is reduced compared to separations in presence of reductant, and there is a light shift of the basic pIs towards basic pH because of the missing negative charges of the thiols.

When DeStreak (HED) is used, it is highly recommended to use cup- or paper bridge-loading at the anodal end of the IPG strip instead of rehydration loading. More information can be found on pages 79 and 85 where rehydration and sample loading is explained. If rehydration loading is needed, the DTT content of the final rehydration solution (sample diluted with rehydration solution) must not contain more than 1 mmol/L DTT.

If DeStreak is mixed with a reductant, it will become reduced to 2-mercaptoethanol. As described above, 2-mercaptoethanol causes horizontal streaking.

An alternative way to adequate and reproducible 2-D pattern in basic gradients has been suggested by Hoving *et al.* (2001): the addition of higher amounts of DTT to the gel, adding more DTT to a cathodal paper strip, and a few more measures described later in the isoelectric focusing chapter.

Hoving S, Gerrits B, Voshol H, Müller D, Roberts RC, van Oostrum J. Proteomics 2 (2002) 127–134.

Carrier ampholytes Carrier ampholytes, which had been designed for generating pH gradients, improve the solubility of proteins considerably by substituting ionic buffers. In a mixture they are charged. They do not disturb IEF like buffer addition, because they migrate to their pIs, where they become uncharged. Dedicated pH intervals, prepared for the addition to immobilized pH gradients, are called *IPG buffers*.

Note: The composition of carrier ampholytes or IPG buffers used will influence the result.

Various IPG buffer mixtures are designed for the respective pH gradients. Sometimes in practice, the use of carrier ampholyte mixtures for wide gradients, like Pharmalytes pH 3–10, instead of dedicated IPG buffers, has shown better results for narrow gradients.

This should be checked in a practical optimization experiment for a different sample type.

Dyes The anionic dye Bromophenol blue is very useful as a control for the start and running conditions. The low amounts used do not disturb the analysis.

Bromophenol blue can be interchanged with Orange G.

1.5.1.4 Alternative Lysis Solutions

Rabilloud T. Electrophoresis 19 (1998) 758–760.

Thiourea / urea solution For the extraction and solubilization of highly hydrophobic proteins like membrane proteins a combination of 7 mol/L urea plus 2 mol/L thiourea lysis solution can be very helpful to get more proteins into solution (Rabilloud, 1998).

> Composition of the alternative lysis solution:
> ***7 mol/L urea, 2 mol/L thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 2% (v/v) carrier ampholytes, 0.002% (w/v) Bromophenol blue.***

This streak and the blurred spots are not sample-specific. It seems that thiourea starts to focus itself.

Generally, extraction with urea and thiourea combined increases the number of spots considerably. It can be observed in the literature that many researchers have started to use this combination of chaotropes for all types of samples. However artifacts can be observed as well. Figure 1.15 shows the results of 2-D electrophoresis of rat liver extracts: the lysis buffer differed only in the chaotropes used. The thiourea-containing sample shows many more spots, but also a phenomenon typical for thiourea gels: a vertical streak in the acidic area and blurred spots in the pH range below the streak. This effect becomes more pronounced with increased focusing times. So far a remedy for this effect has not been found.

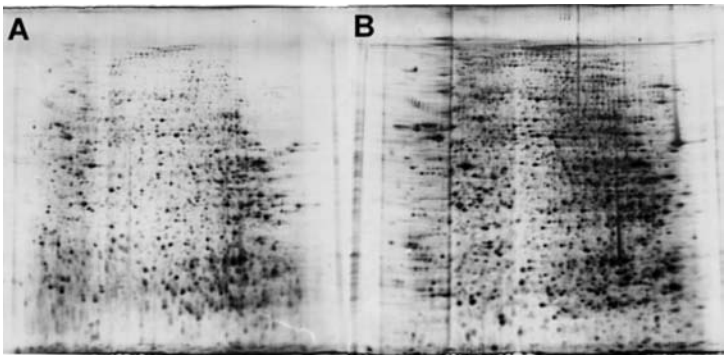


Fig. 1.15: 2-D electrophoresis of rat liver extracts.
A: Lysis solution contained 8 mol/L urea.
B: Lysis solution contained 7 mol/L urea and 2 mol/L thiourea.
Gels: IEF in 18 cm IPG 3–10 non-linear, SDS PAGE in 20×20 cm gels of 12.5% *T*, 1 mm thick. Silver staining.

The precipitation clean-up procedure described below can also remove the SDS from the proteins.

SDS procedure In some cases the anionic detergent SDS can be employed for sample preparation: up to 2% SDS has been used. Before the sample is applied to denaturing isoelectric focusing the SDS sample has to be at least 20-fold diluted with urea and a non- or

zwitterionic detergent containing solution. In the electric field the SDS will separate from the proteins and migrate into the anode. The major reasons for using SDS are:

- In human serum and plasma the formation of oligomers are only prevented by boiling the sample in SDS.
- Organisms with tough cell walls sometimes require extraction with 1% SDS before they are diluted with lysis buffer.
- Some very hydrophobic proteins may require extraction with high percentage of SDS.
- It is also applied for plant protein extraction (see below).

Hughes GJ, Frutiger S, Paquet N, Ravier F, Pasquali C, Sanchez JC, James R, Tissot JD, Bjellqvist B, Hochstrasser DF. Electrophoresis 13 (1992) 707–714.

Görg A, Drews O, Weiss W. In Simpson RJ, Ed. Purifying proteins for proteomics: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2003) 407.

Unfortunately SDS does not always completely separate from the proteins, even under high field strength. This can result in the shift of some isoelectric points to a more acidic value.

New zwitterionic detergents and sulfobetains A series of novel zwitterionic detergents (Chevallet *et al*. 1998) and non-detergent sulfobetains (Vuillard *et al*. 1995) have been tried. Some hydrophobic membrane proteins could be solubilized, which otherwise would have been lost. Molloy (2000) has reported that membrane proteins go better in solution with 7 mol/L urea plus 2 mol/L thiourea and alternative zwitterionic detergents like ASB 14 or sulfobetain in the lysis solution. Nevertheless, up to now none of these additives has performed so well in general that it has replaced CHAPS in the standard cocktail. A perfect solution has not yet been found.

Chevallet M, Santoni V, Poinas A, Rouquie D, Fuchs A, Kieffer S, Rossignol M, Lunardi J, Gerin J, Rabilloud T. Electrophoresis 19 (1998) 1901–1909.

Vuillard L, Marret N, Rabilloud T. Electrophoresis 16 (1995) 295–297.

Molloy MP. Anal Biochem 280 (2000) 1–10.

1.5.1.5 **Protease Inactivation**

Some proteases are also active in presence of urea and detergents. Protease inhibitors can inactivate most of the proteolytic activities, however in some cell lysates not completely. *PMSF* is frequently used (8 mmol/L), but it is a toxic compound. It has to be added to the sample prior to the reductant, because thiols deactivate PMSF. *Pefabloc* (AEBSF) applied as 5 to 10 mmol/L is less toxic, but might lead to charge modifications of some proteins (Dunn, 1993). This can also happen with application of protease "cocktails". Some proteases are inhibited by the denaturing conditions, some by basic pH. Therefore Tris base – below 40 mmol/L – is sometimes added to the lysis solution.

There are no protease inhibitors, which can stop all protease activities completely.

Dunn MJ. Gel electrophoresis of proteins. Bios Scientific Publishers Alden Press, Oxford (1993).

Proteases can be inactivated by boiling the sample in SDS buffer for a few seconds prior to the addition of urea-containing lysis solution. Completely irreversible protease inactivation is only obtained

In practice the sample is extracted with a protease inhibitor cocktail, and then the sample is precipitated.

with immediate precipitation of the extracted proteins. This can be done with a 2-D cleanup kit, with the methanol chloroform method, or at –20 °C with TCA acetone (see below).

1.5.1.6 Phosphatase Inactivation

The determination of phosphorylations and their locations on the protein molecule reveals very valuable information on biochemical processes. This is done with tandem mass spectrometry with several fragmentations of the tryptic peptides. To maintain the phosphorylation status two phosphatase inhibitors are added: 1–10 mmol/L Na_3VO_4, a phospho-tyrosine inhibitor, and 1–10 mmol/L NaF, which is a phospho-serine/threonine inhibitor.

1.5.1.7 Alkaline Conditions

Rabilloud T, Valette C, Lawrence JJ. Electrophoresis 15 (1994) 1552–1558.

Tris base up to 40 mmol/L or 25 mmol/L spermine base (Rabilloud *et al*. 1994) is sometimes added to the lysis buffer to maximize protein extraction, precipitate nucleic acids, or to keep protease activities low. This should not be done, when basic proteins are of interest and must be displayed in the gel. Care should be taken when preparative sample loads need to be analyzed: the ionic contamination can become too high.

Yan JX, Devenish AT, Wait R, Stone T, Lewis S, Fowler S. Proteomics 2 (2002) 1682–1698.

For labeling with modified CyDye fluors for fluorescence 2-D difference gel electrophoresis (DIGE), the pH of the sample must be higher than pH 8. This is achieved by adding 30 mmol/L Tris to the lysis solution. Sometimes the sample solution pH is adjusted to pH 8.5 with NaOH solution. This pH value is the optimum for dye labeling (Yan *et al*. 2002).

1.5.1.8 Frequently Applied Sample Treatments

It is important that the washing solution contains enough osmoticum to avoid cell lysis at this stage. Sorbitol does not work as well as sucrose.

Cell washing For the analysis of cell extracts washing of cells with PBS (phosphate buffered saline) is proposed. Unfortunately PBS contaminates the cell surfaces with salt, which leads to horizontal streaking. Either it is possible to remove the PBS solution completely, or the cells are washed with Tris-buffered sucrose solution (10 mmol/L Tris, 250 mmol/L sucrose, pH 7). Often cells are first washed with PBS solution, and then as a last washing step Tris-buffered sucrose solution is used.

Cell disruption The choice of disruption methods is dependent on the type of sample. The easiest way is osmotic lysis of cultured cells, using the standard lysis solution defined above. Sometimes lyophilized cells are brought into solution with lysis solution. For analytical

applications 5 to $10{\times}10^6$ cell equivalents are usually applied. Bacteria cells can be extracted by repeated freezing at –20 °C and thawing. For some organisms detergent lysis or sometimes even enzymatic lysis is necessary, for instance for tough cell walls of yeast and fungi. When samples are treated with the anionic detergent SDS, the solution must be diluted with lysis solution to a less than 0.1% (w/v) SDS with at least 8 × the concentration of zwitterionic detergent prior to IEF. Sonication with a probe is very helpful for solubilization. French pressure cells and mechanical homogenizers, like bead beaters, are employed to burst tough cell walls from yeast or plant cells.

When a sonicator is used, the procedure should be performed on ice. Only short bursts should be applied in order to avoid heating of the urea.

Tissues and samples with tough cell walls are often treated with mortar and pestle at sub-zero temperatures. The handling of small sample amounts requires smaller tools. A new sample grinding kit is designed for the effective grinding of small samples for the purpose of protein extraction. The kit consists of microcentrifuge tubes each containing a small quantity of abrasive grinding resin suspended in water. Extraction solution of choice is added to the tube along with the sample to be ground. A grinding pestle is used to grind the sample. Cellular debris and grinding resin are removed by centrifugation. Intracellular organelles are also disrupted, resulting in the liberation and extraction of all proteins soluble in the extraction solution.

General The disruption method used can influence the 2-D pattern. Therefore it has to be employed in a reproducible way and be thoroughly described in the analysis protocol.

1.5.1.9 **Removal of Contaminants**

A crude extract can be contaminated with salt ions, phospholipids and nucleic acids, leading to disturbances or background streaking.

Nucleic acids Nucleic acids are visible after silver staining as disturbing horizontal streaks in the acidic part of the gel. Furthermore, they can precipitate with the proteins when the sample has to be applied on the basic end of the IEF gel. They can be removed with DNAse and RNAse treatment, or with benzonase. The easiest technique is sonication, which breaks nucleic acids into little fragments. Precipitation also removes nucleic acids.

Heating must be avoided when sonicating. Proteins are not destroyed with sonication.

Lipids Lipids are removed with an excess of detergent (>2%) or with precipitation. *Proteases* are irreversibly inactivated by precipitation. *Solid material* is removed by spinning it down by centrifugation. *Salts* can be dialyzed away or removed by precipitation. In the following pages the procedures are explained more detailed.

Salts When a sample contains too much salt, the conductivity will be too high for isoelectric focusing.

Microdialysis Desalting with microdialysis tubes shows less protein losses than with gel filtration. A mini dialysis kit has been designed for the dialysis of small samples with minimal handling and without sample loss. Each dialysis tube consists of a sample tube with a cap that incorporates a dialysis membrane. Usually the membrane with a molecular cut-off of 8,000 Da is used. The sample is pipetted into the tube, which is capped and inverted in a beaker containing urea–detergent solution. The tubes are held in place with floats. Salts and molecules smaller than the molecular weight cut-off of the dialysis membrane rapidly exchange through the membrane. Following dialysis, the tube is centrifuged briefly to recover the contents. Then the reductant and IPG buffer are added to the sample again. Dialysis time is 2 hours to overnight.

Görg A, Boguth G, Obermaier C, Posch A, Weiss W. Electrophoresis 16 (1995) 1079–1086.

Electrophoretic desalting There are some cases where the sample must not be dialyzed. For example: Proteins in halobacteria lysate will not be soluble if the salt is removed. Another example: if the extraction solution containing bovine vitreous proteins would be desalted, they would form a gel. Görg *et al.* (1995) showed that the sample can be electrophoretically desalted during the first IEF phase in the immobilized pH gradient strip: the applied voltage has to be limited to 100 V for 5 hours.

1.5.1.10 Precipitation Methods

Note: Protein losses can never be completely prevented.

There are several reasons to apply a protein precipitation procedure:

- Concentration of low concentrated proteins, like in plant tissue;
- Removal of several disturbing compounds at the same time;
- Irreversible inhibition of protease activity;
- Disintegration of complexes;
- Removal of endogeneous small peptides, which would interfere with DIGE labeling.

The proteins are precipitated while interfering substances such as detergents, salts, lipids, phenols, and nucleic acids are left behind in solution. The proteins are then resuspended in lysis solution.

Wessel D, Flügge UI. Anal Biochem 138 (1984) 141–143.

Method by Wessel and Flügge (1984) Methanol and chloroform are added to the sample. After adding water the phases are separated and

the proteins are precipitated at the chloroform–methanol–water interphase. Excess methanol is added, followed by centrifugation. This precipitation method holds one of the best yields.

Method by Damerval et al. (1986) The frozen plant material is ground in a pre-frozen mortar. The powder is mixed with 10% TCA in cold acetone (–20 °C) containing 0.07% 2-mercaptoethanol. Precipitation occurs overnight in a freezer, followed by centrifugation and repeated washing with acetone (–20 °C) containing 0.07% 2-mercaptoethanol. The pellet is resuspended in lysis buffer. The disadvantage is, however, that some acidic proteins get lost with this procedure, because they are not precipitated into the pellet. The yield of proteins with pIs higher than pH 7 is much higher when extracted under acidic conditions than with lysis buffer. Görg *et al*. (1997, 1998) have therefore employed this procedure – with slight modifications – to extract more basic proteins from bacterial cells, yeast, and animal tissue. They used 20% TCA and replaced the 2-mercaptoethanol by 0.2% DTT.

Damerval C, DeVienne D, Zivy M, Thiellement H. Electrophoresis 7 (1986) 53–54.

Görg A, Obermaier C, Boguth G, Csordas A, Diaz J-J, Madjar J-J. Electrophoresis 18 (1997) 328–337.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W: Electrophoresis 21 (2000) 1037–1053.

Method by Mastro and Hall (1999) This procedure is applied for delipidation and precipitation of lipid-rich samples. Biological extracts with a lipid content of over 20% of dry matter are delipidated and precipitated with a mixture of tri-*n*-butyl phosphate, acetone and methanol at 4 °C, centrifuged, washed with the same mixture and air-dried.

Mastro R, Hall M. Anal Biochem 273 (1999) 313–315.

2-D clean-up kit This procedure can be completed in one hour and does not result in spot gain or loss. The principle is similar to the method by Damerval *et al*. (1986), but with a detergent co-precipitant proteins are more efficiently and completely removed from the solution. The wash buffer contains some organic additives that allow rapid and complete resuspension of the proteins with lysis buffer. The first experiences using this procedure were reported in a paper by Stasyk *et al*. (2001): The spot resolution is improved and the number of spots higher compared to crude extracts or other precipitation methods. In extreme cases no sample proteins at all can migrate into the IPG strip because of aggregating proteins and contaminating molecules. Figure 1.16 shows such a case: only after a clean-up procedure is a 2-D protein pattern obtained. When the crude sample was applied, the Bromophenol blue band did not start to migrate in the electric field, indicating a very high salt content.

Stasyk T, Hellman U, Souchelnytskyi S. Life Science News 9 (2001) 9–12.

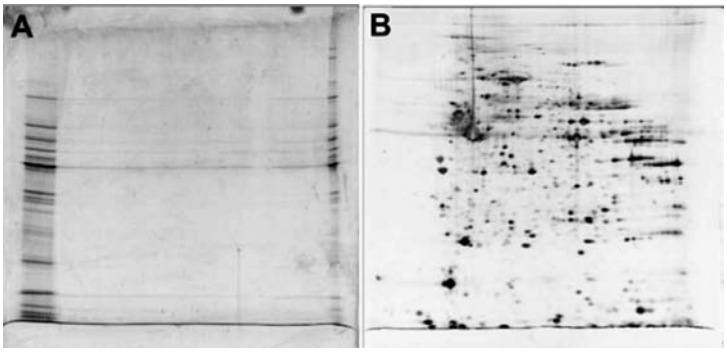


Fig. 1.16: 2-D electrophoresis of rat tongue tissue.
IPG 3-10L 13 cm. Silver staining.
A: Crude extract of rat tongue tissue.
B: Rat tongue tissue extract after treatment with the 2-D clean-up kit.

The pellet must never become completely dry; this is the most frequent reason for problems with resolubilization.

Resolubilization In practice the 2-D clean-up kit and the method according to Wessels and Flügge hold the highest yield among the precipitation methods. Nevertheless, there are sometimes difficulties to get all precipitated proteins solubilized again. In the practical Part II on page 295 a number of procedures are proposed for getting the protein pellet completely back into solution.

Immunoprecipitation A widely used procedure to reduce the protein complexity for subsequent 2-D electrophoresis is immunoprecipitation (IP) with agarose beads-bound antibodies. Elution with urea solution should be avoided, because with this measure antibodies can get removed from the beads. The standard elution buffer is 200 mmol/L glycine, titrated to pH 3 with HCl.

1.5.1.11 High Molecular Weight Proteins

The transfer of HMW proteins from the first to the second dimension is improved, when the electric field is kept very low during the first two hours (see below).

It seems that this subject may not fit into the sample preparation part, because nothing can be done here to improve the performance of 2-D electrophoresis for displaying high molecular weight proteins with sizes above 150 kDa. It is known that HMW proteins can get lost during the isoelectric focusing step or between the first and second dimensions. But they are included, when they are just run in a one-dimensional SDS gel.

In general the number of HMW sample is not so high that they could not be resolved in a one-dimensional SDS PAGE separation. If HMW proteins are of interest, there is the possibility to prepare a portion of the sample with SDS sample buffer and run it as a 1-D sample, either on the 2-D gel together with the IPG strip or on a separate SDS gel.

1.5.1.12 **Overloading Effects**

When a sample contains one or a few highly abundant proteins, an overloading effect can occur: When the electric field focuses the protein at the isoelectric point, the gel will build a high ridge at this point. When the amount of a protein is too high, the focusing force will exudate a part of the protein through the gel surface of an IPG strip. The protein will diffuse over the surface (see Figure 1.17). The effect will be visible as strong horizontal streaks over the 2-D gel.

Therefore estimation of the protein content in the sample is very important.

This phenomenon is particularly intensified, when the IPG strip is run with the surface downward, because there is additional mechanical pressure acting on the ridges.

Fig. 1.17: Effect from overloading a protein in an IPG strip.

1.5.1.13 **Quantification Methods**

The usually applied additives, like urea, detergents, and reductants, interfere with the standard protein assays (e.g. Bradford, Biuret, Lowry). False values often lead to over- or under-estimation of the protein load applied on gels. Ramagli (1999) had modified an existing method to a relatively reliable procedure by acidifying the sample with 0.1 mol/L HCl.

Ramagli LS. In Link AJ. (Ed) 2-D Proteome Analysis Protocols. Methods in Molecular Biology 112. Humana Press, Totowa, NJ (1999) 99–103.

A more reliable protein assay is based on precipitation to get rid of the additives. The subsequent assay is based on the specific binding of copper ions to protein. Precipitated proteins are resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The color density is inversely related to the protein concentration. The assay has a linear response to protein in the range of 0 µg to 50 µg.

The precipitation reagents are the same like in the 2-D clean-up kit.

Another possible method is the labeling of the proteins with a fluorescent tag and dotting a dilution series on a low fluorescent blotting membrane (Mackintosh *et al.* 2005). The membranes are washed, and then scanned with a fluorescence imager.

Mackintosh JA, Veal DA, Karuso P. Proteomics 5 (2005) 4673–4677.

For a reliable comparison of 2-D gels the protein load should be similar for all IPG strips run in one gel. Therefore the protein amount needs to be checked. Variations of protein loads must be in certain limits to allow for corrections by the normalization function of image analysis (see pages 131 and 133). The knowledge of the protein content of a sample is very useful to avoid under- or overloading.

If possible, these highly abundant proteins should be depleted with affinity media, or the sample should be pre-fractionated.

However, when the protein composition is unbalanced, this information does not help very much. A typical example is serum or plasma, where albumin makes up ca. 60% and the gamma globulins ca. 20% of the total protein. These highly abundant proteins can easily cause overloading effects (see above). Thus for such sample types the total protein loading capacity of an IPG strips is considerably reduced.

1.5.1.14 Analytical Versus Preparative Sample Load

Sample loads are often classified into *analytical* or *preparative* loads. In practice it is not easy to differentiate clearly between these terms: A strong protein spot in an analytical gel can contain enough material for further analysis whereas a weak spot in a preparative gel can be insufficient. Roughly, when 50 to 150 μg of total protein is applied on a gel, and silver or sensitive fluorescent staining methods need to be employed, it is an analytical gel. Preparative gels are loaded with 500 μg to 1 mg total protein or more. These gels are usually stained with Coomassie brilliant blue, medium sensitive fluorescent dyes, or zinc–imidazol.

Anderson L, Anderson NG. Proc Nat Acad Sci USA. 74 (1977) 5421–5425.

Anderson NG, Anderson NL, Tollaksen SL. Clin Chem. 25 (1979) 1199–1210.

Hughes GJ, Frutiger S, Paquet N, Ravier F, Pasquali C, Sanchez JC, James R, Tissot JD, Bjellqvist B, Hochstrasser DF. Electrophoresis 13 (1992) 707–714

Harrington MG, Merrill C. J Chromatogr 429 (1988) 345–358.

Yun M, Mu W, Hood L, Harrington MG. Electrophoresis 13 (1992) 1002–1013.

Zerr I, Bodemer M, Otto M, Poser S, Windl O, Kretzschmar HA, Gefeller O, Weber T. Lancet 348 (1996) 846–849.

Burkhard PR, Rodrigo N, May D, Sztajzel R, Sanchez J-C, Hochstrasser DF, Shiffer E, Reverdin A, Lacroix JS. Electrophoresis 22 (2001) 1826–1833.

Chromy BA et al. J Proteome Res , 3 (2004) 1120–1127.

1.5.1.15 Special Cases

Human body fluids Body fluids are an important source for detection and monitoring of disease markers. The Anderson group had started many years ago to collect data for the Human protein index with 2-D electrophoresis, for instance analyzing plasma and urinary proteins (Anderson *et al.* 1977, 1979). A plasma protein map produced with immobilized pH gradients has been published by Hughes *et al.* (1992).

Besides plasma and serum, cerebrospinal fluid is frequently used as a sample for detecting and monitoring prognostic and diagnostic markers. Here is a selection of papers on 2-D electrophoresis of cerebrospinal fluid: Harrington and Merrill (1988), Yun *et al.* (1992), Zerr *et al.* (1996), Burkhard *et al.* (2001).

Unfortunately most of the body fluids are loaded with some highly abundant proteins and salt ions, which interfere with the first dimension. Microdialysis and/or low-voltage start conditions for isoelectric focusing are required to avoid distorted patterns.

Albumin For serum and plasma usually the high abundance of albumin and globulins limit the loading capacity for the rest of the proteins. When the albumin and IgG content in samples like plasma, serum, and cerebrospinal fluid are removed with affinity media, the sensitivity of detection for other proteins is considerably improved (see, for instance, Chromy *et al.* 2004). However, as albumin is a

transport protein, other proteins, which stick to albumin, are removed at the same time (Granger *et al*. 2005). Some research groups have already started to study the so called "Albuminome" (Gundry *et al*. 2007). Currently there is no procedure available to get rid of albumin without losses of other proteins.

Granger J, Siddiqui J, Copeland S, Remick D. Proteomics 5 (2005) 4713–4718.

Gundry RL, Fu Q, Jelinek CA, Van Eyk JE, Cotter RJ. Proteomics Clin Appl 1 (2007) 73–88.

Plant proteins Sample preparation from plant tissues is particularly troublesome, because interfering substances such as polysaccharides, nucleic acids, lipids and phenolic compounds are present in high concentrations, whereas the proteins exist in low abundance. The classic procedure for protein extraction from plant tissue is the precipitation with TCA and acetone according to Damerval *et al*. (see above). Meanwhile some more work has been invested for more efficient extraction procedures for recalcitrant plant tissue (Giavalisco *et al*. 2003; Méchin *et al*. 2003; Carpentier *et al*. 2005). Relatively elaborate procedures are also required for the analysis of plant membrane proteins (Hurkman and Tanaka, 1986). Here the protein pellet has to be extensively washed before it can be resuspended in lysis buffer for IEF. Delaplace *et al*. (2006) could show for the example of potato tuber proteins that the phenolic phase extraction method according to Hurkman and Tanaka and an extraction with SDS lysis solution are complementary regarding the M_r range of preferentially extracted proteins.

Another challenge for plant proteomics researchers is the high abundance of Rubisco (ribulose-1,5-bisphosphate), the most abundant enzyme on earth, and making up about 60% of soluble leaf protein.

Méchin V, Consoli L, Le Guilloux M, Damerval C. Proteomics 3 (2003) 1299–1302.

Giavalisco P, Nordhoff E, Lehrach H, Gobom J, Klose J. Electrophoresis 24 (2003) 207–216.

Carpentier SC, Witters E, Laukens K, Deckers P, Swennen R, Panis B. Proteomics 5 (2005) 2497–2507.

Hurkman WJ, Tanaka CK. Plant Physiology 81 (1986) 802–806.

Delaplace P, van der Wal F, Dierick J-F, Cordewener JHG, Fauconnier M-L, du Jardin P, America AHP. Proteomics 6 (2006) 6494–6497.

1.5.1.16 Integration of Sample Preparation into a Laboratory Workflow System

It is very important to link the sample preparation procedure to the result data even down to mass spectrometry analysis. A good example case for this need is described in the – already mentioned – paper by Parness and Paul-Pletzer (2001), where the origin of the keratin of the protein spots has been found in the reductant.

Parness J, Paul-Pletzer K. Anal Biochem 289 (2001) 98–99.

Peculiar results in mass spectrometry analysis must initiate an automated search in the entire laboratory workflow. Therefore all data, including the source and the LOT number of each reagent used, have to be entered together with the sample code into the database of the laboratory workflow system.

1.5.1.17 Further Developments in Sample Preparation Procedures

Whereas the methodology for the separation and further analysis of the proteins has become much easier, reliable, and reproducible, there is still a substantial wish list for improvements of sample preparation:

- A very important goal in for creating reliable data is a certain standardization of sample preparation and a systematic reduction of protein complexity prior to 2-D electrophoresis.
- A wider choice of detergents and other additives, which support the solubility of hydrophobic proteins, and would reduce the losses of proteins.
- Multiple automated sample preparation will reduce the manual influence and allow higher throughput. Because most of the problems in practice are created by contaminants of all different kind, the 2-D clean-up precipitation and resolubilization method should be applied to many sample types.
- Pre-fractionation of proteins according to their isoelectric points into defined packages, which are applied on narrow pH interval gels can substantially reduce the load of sample not displayed in these gels, and in this way reduce losses of proteins.
- More efficient methods for depletion of highly abundant proteins need to be developed.

1.5.2 Pre-labeling of Proteins for Difference Gel Electrophoresis

The separation conditions for labeled proteins are exactly the same as for non-labeled.

With the difference gel electrophoresis (DIGE) approach the proteins of several samples are labeled prior to the electrophoretic separation with different – spectrally distinct – fluorescent dyes. The samples are mixed together and run on the same 2-D gel. In this way the proteins of different samples are migrating under identical conditions, because the gel-to-gel variations are eliminated. For this method the cyanine dyes (CyDye) are chemically modified in a way that they bind to proteins and allow precise co-migration of the same proteins in both dimensions (Ünlü *et al*. 1997). After electrophoresis the gels are scanned with a fluorescent imager at different wavelengths. There is a linear relation between a labeled protein and the signal measured with the imager, because on excitation of the dye by monochromatic

Ünlü M, Morgan EM, Minden JS. Electrophoresis 19 (1997) 2071–2077.

light, the dye emits light in proportion to the amount of labeled compound in the sample.

Laser scanners with photomultipliers are more expensive than white light images with CCD cameras, but they are more precise and sensitive, and have a broader dynamic range.

Three spectrally distinct dyes are available: Cy2, Cy3 and Cy5. They can be excited with a blue, red and green laser, respectively. In the scanner the emitted light is amplified with photomultiplier tubes and recorded with the computer. As an alternative a white light source can be used, and the monochromatic light is created with particular narrow band pass excitation filters. In this case the emitted light is collected with a scanning CCD camera. In order to prevent crosstalk between the different sample signals, in both cases the different channels are scanned sequentially, and narrow band pass emission filters are inserted for the respective fluorophores.

Often the differences are subtle; they are not detectable with the eye.

The images can be displayed on a computer screen in a false color representation. This allows a preview on the results. Using an image-editing program the false color pattern can be overlaid, which is an easy way to visualize differences in protein expression. Figure 1.18 shows such an overlay for two samples. Yellow spots indicate equal expression levels, green or red spot up- and down-regulations respectively. This visual inspection is very useful as a control over the separation quality and for optimization of sample preparation and labeling. For the final evaluation of the results dedicated image analysis software, DeCyder™, has to be employed.

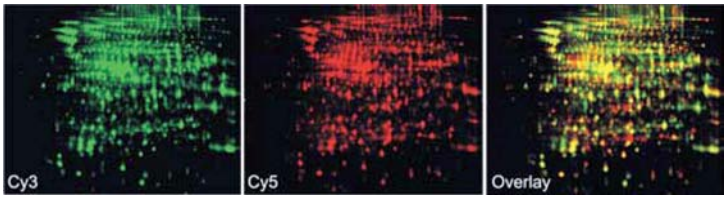


Fig. 1.18: False color representation of 2-D electrophoresis separations of two samples labeled with Cy3 and Cy5. The overlay image visualizes up and down-regulated proteins.

Two different ways of labeling are employed: lysine and cysteine tagging. The by far mostly applied method is labeling of the lysine, because its ε-amino side group is easily accessible without derivatization of the proteins. Also, lysine is one of the most frequently present amino acids in proteins. The labeling occurs via NHS ester chemistry. Prior to cysteine labeling the disulfide bridges have to be cleaved with a reductant. In this case maleimide chemistry is applied. However, cysteine is markedly less frequently present than lysine.

1.5.2.1 Lysine Labeling (Minimal Labeling)

In downstream analysis the non-labeled peptides are in a great majority versus the labeled ones, therefore only the non-modified peptides will be detected with mass spectrometry.

It must be avoided to block all lysines of the proteins with a label, because the proteins would become very hydrophobic and would not stay in solution. Therefore only a small portion of the proteins is labeled. In practice only 400 pmol/L of dye is added to 50 µg of protein. In this way only 3 to 5% of the proteins will receive a tag; the other 95–97% proteins remain unlabeled. This measure, which is called "minimal labeling", prevents also the appearance of multiple labels: the occurrence of multiple labels on proteins with several lysines will statistically be so low that these molecules are below the limit of detection. Overlabeling with minimal dyes could be recognized very easily in the false color image (see above): Two and more dye molecules per protein would cause vertical streaking due to increased molecular weight of a part of the molecules of the labeled protein.

In the lower molecular weight area of the SDS gel an offset of non-labeled from labeled protein spots can be observed. Therefore, when spot picking is intended, it is recommended to stain the gel after scanning with a fluorescent dye and match the spots with the labeled spots using the spot matching algorithm of the evaluation software.

It is, however, very important that these dyes are charge and size matched. Therefore a basic buffering group is added to each of the dyes, thus the isoelectric points are not changing. Also in the second dimension the proteins migrate to the same point independently of their label, because the molecular weights added are in the range between 434 and 464 for the three dyes Cy2, Cy3, and Cy5. This little difference cannot be resolved by SDS electrophoresis. The 2-D pattern obtained with minimal labeling does not differ from a pattern of post-stained 2-D gels of non-labeled proteins.

The sensitivity of minimal labeling is comparable to good quality silver staining, however exhibiting a much wider dynamic range and linearity. It is very rare that a protein does not pick up a label or does not contain any lysine. To be on the safe side it is recommended to stain a gel with a sensitive fluorescent dye to detect such proteins.

Underlabeling is not an issue: if more protein is labeled with the same amount of dye, the scanned image will be the same. However, when proteins are overlabeled, multiple labels can become visible, particular in the cathodal area of the 2-D gel.

The distribution of dyes is uniformly and quantitative across all the different proteins.

In order to make the method reliable and reproducible, the labeling has to be performed under optimum conditions and needs to be well controlled (see Figure 1.19). The proteins are solubilized and denatured with high molar urea or thiourea/urea. The optimal concentration of the protein lysate is between 5 and 10 mg/mL. But also samples containing from 1 mg/mL to 20 mg/mL have been successfully labeled. If the sample is too diluted, labeling will be less efficient. Lysine labeling is performed on ice and in absence of IPG buffers or carrier ampholytes and reductants: The primary amino groups of amphoteric buffers would be labeled as well; the reductant would interfere with the reaction. These compounds are added after labeling had been completed. A sample pH value above pH 8.0 is essential, the optimum lies at pH 8.5. Therefore the sample solution should always contain 30 mmol/L Tris-base. Samples, which have been

extracted with TCA acetone or have been cleaned up with precipitation, are often very acidic. In this case it can be necessary to adjust the pH value by adding 50 mmol/L NaOH solution. The reaction is carried out on ice for 30 minutes and stopped by adding a 0.1% (w/v) lysine solution.

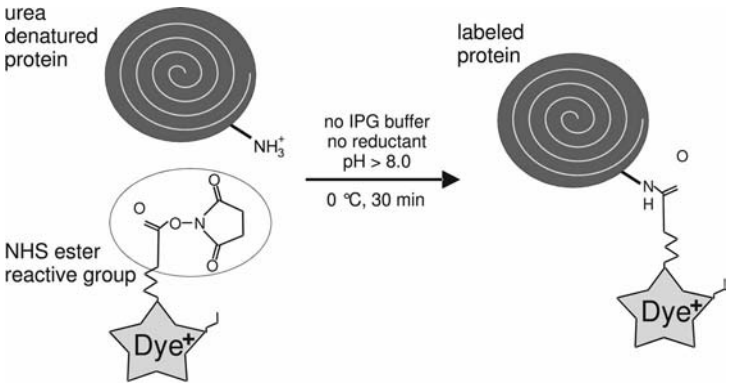


Fig. 1.19: Simplified diagram of lysine labeling for DIGE. No derivatization is needed, because the ε-amino group of the lysine is readily accessible.

Subsequently the samples are combined and either submitted directly to 2-D electrophoresis or stored in a deep freezer until use.

1.5.2.2 Cysteine Labeling (Saturation Labeling)

In contrast to lysine labeling here all available cysteines will be tagged. Labeling the cysteines is pH neutral and does not increase the hydrophobicity. However the resulting 2-D electrophoresis pattern looks different from those originating from minimal labeled or non-labeled proteins: proteins with many cysteines will pick up many labels, which results in slower migration in the SDS gel and a much stronger light emission signal. The increase of molecular weight is 650 Da per label. Because of multiple labels, here it is particularly important that the dyes are size matched. Not all proteins contain cysteine. It is estimated that in human, animal, and plant proteomes about 95% of the proteins possess at least one cysteine, and can therefore be detected with this method.

However, there are bacteria species, which do not contain a single cysteine. Needless to say, the method cannot be applied for those.

The sensitivity of this method is very high. The application of down to 1,000 human cells (equal to 2.5 µg protein) provides still very good 2-D gels, as shown in a paper by Sitek *et al.* (2005).

Sitek B, Lüttges J, Marcus K, Klöppel G, Schmiegel W, Meyer HE, Hahn SA, Stühler K. Proteomics 5 (2005) 2665–2679

Cysteine labeling requires more work than lysine labeling. First the disulfide bridges are cleaved with the reductant TCEP, thereafter the

This technique is mainly applied for very scarce samples.

dye with the maleimide reaction group is added (see Figure 1.20). The temperature is higher and the reaction time is longer than for minimal labeling in order to achieve complete labeling of all cysteines. To avoid bleaching of the dyes these steps are therefore performed in the dark. Furthermore, some optimization for certain sample types may be necessary, because the quantitative amount of cysteines in the protein mixture cannot be predicted. If more dye had been applied than cysteines were available, non-specific labeling of some lysines can be observed, indicated by a pI shift of some spots towards the acidic side. If there was not enough dye for all the cysteines, some proteins will partly have lower molecular sizes and will migrate a little faster, resulting in vertically elongated spots or vertical streaks. The optimal concentrations can be assessed by a same–same experiment with different label concentrations (see also Sitek *et al*. 2005). The applied ratio of reductant to dye is constant.

Here is one of the major differences between minimal labeling and cysteine labeling: overlabeling causes vertical streaks in minimal labeling, but horizontal displacement in cysteine labeling.

Cy2 shows too strong self-quenching effects, when dye molecules are in close proximity to each other.

For this method only the two dyes Cy3 and Cy5 can be used, because Cy2 does not work properly for this application.

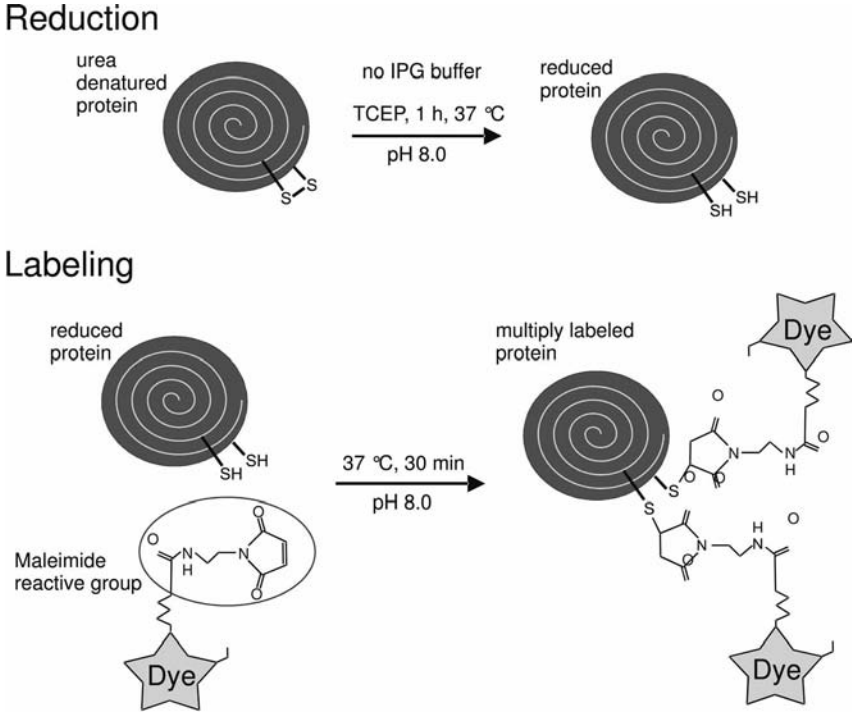


Fig. 1.20: Simplified diagram of cysteine labeling for DIGE. The more cysteinyls are available after reduction, the more dye molecules will become attached to a protein molecule.

For downstream analysis the spots could in principle be picked directly from the image file (see spot picking below). However, because the method is so sensitive, there will not be enough protein material in a spot for mass spectrometry analysis. Therefore a preparative 2-D gel of a reference proteome loaded containing about 300 µg protein has to be run. Also for this gel the cysteines must carry a label; otherwise the spots to be excised cannot be matched with the spots in the analytical gel. The selection of the optimal reference proteome, which should show the most similarities with the analytical sample, is dependent on the type of sample. Labeling of the preparative sample proteins is performed with Cy3.

Because in this case all cysteine-containing proteins are modified, the molecular weight added to the cysteines needs to be entered into the database search definitions, analogous to alkylation of cysteines.

1.5.2.3 The Internal Standard

The multiplex feature of DIGE offers the unique possibility to reserve one channel for an internal standard. Some papers are describing the application of a mix of labeled commercial standard proteins. However, by far the greatest effect is obtained, when the internal standard is a pool of aliquots taken from each sample of an experiment, and labeling this mixture with one of the dyes. This concept is shown in a simplified schematic diagram in Figure 1.21. Of course, "control" and "treated" can be replaced by "healthy" and "diseased" or by "wild type" and "mutant".

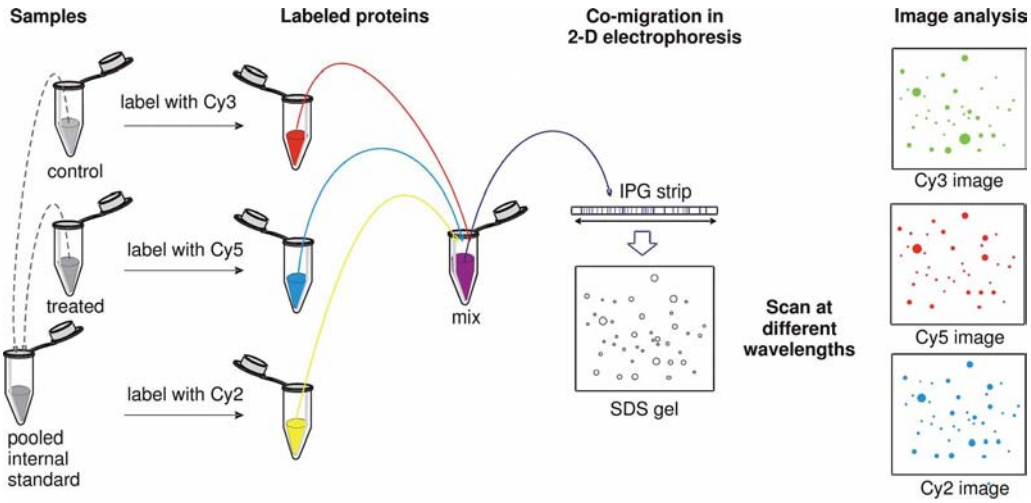


Fig. 1.21: Simplified schematic representation showing how to create a pooled internal standard in difference gel electrophoresis (DIGE). The sample proteins are labeled with Cy3 and Cy5, the internal standard with Cy2. From Westermeier and Scheibe (2007).

Westermeier R, Scheibe B. In Posch A. Ed. Sample Preparation for 2D PAGE. Methods in Molecular Biology, Humana Press, Totowa, NJ (2007) in press.

When minimal labeling is employed, the standard is always labeled with Cy2, for saturation labeling it is Cy3.

In reality it makes no sense to run only two samples in an experiment. As already mentioned in the introduction, in order to find significant protein changes with acceptable statistical confidence, at least three biological replicates are required. This means that the internal standard will be composed of aliquots from six samples: three controls plus three treated. It is important to create enough pooled internal standard to be able to apply it on every gel. With minimal labeling each gel contains two samples and one standard, with saturation labeling only one sample and the standard are run together.

All this will be described in more detail in the chapter on image analysis.

Because the proteins are co-migrating in the gel, no spot matching is needed within the gel. The sample spot volumes can directly be compared to the spot volumes of the standard, which is the mixture of all samples. The dedicated software, DeCyder, does this automatically, employing a co-detection algorithm. The result is expressed in spot volume ratios. For the comparison of several gels the spot matching procedure is highly facilitated, because the images of the standard are matched, which originate from the identical protein composition. In this way it is possible to perform a fully automated evaluation of a high number of images. Furthermore image analysis can be carried out fully automatically with a batch processor, which reduces the user bias to a minimum.

Artifactual spot volume variation can be caused by slight different conditions during sample application.

Note: ***the result issued by DeCyder consists of spot volume ratios to the internal standard instead of absolute spot volumes. In this way artifactual spot volume variations caused by gel-to-gel variations are eliminated.***

The use of the pooled internal standard makes the evaluation of the results much easier and considerably more reliable:

- Every protein in the population must appear on the standard image in each gel, otherwise it will not be included in the result.
- The sample spot volumes are normalized against the internal standard.
- Variations of spot volumes due to gel-to-gel variations are eliminated.
- Each sample is compared internally with the same standard.
- It enables fully automated accurate spot statistics.
- Gel-to-gel matching becomes much easier.

1.5.2.4 **Experimental Design**

First of all, the pooled internal standard should be labeled with one and the same dye within an experiment. For spot matching it is important that the conditions for the internal standard are identical.

For 3-color experiments the standard is labeled with Cy, for 2-color experiments with Cy3.

In order to obtain statistically significant results, the distribution of the labels among samples has to be well planned. For instance, any preferred labeling of certain proteins by one of the dyes must be statistically excluded. If the experiment consists only of a small number of samples, it is recommended to run the samples two times, but with inverse labeling. For larger experiments, no replicates are required, but a "planned" randomization of the labels and sample application is proposed:

The statistics algorithms of the DeCyder™ software take these measures in calculation.

- The two available dyes should be evenly distributed between treated sample and control.
- Treated sample and control from separate origins should be applied to each gel.

An example for randomization of sample application and inverse labeling is shown in Table 3.1 on page 309.

To minimize methodical variation all samples of an experiment should be run together in one instrument in the first and also the second dimension.

Experiments with more than 12 gels at a time are more difficult to evaluate.

Typically the amount of three times 50 µg protein is applied, which means 150 µg total sample load. Because the detection is very sensitive, it is possible that a small spot does not contain enough protein for mass spectrometry analysis. If more protein is required in a gel, it is suggested to spike non-labeled pooled standard into the 150 µg labeled sample to the desired protein amount.

The original suggestion to run a separate preparative gel is less practical.

It should also be mentioned that the DIGE technique is very useful for checking the quality of sample preparation:

Some samples contain protein individuals with peculiar behavior. Those are detected with this strategy.

- First the same sample is labeled with two different dyes and separated in one gel;
- In a second step three sample preparation replicates of the same sample source labeled with three different dyes are run in a gel.

1.5.2.5 **Major Benefits of 2-D DIGE**

These points have been ranked according the comments of an adequate number of DIGE users.

In short the advantages of the DIGE approach over conventional 2-D electrophoresis are ranked in the order of their importance to practice:

- It saves time: differentially expressed proteins are detected very rapidly.

No gel replicates, automated image analysis.

This reduces also costs for consumables, equipment, and labor.

Gels do not swell, shrink, or break.

Image analysis is the bottle neck in conventional 2-D electrophoresis

In contrast replicate conventional gels do not eliminate these variations, they just average them.

Note that those are still relative quantitative results.

- It saves work: a smaller number of gels has to be run, because samples are combined on gels, and no gel replicates are necessary.
- It eases handling of gels: the gels are analyzed while they are still in the cassettes.
- It simplifies the evaluation: image analysis with DeCyder software runs automatically and is simple to carry out.
- It improves the quality of the results: gel-to-gel variations are eliminated from the result because it measures spot volume ratios relative to the pooled internal standard, which is included in each gel.
- It delivers quantitative values: the internal standard and the wide linear dynamic range of the fluorescent dyes allow the quantitative measurement of minor changes of expression levels with high statistical confidence.
- It affords very sensitive detection: with cysteine labeling samples containing less than 1 μg total protein can be analyzed with 2-D electrophoresis.

Sitek B, Scheibe B, Jung K, Schramm A, Stühler K. In: Proteomics in Drug Research (M Hamacher et al. Eds.) Wiley-VCH, Weinheim (2006) pp 33–55.

Perales M, Eubel H, Heinemeyer J, Colaneri A, Zabaleta E, Braun H-P. J Mol Biol 350 (2005) 263–277.

Helling S, Schmitt E, Joppich C, Schulenborg T, Müllner S, Felske-Müller S, Wiebringhaus T, Becker G, Linsenmann G, Sitek B, Lutter P, Meyer HE, Marcus K. Proteomics 6 (2006) 4506–4513.

Mayrhofer C, Krieger S, Allmaier G, Kerjaschki D. Proteomics 6 (2006) 579–585.

GE Healthcare Application Note: Selective labeling of cell-surface proteins using CyDye DIGE Fluor minimal dyes. (2005) 11-0033-92.

The DIGE concept is not limited to high resolution 2-D electrophoresis with immobilized pH gradients: it has also been successfully applied on 2-D gels based on carrier ampholytes IEF (Sitek *et al*. 2006), blue native electrophoresis (Perales *et al*. 2005), and acidic electrophoresis with cationic detergent (Helling *et al*. 2006).

1.5.2.6 Specific Labeling of Cell Surface Proteins

Usually the proteins of cell or tissue lysates are labeled. It is, however, possible to label the proteins on the surface of the intact cell (Mayrhofer *et al*. 2006). In a GE Healthcare Application Note (2005) it is shown that after cell lysis and 2-D electrophoresis only the specifically labeled surface proteins are visualized. The proteins inside the cell do not become labeled, because of unfavorable conditions: the pH value in the cytosol of an intact cell is usually below pH 7.4; the labeling time is short and performed on ice. This approach is much easier to carry out than using the standard protocol of biotin labeling. As shown in Figure 1.22, the differential comparison of cell surface proteins and whole cell lysate labeled with the second dye in the same gel, is a very straightforward way to identify outer membrane proteins against the background of the total proteins.

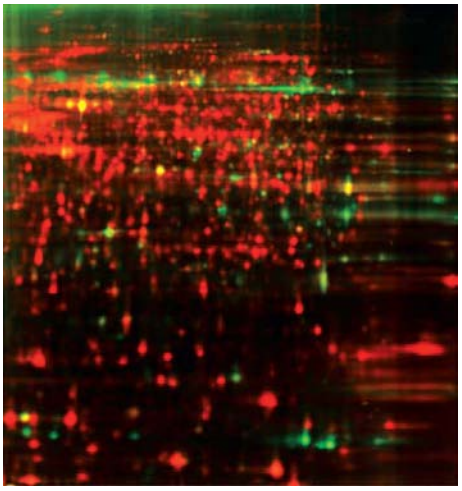


Fig. 1.22: False color representation of 2-D gel images of a CHO-K1 Cy5 cell-surface labeled sample (green spots) and a Cy3 sample (red spots) labeled according to standard Ettan DIGE protocol run in the same 2-D gel. From GE Healthcare Application Note 11-0033-92.

Note: ***The multiplex approach of DIGE has some additional benefits for the conditions during isoelectric focusing: the conductivity differences between the strips are lower, because several samples are mixed together. This leads to more reproducible spot pattern across the gels.***

1.5.3 First Dimension: Isoelectric Focusing in IPG Strips

When detection is performed with western blotting, small formats are preferred.

IPG strips are available with many different lengths and gradients. Optimally the resolution of the two-dimensional gel should be sufficient to get every protein separated from each other. If a protein spot contains more than one protein, the proteins can still be identified and characterized with mass spectrometry, however quantitative assessments are not possible. Therefore large gel formats are preferred. If the resolution of such a gel is insufficient, the sample can be applied on several IPG strips with narrow, overlapping pH gradients. Most frequently wide gradients with pH 3 to 10 or 3 to 11 are run. When higher resolution is required, a combination of an acidic gradient pH 4 to 7 (or 3 to 7) and a basic gel pH 7 to 11 are employed. IPG strips with printed serial numbers are very useful for tracing the samples through the 2-D electrophoresis procedure.

For proteomics studies large formats with 18 or 24 cm strip lengths are preferred because of several reasons:

Ideally one protein/spot. Abundant proteins need more space.

- To reach sufficient resolution.
- To be able to load higher protein concentrations for detection of as many spots as possible and subsequent mass spectrometry analysis of spots.

The smaller the gel, the higher the fringe effect.

- To obtain sufficient evaluative gel area.

1.5.3.1 **Rehydration**

The dry IPG strips need to be reconstituted to their original thickness of 0.5 mm with rehydration solution before use. There are two possibilities:

- Pre-rehydration of the strip without sample; the sample is loaded either on the anodal or cathodal end of the IPG strip with a cup or paper bridge.
- Rehydration with the sample mixed into the rehydration solution.

This solution is suitable for samples without thiourea.

Composition of the standard "rehydration solution": ***8 mol/L urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT, 1.25% (v/v) carrier ampholytes, 10% (v/v) glycerol, 0.002% Bromophenol blue.***

For electroendosmosis see page 21 f or glossary.

- Glycerol reduces electroendosmotic effects, prevents drying of the gel, and urea crystallization. It is not always necessary.
- The concentrations of the additives are lower than in the lysis buffer, in order to reduce unwanted side effects like:
  - Crystallization of urea;
  - Instability of the patterns due to conflicts of the buffering groups, caused by overloading with carrier ampholytes;
  - Formation of micelles between the different detergents in the second dimension, which can cause dark and dirty background with some of the staining techniques (Görg et al. 1987a);
- Bromophenol blue allows a good control of the liquid distribution during rehydration.

See also page 79 for choice of IPG buffers or carrier ampholytes.

Görg A, Postel W, Weser J, Günther S, Strahler JR, Hanash SM, Somerlot L. Electrophoresis. 8 (1987a) 45–51.

When a sample has been extracted with urea *and thiourea*, the rehydration solution must *also* contain thiourea.

- Composition of the thiourea rehydration solution: ***7 mol/L urea, 2 mol/L thiourea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT, 1.25% (v/v) carrier ampholytes, 0.002% (w/v) Bromophenol blue.***

This is the more universal rehydration solution.

Samples without thiourea can also be applied to strips rehydrated with such a solution.

Basic pH gradients It is not recommended to apply rehydration loading to basic pH gradient strips. Obviously some proteins start to aggregate in presence of high pH values. The samples are loaded at the anodal end of the strip with a cup or paper bridge. As already explained above in context with the reductant (see page 56 f), the best remedy against horizontal streaking in basic gels is the rehydration of IPG strips with HED ("DeStreak™") instead of a reductant.

At the anodal end of the strip there are milder pH conditions for the protein mixtures. Rehydration loading on basic strips leads to loss of proteins, which stay outside of the gel.

- Composition of the DeStreak rehydration solution: ***7 mol/L urea, 2 mol/L thiourea, 0.5% (w/v) CHAPS, 100 mmol/L DeStreak reagent, 1.25% (v/v) carrier ampholytes, 10% (v/v) glycerol, 0.002% Bromophenol blue.***

This solution can also be used for broad pH gradients, like pH 3–10 and pH 3–11.

Ready-made rehydration solution containing the DeStreak reagent and all other additives except the IPG buffer are available. This is called "DeStreak solution".

The IPG buffer must be chosen according to the used pH gradient.

For the SDS electrophoresis in the second dimension the IPG strips are equilibrated in the conventional way with SDS, Tris-Cl, urea, glycerol, and reduced with DTT, followed by alkylation with iodoacetamide.

Rehydration cassette In the original rehydration procedure, which is still in use in some laboratories, the strips are placed into a glass cassette, in order to control the gel thickness to prevent over swelling (see Figure 1.23). An additional U-shaped gasket cut from 0.2 mm thick film is added to achieve 0.5 mm thick gels on the 0.2 mm thick film support.

If this U-shaped film is not added, the pore size of the strips will be too small for high-molecular-weight proteins to enter the gel.

With this procedure many strips can be rehydrated under identical conditions. The disadvantages of the cassette method:

Some laboratories claim that they obtain the best results when they rehydrate the strips with this method.

- High volume of rehydration solution is needed.
- Cassettes sometimes leak because of the urea and the detergent in the liquid.
- Rehydration loading of different samples is not possible.

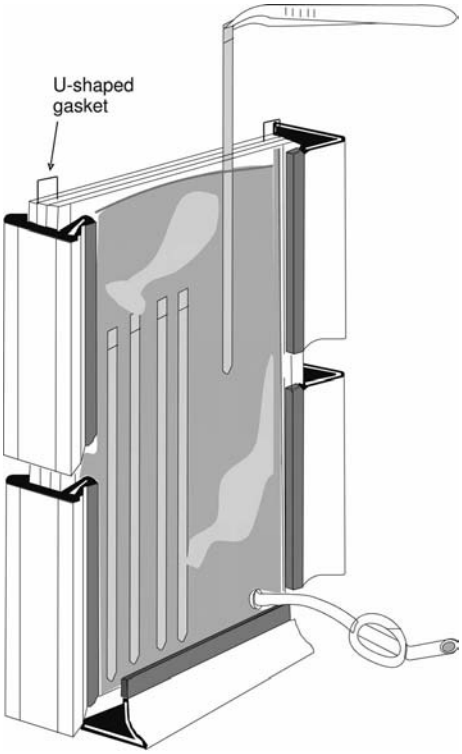


Fig. 1.23: Rehydration cassette with 0.5 mm gasket. Note the protruding ends of the U-shaped film added to achieve 0.5 mm thick gels on film supports.

Sanchez J-C, Rouge V, Pisteur M, Ravier F, Tonella L, Moosmayer M, Wilkins MR, Hochstrasser DF. Electrophoresis 18 (1997) 324–327.

Over-swelling of a strip results in liquid exudation during IEF. This would cause protein transport to the surface, resulting in background smearing.

And the separation time will be longer.

Reswelling tray The disadvantages of the cassette technique are avoided when using a reswelling tray (see Figure 1.24), as suggested by Sanchez *et al.* (1997). The liquid volume offered to the strips must be exactly controlled:

- If the liquid volume is too big, the strip will preferably reswell with the low molecular weight compounds and leave compounds with higher viscosity and higher molecular weights outside. There would not be any control over the reagent concentrations inside the strip.
- If the liquid volume is too small, the resulting pore size will be too small to allow high molecular weight proteins to enter the gel.

According to a theoretical calculation the volumes for the 3 mm wide and 0.5 mm thin strips would be 360 µL for a 24 cm and 270 µL for an 18 cm strip. In practice the optimal reswelling volumes are:

These values are empirical and have been determined and optimizes with practical experiments.

7 cm strip	125 µL
18 cm strip	340 µL
24 cm strip	450 µL

The solutions are pipetted into the grooves as streaks; the strips are placed on top of the solutions, and are covered with cover fluid (paraffin oil) to prevent crystallization of the urea. Reswelling is performed at room temperature, because the urea would crystallize in a cold room. Figure 1.24 shows a reswelling tray for up to 12 strips of different strip lengths up to 24 cm.

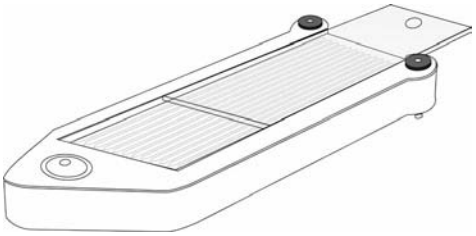


Fig. 1.24: IPG Dry strip reswelling tray for rehydration of IPG strips in individual grooves.

Cover fluid The gel strips are covered with *paraffin oil* during rehydration and IEF to prevent drying of the strips, crystallization of the urea, and oxygen and carbon dioxide uptake. It has never been observed that hydrophobic proteins change over from the rehydration solution into the oil phase.

Silicon oil is not recommended, because it can contain dissolved oxygen and it is not easy to remove from the instruments and the benches.

Rehydration time As mentioned above, there are two ways to apply the sample to these immobilized pH gradient strips rehydration loading and loading the samples on pre-rehydrated IPG strips.

Rehydration time:

Without sample	>6 h
Including sample	>12 h or overnight *)

**) The large protein molecules need a long time to diffuse into the strip.*

1.5.3.2 Sample Application onto IPG Strips

As shown in Figure 1.25, there are several ways to apply the sample to immobilized pH gradient strips:

- *Rehydration loading*. The extracted or solubilized sample is diluted with rehydration solution to the wanted amount of protein. Rehydration of a strip can be carried out in an individual strip holder or in a reswelling tray in an individual groove with a defined volume (see above, rehydration). The dry gel matrix takes up the fluid together with the proteins. Because the pores are very small in the beginning, the smaller molecules (water, urea, detergent, and reductant) go into the gel matrix faster than the proteins. Many of the proteins diffuse into the fully rehydrated gel later. This is the reason for the much longer rehydration time required for rehydration loading (see above). IEF can be performed with the gel surface up or down depending on the accessory or equipment used. Figure 1.29 shows the strip holder concept, where active rehydration (rehydration under low voltage) can be performed, and the voltage steps and gradients for IEF can automatically applied.

Note: the more diluted the sample, the easier the proteins enter the gel.

- *Cup loading*. The strip is pre-rehydrated with rehydration solution. The sample is applied into a loading cup either on the anodal or the cathodal end. Depending on the equipment a maximum of 100 µL or 150 µL sample can be applied. The optimal application point is critical, and has to be determined for each sample type and the gradient used. The proteins are transported into the strip electrophoretically. In this case IEF is always performed with the gel surface up. Between the electrodes and the IPG strip ends filter paper pads are applied. The electrode pads are soaked with distilled water and blotted with a tissue paper to become damp, not wet.

Sabounchi-Schütt F, Aström J, Olsson I, Eklund A, Grunewald J, Bjellqvist B. Electrophoresis 21 (2000) 3649–3656.

- *Paper bridge loading*. For application of very high sample volumes a modification of the cup loading principle has been proposed: paper bridge loading. Solution containing up to 5 mg protein can be loaded on an 18 cm long narrow pH

range IPG strip (Sabounchi-Schütt *et al.* 2000). A large sample volume requires that a large paper pad be applied at the other end of the IPG strip to absorb excess water. Figure 1.25 D shows the arrangement used when sample is applied to a paper bridge positioned between the anode and an IPG strip. The electrode pads are soaked with distilled water and blotted with a tissue paper to become damp, not wet. Sample solution is applied to the paper bridge (500 µL). With anodic application the anode is positioned as far out as possible in the electrode holder, while the cathode is positioned close to the end of the IPG strip to ensure good contact between electrode pad and IPG strip.

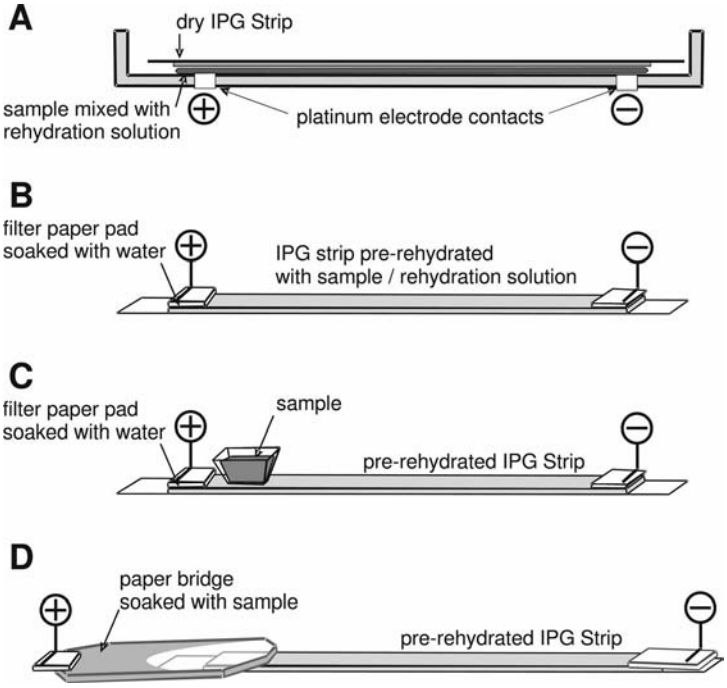


Fig. 1.25: Schematic representation of the different ways of sample application on an IPG strip: A. Rehydration loading in a strip holder for active rehydration and impress the voltage on it for IEF. B. Passive rehydration loading in a reswelling tray. C. Cup-loading for up to 150 µL sample solution. D. Paper bridge loading for up to 500 µL sample solution. For C and D anodal application is shown, for some samples and gradients it might be better to apply the sample at the cathodal end.

Kane LA, Yung CK, Agnetti G, Neverova I, Van Eyk JE. Proteomics 6 (2006) 5683–5687.

Kane *et al.* (2006) have reported that for basic pH gradients they get consistently better results with paper bridge loading than with cup-loading. Furthermore, the proteins with lower isoelectric points stay in the paper bridge and can be reloaded on acidic IPG strips – with application at the cathodal side. In this way it is possible to apply precious, but limited samples onto several gels with different pH ranges.

Note: ***The application point (anodal or cathodal) is of importance to obtain good results.***

See page 40: titration curves.

The way of sample loading will have an effect on the 2-D electrophoresis pattern. This is easy to understand: Each protein has an individually shaped titration curve; the mobility of the protein is usually different, depending on whether it is negatively or positively charged.

- Rehydration loading: Each protein exists in both ionic forms: negatively and positively charged, the proteins approach their pI from both sides with different mobilities.
- Cup or paper bridge loading: All proteins are charged in the same way, all proteins approach their pIs from the same side.

See Table 1,2 below, under "Pro, cup loading"

These facts can explain why some samples show better results with cup loading compared to rehydration loading.

The following table lists the advantages and disadvantages of the two methods.

Tab. 1.2: Comparison of rehydration and cup / paper bridge loading.

Rehydration loading	Cup loading and paper bridge loading
Pro	**Pro**
The proteins are distributed evenly over the gradient, precipitation at the sample application point cannot happen.	IEF in very acidic narrow gradients, in basic pH gradients, and HED containing strips work much better.
Rehydration, sample application and IEF can be combined to one single step, resulting in less manipulation steps.	The sample gets faster transported into the gel and becomes separated, thus the chances of protein interactions are reduced.
When the sample is diluted, a higher sample volume can be applied.	If necessary, the proteins can be loaded onto both ends of the strip in order to include proteins with pIs close to the pH of their application points.
Voltage can be applied during rehydration, which improves the entry of high molecular weight proteins.	For some sample types it is beneficial, when all proteins are charged in the same way, the molecules repel each other and do not form aggregates.

Rehydration loading	Cup loading and paper bridge loading
Con	**Con**
When basic gradients like pH 6–9, pH 6–11, or pH 7–11 are employed, rehydration loading leads to severe protein losses, because several proteins aggregate in basic environment.	Proteins with isoelectric points close to the pH of the application point have low mobility and solubility, they tend to precipitate on the surface and build a vertical streak in the second-dimension gel.
If DeStreak is added to the rehydration solution, the final concentration of DTT must not be higher than 1 mmol/L the use of reductant is thus limited.	The procedure is more laborious and takes longer time than rehydration loading.
The protein mixture is kept for many hours at room temperature, thus the danger of exposure to protease activities can increase.	Also when proteins are applied on both ends, proteins precipitate at the application points.
During the rehydration process the protein concentration outside the gel strip increases, which can lead to aggregation of proteins; those cannot enter the gel. Often they become visible in the 2nd dimension as horizontal streaks.	During the first phase of IEF the proteins become concentrated at the point of entry and can form aggregates and precipitate.

In several papers the statement can be found that higher protein loads can be applied with rehydration loading than with cup loading. This should be checked for each sample type, for some samples it might be vice versa.

A strategy for selection of the optimal procedure is described later.

Gels with high protein loads should always be run with the surface up, also when rehydration loading is performed, see page 95.

See page 97.

1.5.3.3 IEF Conditions

In general, separation conditions have to be adjusted to the nature and composition of the sample. Horizontal streaking in the 2-D pattern, for instance, can have many different reasons: overloading effects, too short focusing time, too long focusing time (some proteins became unstable at their pIs), oxidation of cysteines, too much salt, nucleic acids, phospholipids etc.

For some sample types optimization by a series of pre-experiments can be necessary.

Gel surface up or down Running IPG strips with the gel surface up – as shown in Figure 1.25B, C, D – has the advantage of employing filter pads at the gel ends, which take up salt ions and proteins with pIs lying outside the pH gradient of the IPG strip. Another benefit is the open surface just covered with paraffin oil: when highly abundant

This can reduce horizontal streaking considerably.

proteins form ridges, there will not be any mechanical pressure on them (see Figure 1.32).

Görg A, Postel W, Friedrich C, Kuick R, Strahler JR, Hanash SM. Electrophoresis 12 (1991) 653–658.

Temperature It is very important to run IEF at a defined temperature, even when the proteins are denatured. It had been demonstrated that spot positions of certain proteins can vary along the *x* axis of the gel dependent on the temperature (Görg *et al.* 1991). Running the strips at 20 °C is optimal, because it is above the temperature where crystallization of urea can be critical, and it is below overheating temperature, which can cause carbamylation of proteins during IEF. Even, when the applied electric power, the product of current and voltage, is very low in IPG strips, it is not sufficient to run IEF just in a thermostated room with 20 °C. Active temperature control is necessary to dissipate local heat developed at the ion fronts in the strips ("hot spots", see Figure 1.26). Aluminum oxide ceramics has the best thermal conductivity, and affords far the best temperature dissipation. It is therefore the preferable material for strip holders and manifold accessories.

Electric conditions and temperature control are closely related.

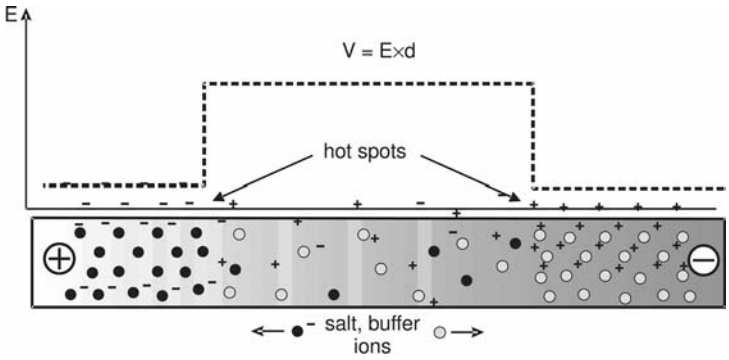


Fig. 1.26: Schematic representation of the distribution of salt and buffer ions in an IPG strip during IEF and the resulting electric field strength. The ion fronts are migrating slowly towards the electrodes. V = voltage, E = electric field strength, d = electrode distance.

Hot spots: In an IPG strip, there are always remains of salt and buffer ions present, which have not been washed out. They are bound to the buffering groups of the immobilized pH gradient. Additionally there are ions, which originate from ionic sample contaminants.

In IEF, all non-amphoteric compounds are transported out of the gradient towards the electrodes.

In the electric field they start to migrate towards the electrodes with the opposite signs. During the run they are forming visible ion fronts between the region with low ion concentration and the regions with high ion concentrations (Figure 1.26), which are moving towards the electrodes. Immobilized pH gradients have very low conductivity, because the buffering groups of the gradient are fixed and cannot dif-

fuse. Because of the differences in conductivity, the electric field strength in these regions is varying considerably. Punctual heat development can be observed at these fronts, so called "hot spots".

Electric conditions As already mentioned above, immobilized pH gradients have very low conductivity. The current is usually limited at 50 to 70 μA per strip. Higher current settings are not recommended, because the strips could start to overheat and burn in certain areas of the gradient. The strips should never be prefocused, because the higher conductivity produced by salt and buffer ions in the beginning is advantageous for sample entry and for the start conditions of proteins.

The electric conditions are controlled with the voltage setting. First low voltage is applied in order to avoid sample aggregation and precipitation in the loading cup or overheating in some strip areas. The voltage is slowly raised to reach electric field strength as high as possible in the focusing phase.

■ Note: ***The set current can limit the achievable voltage, when the sample contains too much salt, or when buffers are included in the rehydration or lysis solution.***

The voltage changes are either programmed in steps or with ramping, applying voltage gradients.

Often the instructions supplied with ready-made IPG strips suggest relatively high voltage start conditions, like 500 V for an hour. These settings can work well for various samples like rat liver extract or *E. coli* lysate, but there are a number of samples which require lower and slower starting conditions. Particularly samples with high contents of high molecular weight and hydrophobic proteins require a low electric field for a relatively long time in the beginning. This is true for all ways of sample application: rehydration, cup, and paper bridge loading.

When a new sample type is separated for the first time, it is highly recommended to apply the safest settings. In Figure 1.30 and in the practical part of this book (see page 314 ff) low voltage settings over longer time are suggested. They represent the "worst case" for unknown samples based on experiences by Burghardt Scheibe.

Burghardt Scheibe: personal communication.

The electric conditions change sometimes dramatically during the first phase as shown in Figure 1.27:

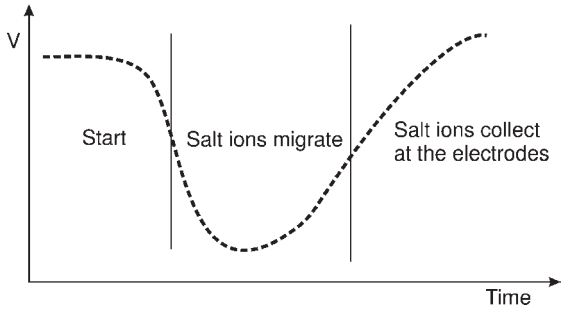


Fig. 1.27: Development of the actual voltage course during the first IEF phase in an IPG strip.

The separation can be speed up with applying higher electric field strength. For these high voltages the instruments have to be designed to comply with a high safety level.

Volt hours The complete voltage load is defined in volt hour integrals (Vh): the amount of voltage applied over a certain time. For example: 5,000 Vh (5 kVh) can be 5,000 V in one hour or 1,000 V over five hours.

- The Vh definition corrects the running conditions for different conductivities in different strips caused by different protein compositions and salt loads.
- In many cases increasing the voltage gradients (ramping) rather than in steps improves the result considerably. In order to apply comparable voltage loads, the Vh value is a good measurement.

Underfocusing When the applied volt hours are insufficient, not all spots are round. Horizontal streaks are produced instead. Higher volt hours loads are needed for samples containing a higher number of high molecular weight proteins, more hydrophobic proteins and for preparative runs.

These effects are result of too long time, not of too high voltage.

Overfocusing When the proteins are focused too long, some other negative effects can happen:

- Cysteines become oxidized, the pI of the protein changes.
- Some proteins become instable at their isoelectric point. The modified proteins or its fragments have different pI and start to migrate

again. The result: some horizontal streaks coming out from a few spots.
- Basic narrow gradients are particularly sensitive to over focusing. Additionally to possible protein breakdowns, the basic extreme (> pH 10) of the gel can become instable because of the very high pH.

For basic pH gradients the best results are obtained with a focusing phase as short as possible at a voltage as high as possible.

Should the optimal Volt hours have been achieved during the night or another time of no attendance, the separation will stop after the programmed value has been reached. Instead of applying a low "cruising voltage" no voltage should be applied on the strip. Prior to removal the strips from the strip holders the highest possible voltage is applied for 15 minutes, in order to refocus the slightly diffused bands.

Some manuals suggest applying a low "cruising" voltage of 500 V to maintain the band sharpness. This is not the optimal procedure.

Sample entry effects During the first phase of IEF with the cup loading procedure complex phenomena are observed:

Josef Bülles, personal communication.

- If the voltage setting in the beginning is low (100 V, 18 cm IPG strip), the proteins enter the gel, but a part of them precipitates or gets stuck within the first few cm of the strip, when the voltage is raised too quickly after this phase. As a result of this a vertical streak can be found in the second-dimension gel, which is displaced a few centimeters from the application point.

It should be continued to use low voltage during the first few hours.

- If the voltage setting for the start is high (like 500 V on a 18 cm IPG strip), a part of the proteins accumulate and precipitate on the surface of the application point and show a vertical streak in the second dimension. Sometimes the precipitated proteins at the application point are washed off during equilibration: and then no streak is visible.

In the second case the 2-D separation shows a clearer pattern than in the first.

These matters are critical: the voltage level during sample entrance and the beginning of protein migration, and the duration of the focusing phase at high voltage.

1.5.3.4 **Instrumentation**

The Multiphor Originally the IPG procedures were optimized on the Multiphor chamber (see Figure 1.28). With this equipment, rehydration can only be performed in the reswelling tray or a rehydration cassette outside the instrument. The separation is performed in a tray for up to twelve strips. Due to safety regulations the maximum voltage allowed is 3,500 V, because in the modular set-up power supply and cooling device are connected to the chamber via cables and tubing. The handling of the IPG strips and the entire IEF procedure were improved by developing a dedicated instrument for IEF in IPG strips for 2-D electrophoresis.

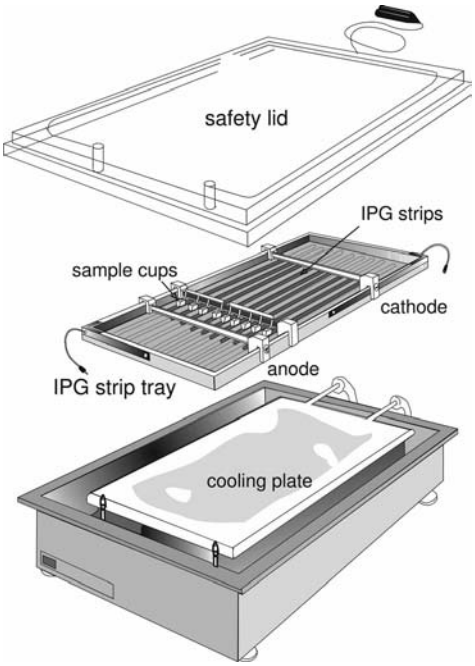


Fig. 1.28: Multiphor chamber with tray for IEF in IPG strips. An external programmable power supply for high voltages and a thermostatic circulator is required.

When everything is integrated in one instrument, much higher voltages can be applied than on modular systems with separate power supplies and thermostatic circulators.

The IPGphor In the instrument shown in Figure 1.29, rehydration and IEF can be combined. In this case the IPG strips are run facing down. The high thermal conductivity of the ceramics material is very important to remove the locally developed heat from some areas of the IPG strips efficiently. The programmable power supply, which generates up to 10,000 V, and a Peltier cooling system are integrated into the electrophoresis chamber.

The strip holders contain platinum contacts at fixed distances. The electric field is applied at the gel strips through these contacts, which are positioned on the respective contact area of the cooling plate. The anodal contact area is large to accommodate all different lengths of strip holders or – alternatively – a Manifold.

Up to 14 regular strip holders fit into the instrument.

At the back of the apparatus – in the older versions at the bottom – is a serial port for possible software updates and to connect a computer or serial printer to the instrument. In this way a report on the electric conditions is obtained after every five minutes of the run. With this interface the instrument can be integrated into the laboratory workflow system and allow procedures according to GLP (good laboratory practice).

For optimization of running conditions the report of the actual electric parameters is very useful.

It is not recommended to run strips with different lengths and different pH gradients at the same time. Also, the temperature setting of 20 °C should not be modified (see above).

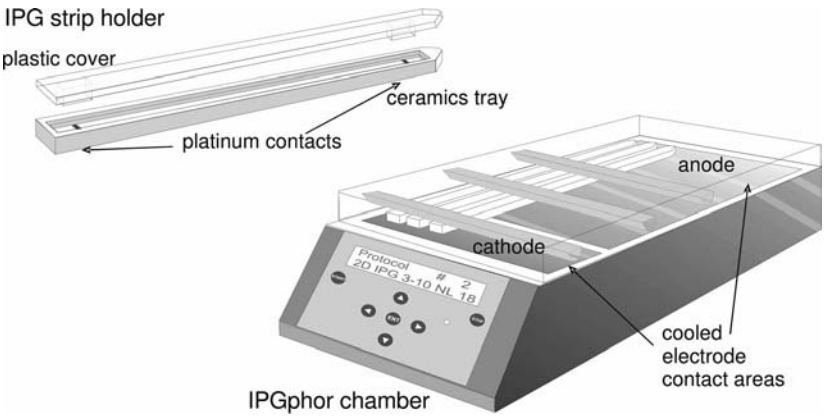


Fig. 1.29: IPGphor strip holder and IPGphor chamber for IEF in IPG strips.

Ideally the electric settings of the IPG strip IEF system are controlled and monitored by an external computer. In this way it is possible to see from the running conditions, whether the isoelectric focusing run will give good or bad 2-D results. Figure 1.30 shows two screen dumps of the programmed and monitored running conditions of two real IPG strip runs. In both cases the current was limited to 50 µg per strip. On the upper graph the really applied voltage followed exactly the programmed voltage, because all samples were well prepared, and thus the running conditions were never limited by maximum current. On the lower graph one or more samples contained too many salt ions: the high conductivity in one or more strips resulted in reaching the current limit several times. The second pro-

Monitoring the IPG strip runs is a valuable tool to control the quality of sample preparation. In some cases the graph can indicate that it is not worthwhile to continue with a second-dimension run. Therefore this function can save a lot of work time and material.

tocol indicates suboptimal isoelectric focusing conditions, which will result in disturbances in the 2-D gel pattern.

good run

bad run

Fig. 1.30: Monitored voltage and current graphs from two IEF runs in IPG strips. For more details see text.

Strip holders Figure 1.31 shows the procedure how to apply the sample and IPG strips. The trays, made from specially treated aluminum oxide ceramics are placed on the thermostated electrode contact areas of the power supply. Rehydration as well as the IEF separation is carried out at +20 °C.

Ceramics material is the optimum choice; plastic does not have a good thermal conductivity. Other materials are not usable, because they exhibit electric conductivity, bind proteins, or show EEO effects.

The advantages of strip holders with platinum contacts are:

- The number of manipulations and hands-on steps is reduced.
- Up to an entire day can be saved.
- Active rehydration loading can be performed under low voltage (30–60 V) for 10 hours, which facilitates the entry of high molecular weight proteins larger than 150 kDa into the strips (Görg *et al.* 1998).

In most laboratories this method has become the default procedure.

Görg A, Boguth G, Obermaier C, Harder A, Weiss W. Life Science News 1 (1998) 4–6.

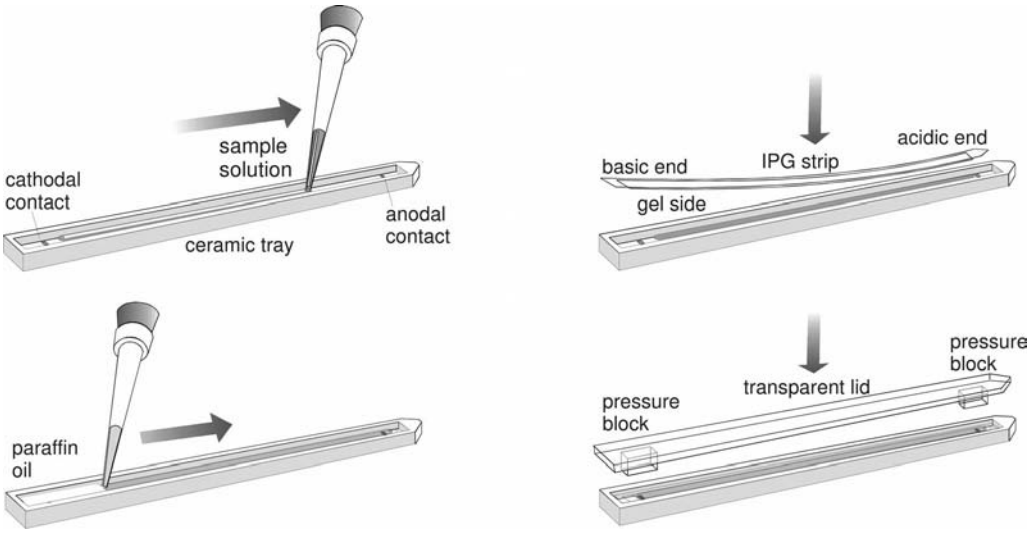


Fig. 1.31: Rehydration loading into IPG strips in individual strip holders of the IPGphor.

The pressure blocks on the cover lids hold the strips down, in order to maintain the contact also when electrolysis gas is produced during IEF. The bars of the safety lid of the chamber press the coverlids down. The most reproducible results are obtained when the acidic end of the strip is always placed in direct contact with the anodal end of the tray.

The distance between the platinum contact and the end of the tray is shorter at the anodal end.

Clean strip holders should be handled with gloves to avoid contamination.

Cleaning of the strip holders The strip holders must be carefully cleaned after each IEF run. The solutions must never dry in the strip holder. Cleaning is very effective, if the strip holders are first soaked a few hours in a solution of 2–5% of the specially supplied detergent in hot water.

Usually the strip holder slot is vigorously brushed with a toothbrush using a few drops of undiluted IPGphor Strip Holder Cleaning Solution. Then it is rinsed with deionized water.

SDS solution in absence of buffer has a neutral pH.

Sometimes protein deposits are left on the bottom of the strip holder after IEF. This happens when highly abundant proteins have been squeezed out of the gel surface at their isoelectric points (see Figure 1.32). It is not always easy to remove these proteins from the strip holder, particularly when they are sticky like serum albumin. In this case the strip holders should be boiled in 2% (w/w) SDS with 1% (w/v) DTT for 30 minutes before the slot is cleaned with the toothbrush.

- Important: ***Strip holders may be baked, boiled or autoclaved. But, because of the specially treated surface they must not be exposed to strong acids or bases, including alkaline detergents.***
- Note: ***The strip holder must be completely dry before use.***

For cup loading the gels are always run gel side up.

For most analytical applications this procedure works very well and delivers qualitative good and highly reproducible results. However, there are a few situations, where running the strips gel side up works better. In some cases cup loading delivers the best results (see also comparison on page 84 f).

Another advantage of running preparative samples with the surface up is that the electrodes are easier accessible for the insertion of filter paper pads between the electrodes and the strip. In this way salt and buffer ions as well as proteins with isoelectric points outside the pH range of the strip's gradient can be collected in the paper pads.

Preparative runs It has been observed that when the sample loads are increased from analytical (ca. 100 µg) to preparative (> 1 mg) amounts, the quality of the 2-D pattern decreases, when the strips are run with the surface down. This phenomenon can be caused by the increasing amount of some highly abundant proteins. In the end phase of IEF every focused protein forms a little ridge. These little ridges can be easily seen on the surface of the strips after IEF. When very high abundant proteins are focused, they form much higher ridges than the other proteins. In the case of regular strip holders the weight of the strips rests on these ridges, and the proteins are partly squeezed out. These proteins diffuse along the interface between the gel surface and the strip holder bottom (see Figure 1.32), and create a smear. When the strips are run with the surface up, no pressure is applied on the ridges. Thus, the results are better, when preparative runs are performed with the surface up, like in the Manifold (see below, Figure 1.33).

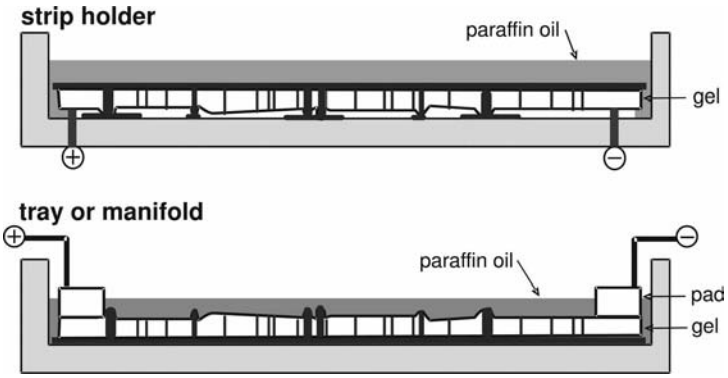


Fig. 1.32: Schematic representation of the running conditions for IPG strips with high protein loads. Comparison of runs with the surface down and with the surface up.

Narrow pH gradients High sample load also means high load of contaminants. This is particularly important for runs in narrow pH intervals. The advantage of narrow pH gradients is the high spatial resolution, but also the high loading capacity. Most of the protein load, however, will accumulate at the electrodes, because most proteins have isoelectric points outside the pH range of the strip. If these proteins cannot migrate out of the gel, they will precipitate there. Because they are charged, they can cause electroendosmotic effects: the resulting local water transport pushes non-precipitated proteins towards the gel center, which are visible as horizontal streaks at the lateral sides of the 2-D gel. As a remedy is suggested to apply filter paper pads soaked with water (damp, not wet) between the electrode contacts and the ends of the strip after the rehydration has been completed or a few hours before the end of the separation.

If the sample has to be electrophoretically desalted (see page 62) the filter pads have to be exchanged several times for new ones.

Manifold The manifold (Figure 1.33) is designed for being used with cup loading or paper bridge loading; but also IPG strips after rehydration loading in the reswelling tray. It is highly recommended to use a manifold made from aluminum oxide ceramics. Plastic material exhibits lower temperature conductivity, and is less efficient in heat dissipation. A few degrees difference between IPG strips can cause some protein spots to change positions, leading to false conclusions.

It cannot be used for rehydration of IPG strips, because the grooves are too wide.

- As already mentioned, for basic and very acidic gradients, and for DeStreak-containing IPG strips *cup loading* should be applied. The samples are applied at the area of the milder pH.

Active rehydration loading can be very useful, but it is only possible in the strip holders.

- High sample loads (>1 mg) often require runs with the gel surface facing up. Several ways of sample applications are possible:
  - *Rehydration loading* in the external reswelling tray (Figure 1.24);
  - *Rehydration loading* in strip holders;
  - *Cup loading*;
  - *Paper bridge loading*.
- Because of its geometry, up to 100 μA per IPG strip can be applied, which also facilitates preparative separations.

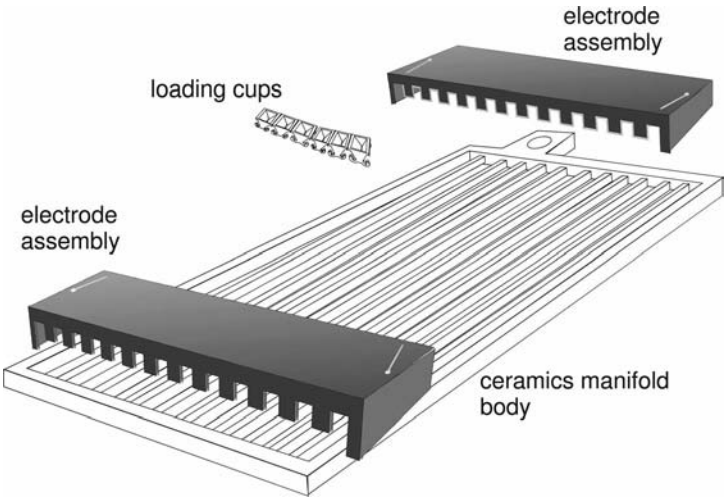


Fig. 1.33: Manifold for running up to twelve IPG strips with cup or paper bridge-loading. IPG strips containing already the sample from rehydration loading in the reswelling tray are run with the gel surface up.

The high volume of oil is also needed, when only a few strips are applied, because it should be avoided that some strips are run with a dry surface.

Protrusions along the grooves inside the Manifold align the rehydrated IPG strips, keeping them straight and centered when placed inside the manifold. Filter paper pads are soaked in deionized water and inserted between the electrodes and the IPG strips to trap salt ions, and those proteins, which possess pIs above and below the pH gradient. After the loading cups are put into position 100 mL paraffin oil is carefully poured into the Manifold, around the cups. This is a practical way to control whether there is a leaking cup. In the manual, which comes with the instruction, it is recommended to pour the oil in first; in this case it is very important to rehydrate the strips with sufficient Bromophenol blue.

The electrode assembly has electrode teeth on one side and hold-down teeth for paper bridge-loading on the other side. Therefore the length of the paper bridge is important (see also page 319 f).

Cleaning of the manifold Cleaning of the manifold is less critical than for the strip holder, because the gel surface and the proteins do not have direct contact with its surface. It is – like the strip holder – brushed with a toothbrush using a few drops of undiluted IPGphor strip holder cleaning solution. Then it is rinsed with deionized water. The loading cups can be reused for most of the samples after thorough cleaning with laboratory detergent.

1.5.3.5 Strategy for IEF Optimization

Start with very low voltage settings for a long time period!

In the following an example is given how to plan optimization work and to check the reliability of the analysis. Details on running conditions are given in the protocol *Step 3: Isoelectric Focusing*, page 317.

18 cm or 24 cm IPG strips should be used from the beginning. Shorter strips do not exhibit sufficient resolution.

Tab. 1.3: Strategy for IEF.

1	Wide gradient pH 3–10 linear or non-linear	Application of ca. 100 µg total protein by rehydration loading at 50 V for 12 hours.
2	Wide gradient pH 3–10 linear or non-linear	Rehydration loading in reswelling tray and IEF in cup loading strip holder.
3	Wide gradient pH 3–10 linear or non-linear	Cup loading on pre-rehydrated strip: one application at the anodal end and one on the cathodal end.
Decision point: selection of the best procedure		
4	Semi-wide gradients pH 4–7, pH 6–11, etc.	Way of loading is dependent on the results in the wide gradients; on basic strips.
5	Narrow gradients, like one pH unit	Way of loading is dependent on the results in the wide gradients.
6	Basic gradients	No rehydration loading; sample is applied at the anodal end.
7	Preparative runs	The strips must be run with the gel surface up.

In general it can be said that the Manifold is the more versatile accessory for the IPGphor.

The only downside is that active rehydration loading cannot be done in the reswelling tray.

Methodology check If the result is not satisfying – streaky and smeary pattern, only few or no spots – it is important to find out whether the problem is caused by inadequate sample preparation or by certain issues during the separation. The trouble shooting guide in the appendix of this book can be very useful here. But a complete run with a test sample – *E. coli* lyophilizate – is highly recommended.

Replicate gels are highly recommended for statistically reliable results.

Reproducibility check When the optimal conditions have been found, samples must be run in at least doublets or triplets, in order to check, whether observed pattern differences are caused by the noise of the system or by variations between different samples.

–20 °C to –40 °C are not enough: some proteins become modified.

After IEF Either the strips containing the focused proteins are equilibrated in SDS buffer and run on the second dimension right away, or they are stored at –60 °C to –80 °C in a deep-freezer.

Patestos NP, Fauth M, Radola BJ. Electrophoresis 9 (1988) 488–496.

Staining of an IPG strip (Acid violet 17) Sometimes it can be useful to check whether the separation in the first dimension worked well before all the work with the second-dimension run is started. Because of the high urea and detergent concentrations in the gel silver and Coomassie brilliant blue staining produce a dark background. The most sensitive technique is Acid violet 17 staining according to Patestos *et al.* (1988). The bands are visible after 40 minutes, during this time the proteins in the other strips can be kept focused with the application of a medium high voltage, like 2000 V.

Of course, the stained strip does no longer release the proteins into the second-dimension gel.

1.5.3.6 Measurement of the pI

The pH gradient in an IPG strip cannot be determined with a surface electrode, because the conductivity is too low. The calculation of the pH gradient based on the pK values and the concentrations of the Immobilines is rather complex, because the presence of urea and the running temperature has to be taken in account. The addition of protein standards is generally not recommended, because absolute purity of these proteins would be required, and adding additional proteins to a complex mixture can easily lead to errors in interpretation of the pattern.

Two different ways of pI measurement are proposed for 2-D electrophoresis.

Method 1. pH gradient graphs Graphs of operational pH gradient profiles are published in a data file by GE Healthcare Life Sciences (2003) as a basis for pI estimation. More information and larger

graphs can be found at this website: http://www.gehealthcare.com/lifesciences

These gradients are the calculated target pH gradients at 20 °C in presence of 8 mol/L urea. The pH values are plotted over the gel length given in per cent. Two examples are shown in Figure 1.34.

GE Healthcare Life Sciences (18-1177-60): Immobiline DryStrip gels (2004).

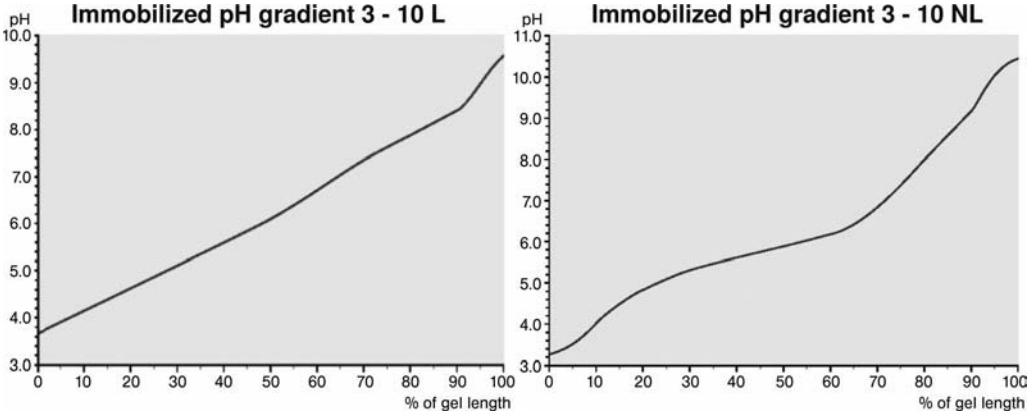


Fig. 1.34: pH gradients in IPG strips 3–10 and 3–10 NL. Downloaded from the following website: http://www.gehealthcare.com/lifesciences

The pI of a protein is estimated by relating the position of the protein in the SDS gel to its original position in the IPG strip, plotting the band position versus pH, and read out the pI. The strips do not contain pH plateaus at the ends: the graph must be aligned from one strip end to the other. Typical standard deviations for different batches of commercial IPG strips are given in the data file, mentioned above, and website.

Note: *Different strip lengths are used, and non-backed gels can swell or shrink.*

This procedure is usually carried out with the image analysis software as a "1-D calibration".

Method 2. Interpolation between identified sample proteins with known pI Prominent spots showing up in each 2-D map of a sample type can be analyzed for identification and amino acid sequence information. The theoretical pIs can then be used as keystones for interpolating the pIs of the other proteins. Bjellqvist *et al.* (1993) have successfully correlated the calculated pIs from the amino acid sequences of proteins with the protein position in immobilized pH gradients and put the procedure into practice for human cells (Bjellqvist *et al.* 1994).

Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez J-C, Frutiger S, Hochstrasser D. Electrophoresis 14 (1993) 1023–1031.

Bjellqvist B, Basse B, Olsen E, Celis JE. Electrophoresis 15 (1994) 529–539.

The second procedure is performed with the image analysis software as "2-D calibration".

This subject will be further explained in the chapter on image analysis of 2-D electrophoresis gels.

1.5.4 Second Dimension: SDS Electrophoresis

1.5.4.1 Gel Types

GE Healthcare Handbook. GE Healthcare Life Sciences 80-6429-60 (2005).

Homogeneous gels For 2-D separations mostly a homogeneous gel with 12.5% *T* and 3% *C* is used; the proteins of major interest in the size range from 10 to 100 kDa are optimally resolved. For special cases a table with optimal gel concentrations for different protein size ranges can be found in the GE Healthcare 2-D Handbook (2005).

Langen H, Röder D, Juranville J-F, Fountoulakis M. Electrophoresis 18 (1997) 2085–2090.

However, as demonstrated by Langen *et al.* (1997), it is not easy to predict the optimal acrylamide concentration according to a mathematical function: In an example shown in this paper a group of proteins with ca. 50 kDa, which were co-migrating in 9–16% *T* gradient gels were best resolved in a gel with 7.5% *T*. This shows that a try and error procedure must be applied for optimization, when certain protein groups need to be completely resolved.

Gradient gels Generally, in gradient gels the overall separation interval is wider; also the linear relation interval between the logarithm of M_r and the migration distance is wider than for homogeneous gels. Also the spots are sharper because the pore sizes are continuously decreasing. This effect can be very useful for separating proteins, which are highly glycosylated. However, when homogeneous gels are run under optimal conditions, resolution and spot definition is high enough: a gradient gel is not necessary.

Gradient gels have the following shortcomings:

- The preparation of gradient gels is much more work.
- It is more difficult to obtain reproducible gel properties than for homogeneous gels.
- The gels swell unevenly during staining, and show uneven background intensity.

Gyenes T, Gyenes E. Anal Biochem 165 (1987) 155–160.

Horizontal flatbed gels Because the IPG strip is just placed on the surface of the horizontal gel, a stacking gel with low acrylamide concentration is necessary for optimal protein transfer and separation (Gyenes and Gyenes, 1987). Resolving and stacking gel is usually polymerized in one piece. These gels can only be cooled from one side; rarely gels thicker than 0.5 mm are used. These gels are polymerized onto a support film.

Görg A, Weiss W. In Rabilloud T, Ed. Proteome research: Two-dimensional gel electrophoresis and identification methods. Springer Berlin Heidelberg New York (2000) 107–126.

Laboratory-made gels are usually run with liquid buffers, connected to the gel with paper wicks. The electrodes are then placed into the buffer tanks. The Laemmli buffers are used. Instructions and

recipes for preparing gels for flatbed systems in the laboratory can be found in several books: Görg *et al.* (2000) and Westermeier (2004).

Westermeier R. Electrophoresis in Practice. WILEY-VCH, Weinheim (2004).

Vertical gels For 2-D electrophoresis in vertical systems the stacking gel is not needed, because:

- The proteins are already pre-separated by IEF and will therefore not aggregate while they enter the resolving gel.
- The proteins migrate from a gel into another gel, and not from a liquid phase into a gel.

Still a discontinuous buffer system is employed: the gel buffer composition is different from the running buffer. The spots are well resolved, because there is a stacking effect happening between the highly mobile chloride in the gel and the lowly mobile glycine in the running buffer.

Therefore the upper running buffer must not contain any chloride ions.

Omitting the stacking gel solves a few technical problems and makes the procedure easier:

- The edge between the stacking and resolving gels contains incompletely polymerized acrylamide monomers and oligomers, which stick to proteins and partly modify proteins.
- Some proteins get caught between the stacking and resolving gels.
- One of the problems sources with reproducibility is abolished.
- Ready-made gels cannot contain a stacking gel with a buffer different from the resolving gel because of diffusion.
- The additional step of polymerizing a stacking gel short before the run is abolished.

The standard thicknesses of SDS slab gels run in vertical equipment are 1.0 mm and 1.5 mm. Thinner gels cannot be used, because the IPG strip including the film support is 0.7 mm thick, and it swells during equilibration with SDS buffer.

The IPG strip is placed onto the SDS gel sideways.

Tab. 1.4: Comparison of gel thicknesses.

Advantages of 1.0 mm gels:	Advantages of 1.5 mm gels:
Faster separations possible, leading to sharper spots	Higher loading capacity
Detection methods faster and more sensitive	Higher mechanical stability
Protein / gel volume ratio more favorable for tryptic digestions resulting in higher peptide yield	

1.5.4.2 **Gel Casting**

First of all, it is very important to clean glass plates and equipment very thoroughly. Mass spectrometry analysis is very sensitive, and every contamination will show up in the mass spectrogram and might lead to wrong results. Keratin stemming from hair and skin is the most frequently found protein contamination; sometimes even in reagents purchased from a supplier. It has become general practice in most proteomics laboratories to filter each monomer and buffer solution through a membrane filter before use.

The author of this chapter could write a detective story on pitfalls caused by dirty or old reagents.

Also for the SDS PAGE step only high-quality chemicals should be used. Particularly for such a sophisticated and multistep procedure like 2-D electrophoresis the search for trouble causing sources is very cumbersome and time-consuming.

When the sample proteins have been pre-labeled with fluorescent dyes, the gels are scanned inside the cassette. Therefore only low-fluorescent glass is used for DIGE experiments.

For accurate spot picking the gel have to be fixed to one of the glass plates, the glass plate without the spacers is therefore treated with Bind-Silane prior to assembling the cassettes. If the Bind-Silane treatment is performed long time before the cassettes are assembled, non-treated and treated glass plates should be kept separate. It has been observed that Bind-Silane molecules diffuse towards the non-treated glass surface and cause strong adherence of the gel to the second glass plate.

> ***Meticulously cleaned instruments and glass plates as well as high-quality reagents are of high importance.***

Gels for miniformat and medium size instruments can be cast as single gels (see Figure 1.35) as well as in multicasters, which are built similar to those used for large gels (see Figure 1.37).

For a high throughput method anyhow multiple gels are needed.

Large format gels, which are usually used in proteomics, can only be prepared in multicasters, because the glass plates of the cassettes would bend out and form a bulbous gel with a higher thickness in the center than along the edges.

For multiple gels it is practical to insert a little piece of paper with a key number for easy identification of the sample.

For the handling of multiple gels it is not very practical to use glass plates with separate spacers and clamps. Therefore the cassettes contain fixed spacers and a hinge on one side, as shown in Figure 1.36. However, when the gel is to be fixed to one of the glass plates with Bind-Silane for spot picking, non-hinged cassettes are used, with the spacers attached to the second glass plates.

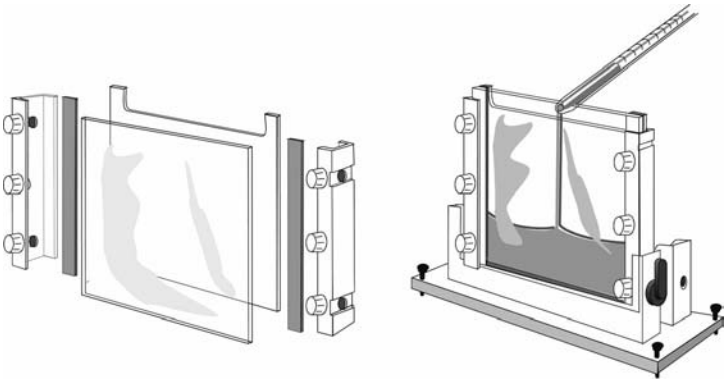


Fig. 1.35: Casting single miniformat and medium size SDS polyacrylamide gels for 2-D electrophoresis

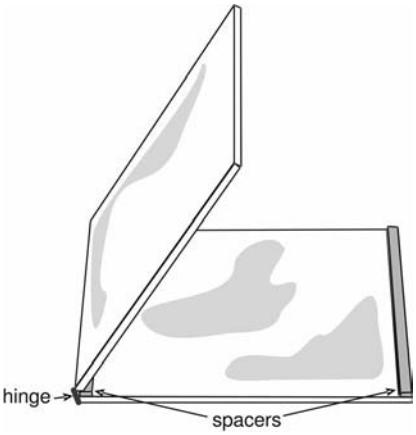


Fig. 1.36: Glass cassette for multiple SDS gel casters and instruments. Spacers are fixed; glass plates are connected with a hinge.

Depending on how many gels to be cast at a time, different types of multiple gel casters have been designed. During pouring the monomer solution into the caster the liquid will not only flow between the spacers, but also between the cassettes. For easy separation after the polymerization plastic sheets have to be inserted between the cassettes. Originally they are designed to introduce the monomer solution into the cassettes from the bottom. This is necessary when gradient gels are prepared.

Without the plastic sheets, the cassettes would stick together because of the polymerized gel layers.

As already mentioned, the polymerization process is an exothermic reaction. When multiple-gel casters are used, the composition of catalysts in the polymerization solution is more critical than for single gel

This practical hint is mentioned here, because some instructions incorrectly suggest the opposite procedure.

casting. If the polymerization runs too fast, very much heat is produced in the center of the multicasters, leading to thermal convection, which results in curved gel edges and irregular polymerization. It is very practical to add the TEMED to the other constituents already when the monomer solution is mixed together. It will not cause any start of polymerization. The ammonium persulfate, however, must not be added before pouring the gel.

Most of the stock solutions are anyhow kept in the refrigerator.

It must be avoided that the start of polymerization begins while the monomer solution is still flowing. Furthermore the multiple solutions in the cassettes need a certain time to level out following the principle of the communicating vessels. The monomer solution should be degassed with a vacuum pump and then precooled in a refrigerator at 4–8 °C. This measure reduces the heat development and delays the start of the polymerization. It has several advantages:

- The solution has time enough to level out between the cassettes and settle until the solution starts to polymerize: this prevents irregular gel composition and variation in gel lengths.
- Local overheating is avoided, which can result in local variations of sieving properties of the gels.
- Gels properties are more reproducible independent of the seasonal temperatures in the laboratory.

When the room temperature is higher than 20 °C, the multicaster should – like the monomer solution(s) – be pre-cooled in a cold room or a refrigerator.

For homogeneous gels it is quicker to fill the caster from the top by pouring the solution directly into the cassettes (see Figure 1.37A).

When alcoholic overlay solutions are used, they need to be removed an hour later, because they gnaw at the plastic surface of the caster box. SDS solution does not need to be removed.

Before the polymerization starts, 0.1% (w/v) SDS–water solution is sprayed into the cassettes with a finger-pumped plant sprayer to achieve straight upper gel edges (see Figure 1.37B). The very small detergent solution droplets glide down on the glass surface very quickly and will not mix with the monomer solution, because they are very small and have a low density. This procedure gives much straighter edges than pipetting water-saturated butanol or 70% (v/v) isopropanol into the cassettes, as it is proposed in most instructions. Pipetting produces larger drops, alcoholic solutions have a high affinity to the vinyl spacer material and can cause bent surfaces.

■ Note: ***It is very important to obtain a straight gel surface, because the IPG strip is placed on the gel edge-to-edge: in this direction the film support is absolutely rigid and cannot be bent to follow a curved edge.***

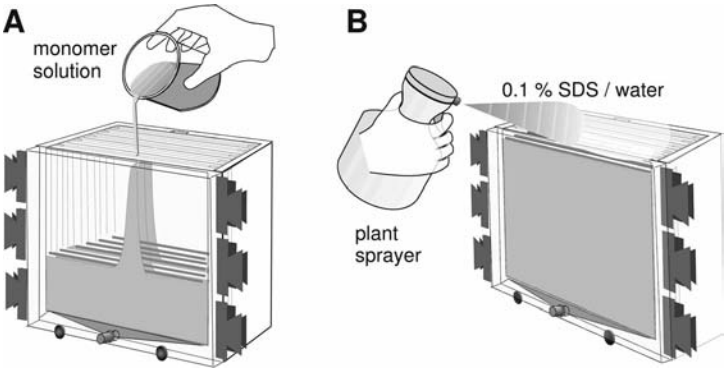


Fig. 1.37: Casting multiple homogeneous SDS gels.
A. Pouring the monomer solution directly into the cassettes.
B. Overlaying the edges by spraying 0.1% (w/v) SDS–water solution into the cassettes.

One should not forget that the quality and the age of chemicals, particularly the catalysts, greatly influence the polymerization efficiency of the monomer solution. For instance: The TEMED can be stable for several years, but it can suddenly decay within a week. Therefore it is advisable to replace the TEMED every year with a new bottle.

Note: The quality of the edge is influenced by the quality of the chemicals.

Gradient gels For the preparation of gradient gels a gradient maker has to be connected to the gel caster. Here there is no other way than casting the gels from the bottom. The gradient solution has to be delivered to the box slowly to avoid mixing – taking 5–10 minutes. The speed can be adjusted by using a laboratory peristaltic pump, or by selecting a certain level for the liquid beaker and reducing the diameter of the tubing with a pinchcock clamp (see Figures 4.4 and 4.6 on pages 334 and 341).

Using a pump means a lot of work. Everything has to be set up very quickly, because the casting and the overlaying procedure must be finished before the gel solution starts to polymerize. The casting procedure must be as exact as possible to prepare reproducible gels.

Gels for spot picking After the 2-D patterns have been compared with image analysis software, proteins of interest have to be identified and characterized. For these further analyses the gel plugs containing these proteins have to be cut out of the gel slab. For high accuracy, reliability, and automation of this procedure robotic spot pickers are employed (see pages 138 and 140 f). The most accurate and reliable way is to use the x/y coordinates of the respective spots from the image analysis results in the automatic spot cutter. It is, however, important that the gel does not change its shape by shrinking or swelling. Therefore gel slabs have to be fixed to a rigid film or glass plate backing.

It is not easy to produce these vertical gels on a film support in the laboratory, because the polyester films are flexible. To set up a casting system for film-supported gels would mean a big investment in equipment.

Gels fixed to a glass plate Because it is not easy to bind a gel to a glass plate after staining, gels are covalently bound to a film support or glass plate during polymerization. Laboratory made gels are usually polymerized onto a glass plate, which has been treated with Bind-Silane. Because staining solutions can diffuse into the gel only from one side, the gel should be as thin as possible for fast and efficient staining. These gels are usually 1.0 mm thick. Fluorescence labeled or fluorescence stained gels must be attached to non-fluorescent glass. Because this type of glass is very expensive, the gel layer is removed from the glass plate after all analysis has been done. When the glass plates are reused, the gel layer is removed with a plastic scraper. Remaining gel pieces disappear after vigorous treatment with a dishwashing brush.

Meanwhile a non-fluorescent support film material has been developed, which allows using ready-made gels for the combination fluorescent detection – spot picking.

It is very annoying, when the valuable sample is not separated well, just because of a little mistake occurring while preparing gels.

Ready-made gels for vertical systems Ready-made gels are more expensive than laboratory-cast gels. However, one should not forget that gel casting is a lot of work – particularly for cleaning the equipment – and working time costs money as well. Commercially produced gels are prepared according to GMP industry standards, and they are quality controlled.

There are two different concepts:

- *Gels in glass or plastic cassettes*. The handling does not differ from that of laboratory-cast gels. The glass cassettes are either sent back to the producer or they are disposed of.
- *Gels on film-support*. The 1 mm thick gels are inserted into specially designed re-usable cassettes (Figure 1.38). Conventional glass cassettes, as shown in Figure 1.36, cannot be used, because air pockets between the glass plate and the backing develop mechanical pressure on the gel, leading to an irregular front.

The sieving properties of a 12.5% T PPA gel are comparable to those of 14% T polymerized with a Laemmli buffer.

These 12.5% homogeneous polyacrylamide gels contain PPA-chloride pH 7.0 instead of the standard Tris-chloride buffer pH 8.8, in order to achieve a long shelf life. Because polymerization at pH 7.0 is more efficient than at pH 8.8, the gels have a very high mechanical stability, and the patterns are highly reproducible. Bis-Tris buffer containing gels are a practical alternative (see above). The gels are packed airtight in flexible aluminum bags.

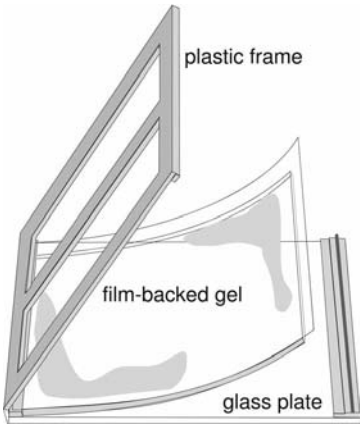


Fig. 1.38: Support cassette and ready-made film-backed vertical gel. The gel is shorter at the upper side to accommodate the IPG strip.

Kits with buffer concentrates and agarose sealing solutions are available. The kit for the film-backed PPA gels contains also a bottle with the gel buffer. The best way to insert the gel into the cassette is to apply a streak of 1 mL of the gel buffer onto the glass plate along the spacer of the closing side, and placing the gel on the glass plate first touching the buffer streak with the respective edge. The gel position can be easily adjusted that the edge at this side is in contact along the entire spacer and the lower gel edge is flush with the edge of the glass plate. When the gel is lowered on the rest of the glass plate carefully, no air bubbles are caught between gel surface and glass plate. The liquid near the closing side and some air bubbles – mostly close to the hinged side – are squeezed out with a roller.

Of course, also these cassettes have to be thoroughly cleaned before and after each use to avoid contaminations.

A liquid layer between gel surface and glass plate has to be avoided. It would cause blurred spots.

A narrow gap of less than 1 mm remains between one spacer and the gel edge: this will be sealed with hot agarose solution after the IPG strip has been inserted.

For Western blotting analysis or for high sensitivity fluorescent staining the support film has to be removed. It can be cut off with a simple instrument using a steel wire (see Figure 1.39). The large format gels are easy to handle also without the film-backing because they are well polymerized.

Note: *For those who have only used film-backed gels, handling of non-backed slab gels requires some practice and skill.*

This procedure works only with flexible support films, not with rigid, Bind-Silane treated glass plates.

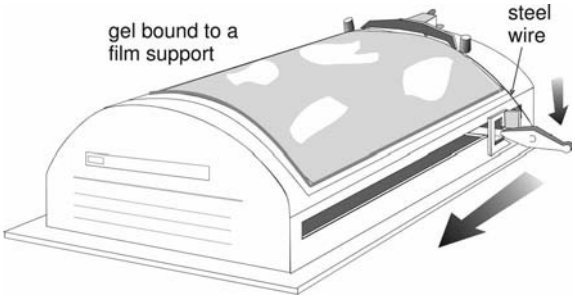


Fig. 1.39: Film remover for complete removal of a flexible film-backing from the gel.

1.5.4.3 Instrument and Gel Setup for the Second Dimension

Figure 1.40 shows a Multiphor flatbed chamber used as SDS PAGE instrument and a mini vertical electrophoresis system for miniformat gels. These two instrument types are not for high throughput and thus mostly employed for optimization work.

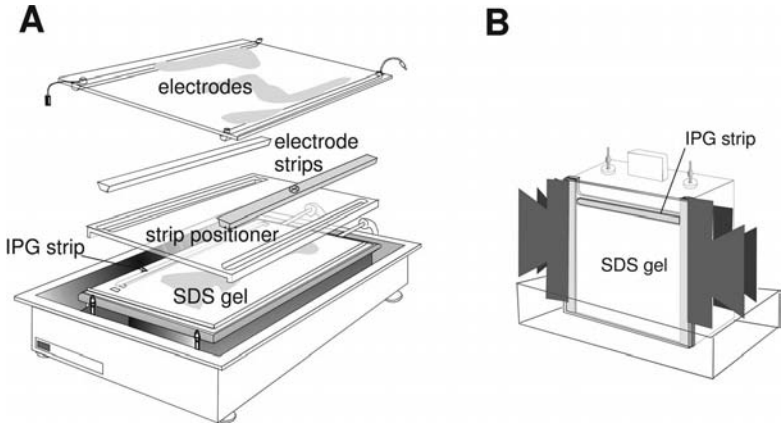


Fig. 1.40: Flatbed and vertical mini electrophoresis apparatus for SDS PAGE.

In the flatbed apparatus the standard gel size is 25×19 cm. The electrode distances can be adjusted to shorter separation distances. For example, SDS electrophoresis of three miniformat IPG strips with 7 cm length can be run together in one gel under identical conditions (see Figure 1.41).

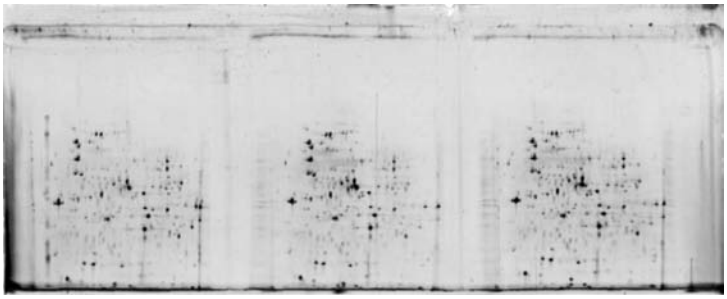


Fig. 1.41: Three miniformat 2-D separations run in one flatbed gel. IEF in three 7 cm IPG strips 4 to 7, SDS PAGE in a 25×11 cm gel with a stacking zone of 5% *T* and a homogeneous resolving area of 12.5% *T*, 0.5 mm thick. Silver staining.

Ready-made gels for vertical miniformat chambers are available from several suppliers. An alternative is to run also several short IPG strips together on a larger vertical gel. Some vertical systems can also be equipped with shorter glass plates.

Tab. 1.5: Comparison of the properties of flatbed and vertical systems.

Flatbed Systems	Vertical Systems	
One gel per instrument.	Multiple gels runs are possible.	
Can also be used for IEF in IPGs.	Dedicated for SDS electrophoresis.	
Gel thickness is limited, because cooling is only possible from one side.	Higher protein loading capacity, because thicker gels can be used, which are cooled from both sides.	*Up to 3 mm thick gels can be used in vertical systems*
Very versatile for different gel sizes and methods.	Gel sizes fixed by glass plate sizes.	
Thin layer gels can be used, easy application of the IPG strips.	Thin gels cannot be used, because the IPG strip would not fit between the glass plates.	*Very thin gels show higher sensitivity of detection, they are easier and quicker to stain.*
Buffer strips (polyacrylamide or filter paper) can be used instead of large volumes of liquid buffers.	Blotting is easier because of higher gel thickness.	*With flatbed systems there is reduced chemical and radioactive liquid waste.*
The IPG strip has to be removed from the SDS gel after 40 minutes because of EEO effects.	The IPG strip does not have to be removed.	*EEO occurs in both systems, therefore urea, glycerol in the equilibration buffer and low initial voltages are needed.*

Görg *et al.* (1995) have performed a systematic comparison of results, obtained with a flatbed and a vertical system for the same

Görg A, Boguth G, Obermaier C, Posch A, Weiss W. Electrophoresis 16 (1995) 1079–1086.

sample: The spot patterns are very similar. It is demonstrated that sharper spots are obtained in thinner gels, which can be employed in the flatbed system, but not in a vertical setup.

Design of vertical instruments The following functions are needed in the instrument for the second dimension:

- High throughput;
- Large gel cassettes;
- Efficient cooling for fast runs with straight front;
- Leakage free;
- Reproducible results independent from gel position;
- Different gel thickness should be possible;
- Easy handling;
- Buffer consumption not too high.

Anderson NG, Anderson NL. Anal Biochem 85 (1978) 341–354.

The first high-throughput chamber had been developed by Anderson and Anderson (1978): the DALT box, where the gel cassettes are tilted by 90 degrees and inserted between insulating rubber flaps (see Figure 1.42). In contrast to conventional vertical systems, the migration direction of the proteins is from left to right instead of from the top to the bottom. Handling of the instrument is easy. As only one single tank is used, there is no problem of buffer leakage from an upper to a lower tank.

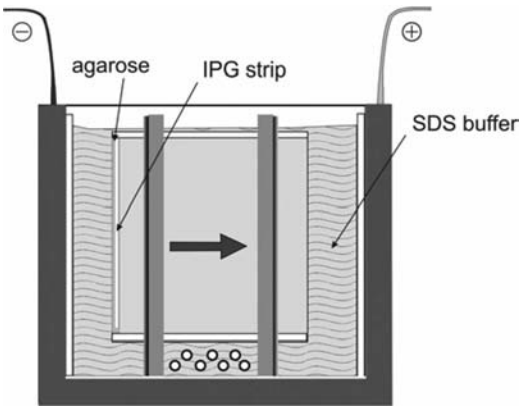


Fig. 1.42: Schematic drawing of the traditional DALT chamber for multiple SDS PAGE runs. The migration direction is from the left to the right. Insulating rubber flaps prevent current flow between the cassettes.

The buffer volume of 20 L is rather high.

The gels are usually run overnight, because the cooling efficiency is limited. The entire box is filled with Tris-glycine buffer. The buffer

should not be used more than once, because it will become enriched with chloride ions from the gel buffer. When the Tris-glycine buffer contains chloride ions, the initial stacking effect is disturbed – resulting in a loss of resolution – and the running time is extended.

A limitation is the design of the buffer tank: the cathodal and anodal buffer reservoir is identical. This makes peptide separations with Tris-tricine buffer very expensive. And – the stable PPA buffer system cannot be applied, because it would require two completely separate tanks for the anodal and the cathodal buffer.

For vertical instruments with completely separate tanks, several construction concepts are possible. Figure 1.43 shows three different designs.

When the buffer tanks are completely separate, the anodal buffer can be used repeatedly, because chloride ions do not disturb there.

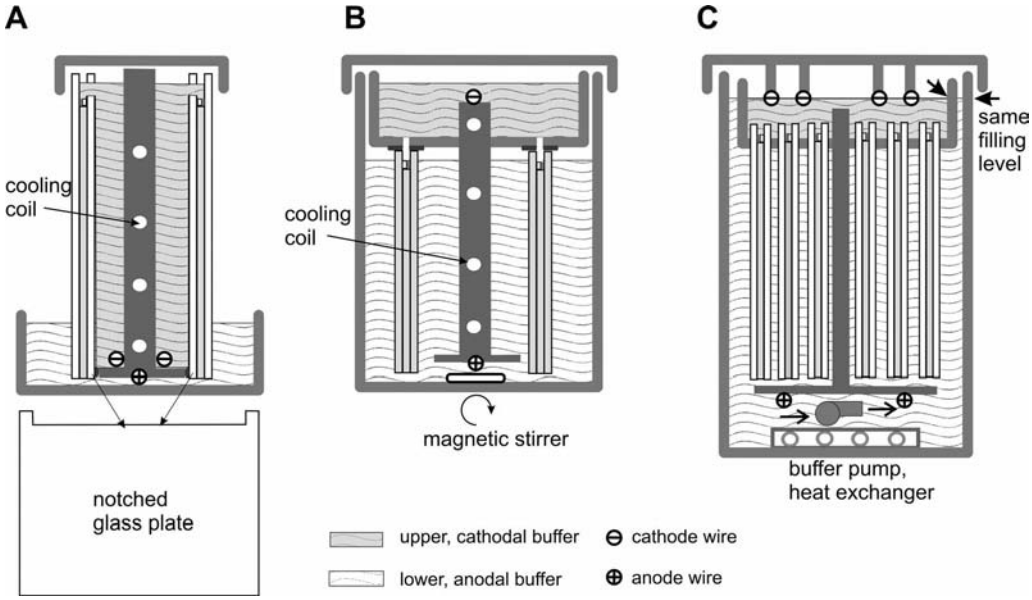


Fig. 1.43: Three different designs of vertical chambers. All of them can be cooled.
A. Buffer-backed chamber.
B. Chamber with tight upper buffer tank.
C. Chamber for fast multiple runs.

Vertical electrophoresis chambers

- In the buffer-back chambers (Figure 1.43, type *A*) the gels are cooled via the cathodal buffer. Notched glass or aluminum oxide ceramics plates are used to enable the contact between the cathodal buffer and the gel.

Maximal two gels can be run; therefore the principle is mostly applied on miniformat apparatus.

In this concept the edges of the glass plates must be absolutely intact to prevent leakage of the upper tank.

- The medium sized chamber with tight upper buffer tank (Figure 1.43, type *B*) can accommodate up to four gels at the same time, when notched glass plates are inserted between each two gel pairs. The gels are cooled via the anodal buffer.
- Multiple runs with large gels can be performed in chambers of type *C* in Figure 1.43. It is, however, important to fill upper and lower buffer to the same level to avoid buffer mixing. The heat removal in this chamber type is very efficient, because the cooling anodal buffer is vigorously pumped around in the lower tank.

The buffer concentration for the upper tank must usually be doubled to prevent depletion of the cathode buffer.

The concept of pumping the anodal buffer around the cassettes is employed for large gel systems: stand-alone instruments (Figure 1.44A) and in integrated systems for running up to 12 gels (Figure 1.44B).

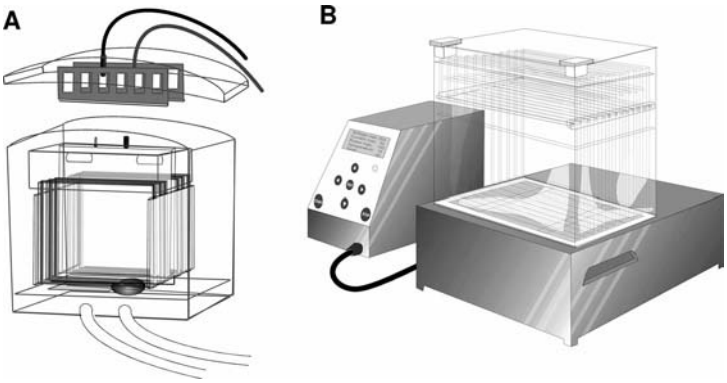


Fig. 1.44: Electrophoresis instruments for large cassettes.
A: Stand-alone instrument for up to six gels.
B: Integrated apparatus Ettan DALT *twelve* for multiple 2-D PAGE. The programmable power supply controls also the Peltier cooling system and the pump for circulating the lower buffer.

At the rear of the separation unit of the integrated system sits a draining valve for convenient removal of used buffer. At the back panel of the control unit of the Ettan DALT *twelve* there is a serial port for possible software updates and to connect a computer or serial printer to the instrument for a report on the electric conditions after every five minutes of the run. With this interface the instrument can

be integrated into the laboratory workflow system and allow procedures according to GLP (good laboratory practice).

■ Note: ***for optimally reproducible 2-D patterns all gels of an experiment should be run together in one chamber.***

Running gels of one experiment in different chambers makes spot matching more difficult.

The following hint goes for all vertical chambers with separate tanks: When the lower – anodal – buffer is mixed with the used upper – cathodal – buffer after each run, the Tris concentration is high enough for repeated use as anodal buffer. This saves work and reagent costs. Of course, the cathodal buffer must be new for each electrophoresis.

The Tris moves from the anodal to the cathodal buffer. Glycine moves from the cathodal to anodal buffer, it is not needed in the anodal buffer.

1.5.4.4 **Equilibration of IPG Strips**

Prior to the run in the second dimension the strips have to be equilibrated with SDS buffer to transform the focused proteins into SDS–protein complexes, which are completely unfolded and carry negative charges only. The following conditions have been optimized by Görg *et al.* (1987b).

Görg A, Postel W, Weser J, Günther S, Strahler JR, Hanash SM, Somerlot L. Electrophoresis 8 (1987b) 122–124

■ Equilibration stock solution: ***2% (w/v) SDS, 50 mmol/L Tris HCl pH 8.8, 0.01% (w/v) Bromophenol blue, 6 mol/L urea, 30% (v/v) glycerol***

Equilibration is performed twice on a shaker:

15 min 10 mL equilibration stock solution	Plus 1% (w/v) DTT
15 min 10 mL equilibration stock solution	Plus 2.5% (w/v) iodoacetamide

Sodium dodecyl sulfate With the amount of 2% SDS there is usually sufficient SDS for preparative protein loads. Some laboratories use 3–6% SDS to facilitate the migration of focused hydrophobic proteins.

Tris HCl buffer pH 8.8 In former protocols it was proposed to add the stacking gel buffer pH 6.8, because the use of a stacking gel was standard procedure. However, it is better to add the more basic resolving gel buffer with a pH of 8.8 to improve the alkylation of the cysteines.

Note: with iodoacetamide the solution can easily become acidic. This would disturb the formation of the SDS–protein complexes.

Bromophenol blue This tracking dye allows a control of the running conditions, the shape of the migration front, and the separation time.

Urea and glycerol These additives have been introduced to keep electroendosmotic effects (see above) as low as possible. Urea is also supporting the solubility of hydrophobic proteins.

This is valid also after running IEF with DeStreak.

Dithiothreitol After the proteins have been focused they have to be treated with the reductant again. When very high protein loads are analyzed, the concentration of DTT needs to be increased.

Iodoacetamide The alkylation agent has several functions:

- Complete alkylation of the cysteines, to avoid partial modification by acrylamide for increased spot sharpness and improved protein identification with mass spectrometry.
- Elimination of point streaking as described by Görg *et al.* (1987b).
- Avoidance of the artifactual horizontal lines across the SDS gel in the size range of 40–50 kDa.

The iodoacetamide functions as scavenger of the excess reductant.

When the cysteines had been pre-labeled with saturation dye for DIGE, the iodoacetamide step is omitted.

Equilibration time Two times 15 minutes seems to be a rather long time, and some might fear considerable losses of proteins due to diffusion. As already mentioned on page 51, immobilized pH gradients keep proteins back like a weak ion exchanger. Thus, only a little amount of proteins – those from the surface – are washed out.

However, the equilibration time must not be extended too far, because this leads to losses of low molecular weight proteins due to diffusion.

The long equilibration time is necessary for the complete formation of SDS–protein complexes, because the negatively charged SDS is repelled by the negative charges on the carboxylic groups of the strips. It has been observed that a too short equilibration leads to vertical streaks and losses of high molecular weight proteins.

Hedberg JJ, Bjerneld EJ, Cetinkaya S, Goscinski J, Grigorescu I, Haid D, LaurinY, Bjellqvist B. Proteomics 5 (2005) 3088–3096.

Modified equilibration Although Hedberg *et al.* (2005) claim that after running the first dimension with DeStreak under oxidative condition equilibration can be shortened and both, equilibration and the second-dimension run can be performed under oxidative conditions, it is not recommended. This procedure is limited just to a few pH gradients. Practice has shown that this method does not give well resolved spot patterns for all sample types.

McDonough J, Marbán E. Proteomics 5 (2005) 2892–2895.

There are cases where increased SDS content facilitates the transfer of hydrophobic proteins from the first to the second dimension. One example is described by McDonough and Marbán, where 10% (w/v) SDS in the equilibration buffer was the only way to move a highly hydrophobic protein into the second-dimension gel.

1.5.4.5 Transfer of the IPG Strip and Running the SDS Gel

In order to make examination and evaluation of 2-D maps easier, the first and second dimensions should always be set together in a standardized way. The majority of the scientific world agrees that it makes sense to orientate the gel in the style of a Cartesian coordinate system: the low values are located at the left bottom and the high values at the right top.

The IEF gel should always be placed on the SDS gel in a standard orientation: low pH to the left, high pH to the right.

Flatbed system It is highly recommended to use the strip positioner plate as shown in Figure 1.40A for:

- Improved alignment of the electrode strips;
- Preventing the electrode strips to slide away;
- Reproducible positioning of the electrode strips and the IPG strip on the SDS gel;
- Achieving a straight front, because the gel surface is covered.

The gels are placed directly on a cooling plate, which is connected to a thermostatic circulator. Usually the running temperature is 15 °C. After equilibration of the IPG strip, the excess buffer is removed by blotting it slightly with clean filter paper. The strip is placed – gel surface down – parallel to and 5 mm apart from the cathodal electrode strip. The acidic end of the strip points to the left side.

No agarose sealing solution is needed.

M_r marker proteins are applied with IEF sample application pieces as shown in Figure 1.45.

After 40 minutes of low voltage – for sample entry – the IPG strip is removed from the gel; the cathodal electrode strip is moved from its original position to the contact area of the IPG strip. This measure prevents drying out and burning of the SDS gel along this contact line. The separation can now be continued with higher voltage settings.

In the flatbed system EEO has stronger effects, because the water is transported out of the SDS gel surface, causing drying of the SDS gel.

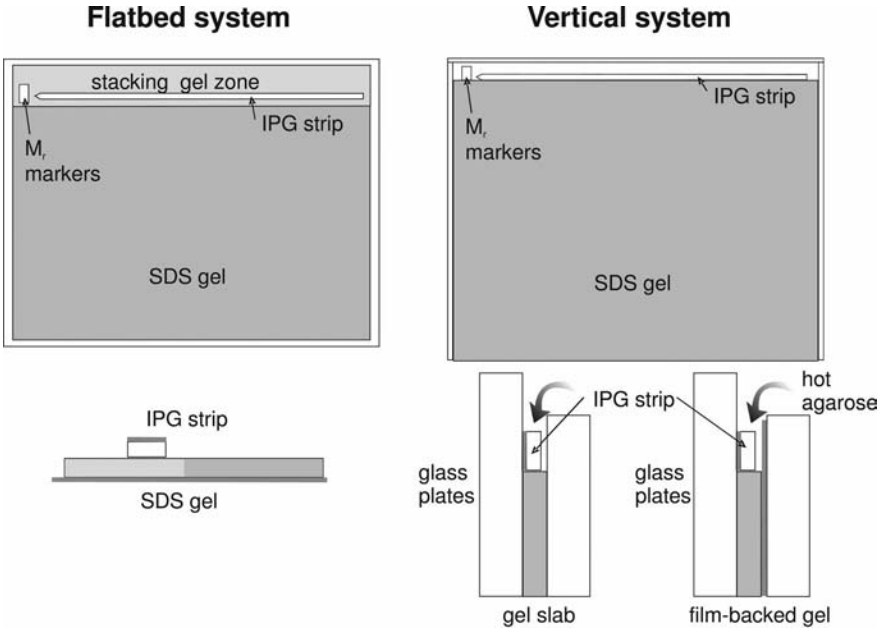


Fig. 1.45: Application of the IPG strip on the different types of second-dimension gels.

Ca. 2 mL of agarose embedding solution is necessary per gel.

Vertical system The IPG strip is placed onto the edge of the SDS gel sideways, with its support film touching one glass plate. Hot agarose embedding solution, pipetted onto the strip has several functions:

- Achieve a gel continuity;
- Prevent air bubbles between the gels;
- Avoid floating of the strip in buffer solution;
- Reduce electroendosmotic effects at very high and very low pH values of the IPG strips;
- Seal the gaps between cassettes spacers and the ready-made gels.

Note: ***The agarose solution should not be hotter than 60 °C; it could cause carbamylation of some proteins because of the presence of urea.***

Composition of the agarose embedding solution: ***0.5% (w/v) agarose, SDS cathode buffer (1× concentration), 0.01% (w/v) Bromophenol blue***

Molecular weight marker proteins.

Tip: ***Best results are obtained when the molecular weight marker proteins have the same start conditions like the proteins from the IPG strips: a IPG strip is rehydrated in the marker protein solution, cut into small pieces, which can be stored in a deep freezer or applied directly to the SDS gel together with the IPG strips of the first dimension.***

The alternative is to apply the markers to an IEF sample application piece directly, in a volume of 15–20 µL and let it dry.

1.5.4.6 Running Conditions for Vertical Gels

Electric conditions Also vertical gels must be run with low voltage at least for the first two hours to reduce the electroendosmosis effects (see page 21). When immobilized pH gradient gels are equilibrated in basic buffers, they become deprotonated: The carboxylic groups become negatively charged, the amino groups are neutral. Thus the IPG strips acquire negative net charges during equilibration with the SDS buffer. It has been observed already during the first approaches utilizing immobilized pH gradients in 2-D electrophoresis that this can cause electroendosmotic effects (Westermeier *et al*. 1983). Those result in protein losses due to a water flow towards the cathode. The water transport is minimized by adding urea and glycerol to the equilibration solution and reducing the electric field during the phase of protein transfer into the second-dimension gel (Görg *et al*.1985, 2003):

Westermeier R, Postel W, Weser J, Görg A. J Biochem Biophys Methods 8 (1983) 321–330.

Görg A, Postel W, Günther S, Weser J. Electrophoresis 6 (1985) 599–604.

Görg A, Drews O, Weiss W. In Simpson RJ, Ed. Purifying proteins for proteomics: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2003) 391–430.

- ***Phase 1: apply only 5 mA (≈ 0.2 W) per gel for 2 hours***
- ***Phase 2: continue with normal SDS electrophoresis settings for fast or overnight runs***

In discontinuous buffer systems, first the conductivity is high, because the gel contains a lot of chloride ions, which have high mobility. When more of the glycine or tricine ions – with low mobilities – are migrating into the gel, the conductivity decreases, and the field strength increases. In order to avoid the production of too much Joule heat at the end of the run, the gels are usually run at low current settings. A possibility to speed up the separation is to adjust the current setting from time to time.

Ideally the power supply offers the feature to run the separation at constant power. Then the current is high at the beginning and low at the end without overheating the gels and the buffers. In this way a separation can be run faster than under conventional conditions.

Temperature Multiple gels produce a substantial amount of Joule heat, which has to be removed. If this is not efficient enough the gels show the "smiling effect": faster migration in the center than on the lateral sides. It was the general opinion that SDS gels should be cooled to 10–15 °C to remove the heat efficiently enough to obtain a straight front. Systematic studies in chambers with more powerful buffer circulation have shown that better results and faster runs are obtained when the gels are run at 25 °C. Of course there are also certain limits how much temperature can be removed from the gels and the buffers.

The high settings are applied after the 2 hours protein transfer.

Benefits of quick runs Fast separations of large gels – within a few hours instead of overnight – result in less diffused spots compared to overnight runs. This is particularly obvious in the low molecular weight area. Less diffused spots have three advantages:

Less need for gradient gels.

- Increased spatial resolution in homogeneous gels;

In comparative experiments over 50% more spots could be detected in these gels.

- Increased detection sensitivity;

Because of the higher protein concentration in the gel plug.

- Improved enzyme kinetics during tryptic in-gel digestion.

1.5.4.7 Molecular Weight Determination

For the determination of molecular weights in 2-D electrophoresis gels two different procedures are used.

Method 1. Co-running molecular weight standard proteins Molecular weight marker proteins can be applied as a separate track to the 2-D gel. The molecular weights of the proteins in the 2-D map are then interpolated with the molecular weight curve obtained from the positions of the marker proteins. This procedure is usually carried out with the image analysis software as "1-D calibration".

The problem with this method is its limited accuracy. The markers have to be applied at the lateral sides of the gels. Often the space is very limited and the bands are curved because of the edge effects in SDS PAGE. Furthermore, the estimation of molecular weights with SDS PAGE is not accurate at all.

Method 2. Interpolation between identified sample proteins with known M_r values Prominent spots showing up in each 2-D map of a sample type can be analyzed for identification and amino acid sequence information. The theoretical M_r values can then be used as keystones for interpolating the M_r values of the other proteins. The second procedure is performed with the image analysis software as "2-D calibration". This method is much more exact than the method described above.

1.5.5 Detection of Protein Spots

There is a demand list of properties required for the ideal spot detection technique in 2-D gels in proteomics: it should:

- Be sensitive enough for low copy number proteins;
- Allow quantitative analysis;
- Have a wide linearity;
- Have a wide dynamic range;
- Be compatible with mass spectrometry;
- Be non-toxic;
- Be environment-friendly;
- Be affordable.

Unfortunately there is no method that affords all these features together. Lately some reviews on detection techniques in proteomics have been published: a "minireview" by Westermeier and Marouga (2005), and a very elaborate compilation by Miller *et al.* (2006). The following table gives an overview over currently used detection principles and their features. Several different protocols exist for most of these methods. Those of major importance are quoted in the table.

Westermeier R, Marouga R. Bioscience Reports 25 (2005) 19–32.

Miller I, Crawford J, Gianazza E. Proteomics 6 (2006) 5385–5408.

1.5.5.1 Comparison of Detection Methods

Tab. 1.6: Detection methods for protein spots.

Method	Advantages	Disadvantages	
Coomassie brilliant blue staining (colloidal)	Steady state method, good quantification, inexpensive, mass spectrometry compatible	Medium sensitivity: LOD ca. 20 ng of BSA; the more sensitive, the slower	*Neuhoff V, Arold N, Taube D and Ehrhardt W. Electrophoresis 9 (1988) 255–262.*

	Method	Advantages	Disadvantages
Jochen Heukeshoven, personal communication.	Coomassie brilliant blue staining (alcohol free, hot, monodisperse)	Steady state method, fast, good quantification, inexpensive, very environment friendly, mass spectrometry compatible	Low sensitivity: LOD only ca. 100 ng of BSA, background destaining necessary
Fernandez-Patron C, Castellanos-Serra L, Hardy E, Guerra M, Estevez E, Mehl E, Frank RW. Electrophoresis 19 (1998) 2398–2406.	Zinc imidazol reverse staining	Medium sensitivity: LOD ca. 15 ng of BSA, fast, very good compatible with mass spectrometry	Bad for quantification, negative staining not easy for documentation
Heukeshoven J, Dernick R. Electrophoresis 6 (1985) 103–112.	Silver staining (silver nitrate)	High sensitivity: LOD ca. 0.2 ng, can be made mass spectrometry compatible	Poor dynamic range, limited quantification possibilities, poor reproducibility because of no endpoint, multistep procedure
Rabilloud T. Electrophoresis 13 (1992) 429–439.	Silver staining (silver diamine)	High sensitivity: LOD ca. 0.2 ng, stains basic proteins better than the protocol above	Poor dynamic range, limited quantification possibilities, poor reproducibility because of no endpoint, multistep procedure, high silver nitrate consumption
Rabilloud T, Strub J-M, Luche S, van Dorsselaer A, Lunardi J. Proteomics 1 (2001) 699–704.	Fluorescent staining with RuBPS	Medium to high sensitivity: LOD ca. 0.4 ng of BSA, very good for quantification, wide dynamic range	Overnight procedure, fluorescence imager necessary, dye particles can cause problems in image analysis, issues with mass spectrometry
Mackintosh JA, Choi H-Y, Bae S-H, Veal DA, Bell PJ, Ferrari BC, Van Dyk DD, Verrills NM, Paik Y-K, Karuso P. Proteomics 3 (2003) 2273–2288.	Fluorescent staining with Deep Purple™	Medium to high sensitivity: LOD ca. 0.4 ng of BSA, very good for quantification, wide dynamic range, mass spectrometry compatible	Fluorescence imager necessary
Ünlü M, Morgan ME, Minden JS. Electrophoresis 18 (1997) 2071–2077.	DIGE: Fluorescent minimal labeling	High sensitivity: LOD ca. 0.5 ng, direct comparison of up to three samples in one gel, good for quantification, wide dynamic range, mass spectrometry compatible	Fluorescence imager necessary
Sitek B, Lüttges J, Marcus K, Klöppel G, Schmiegel W, Meyer HE, Hahn SA, Stühler K Proteomics 5 (2005) 2665–2679.	DIGE: fluorescent saturation labeling	Very high sensitivity: LOD below pg, direct comparison of up to two samples in one gel, good for quantification, wide dynamic range, mass spectrometry compatible	Labeling protocols must be optimized for different sample types, fluorescence imager necessary

Method	Advantages	Disadvantages	
Radioactive labeling	High sensitivity: LOD below pg, good quantification, very wide dynamic range with phosporimager	Limited to living cells, gels have to be dried, phosphor imager necessary, radioactivity needed	*Johnston RF, Pickett SC, Barker DL. Electrophoresis 11 (1990) 355–360.*
Stable isotope labeling	High sensitivity: LOD below pg, wide dynamic range, good quantification	Methods still under development also for 2-D electrophoresis, expensive, mass spectrometer needed	*Smolka M, Zhou H, Aebersold R. Mol Cell Proteomics 1 (2002) 19–29.*
Western blotting	Ideal for highly sensitive and selective detection of specific proteins, general protein staining is also possible, proteins are well accessible on the blotting membrane	Additional electrophoresis step, uneven transfer of proteins, limited mass spectrometry compatibility because of membrane material, specific detection works only when antibodies are available	*Dunn MJ. In. Link AJ. Ed. 2-D Proteome analysis protocols. Methods Mol Biol 112 (1999) 319–329.*

In practice the mostly applied techniques in proteomics laboratories are Coomassie brilliant blue, silver and fluorescence labeling and staining.

1.5.5.2 Staining with Visible Dyes

It is very important to know that different staining techniques attach to proteins differently. There are proteins, which do not stain at all with Coomassie brilliant blue, but with silver and vice versa. Also different silver staining protocols deliver different patterns of the same sample.

Zinc imidazol negative staining When image analysis of the pattern is not an issue, and mass spectrometry analysis of some spots is the major goal, this procedure can be recommended.

Coomassie blue staining The "classic" alcohol/acetic acid Coomassie blue staining recipes should not be used for 2-D electrophoresis, because during destaining with the alcohol-containing solution the protein spots are partly destained as well. Some proteins – for instance collagen – lose the dye before the background of the gel is destained. Because no steady state is reached, quantification is not reliable and not reproducible.

The alcohols used are: methanol, ethanol, and isopropanol.

Colloidal Coomassie blue staining Colloidal Coomassie blue staining according to Neuhoff *et al.* (1988) contains also alcohol, but in presence of ammonium sulfate. Ammonium sulfate increases the strength of hydrophobic interactions between proteins and dye. The

Anderson NL, Esquer-Blasco R, Hofmann J-P, Anderson NG. Electrophoresis 12 (1991) 907–930.

methanol allows a much faster staining process. Coomassie G-250 is used. Repeated staining overnight alternated by fixing during the day with 20% ammonium sulfate in water for several times gives a sensitivity approaching that of silver staining. But this procedure takes a very long time and needs many steps; it is not ideal for high throughput. An easier "walk-away" protocol has been developed by Anderson *et al.* (1991), but it is not quicker.

This is the most environment friendly staining procedure, and it is well compatible with mass spectrometry analysis.

Hot Coomassie blue staining Much quicker results are achieved with a direct fixing/staining procedure using alcohol-free Coomassie R-350 in 10% acetic acid at elevated temperature. The easiest way is to heat the solution to 80–90 °C on a heating stirrer and to pour this solution over the gel, which lies in a stainless steel tray placed on a rocking platform. Staining takes 10 minutes. The gel has to be destained with 10% acetic acid at room temperature for several hours. Staining as well as destaining solutions can be used repeatedly. The dye can be removed from the destainer by adding paper towels to the destainer or filtering it through activated carbon pellets.

No loss of sensitivity has been observed, when glass- or film-backed gels are stained with Coomassie blue. Coomassie blue also exhibits fluorescence. It is possible to scan Coomassie blue-stained gels with a fluorescence imager, when all emission filters are removed.

Silver staining The silver nitrate protocol is mostly preferred to the silver diamine protocol, because it needs only 10% of the amount of silver nitrate, and it is less dangerous to get a silver mirror on the gel surface. When glass- or film-backed gels are stained, the staining steps have to be prolonged, and, unfortunately there is a loss of sensitivity, because the backing blocks one side of the gels for the solutions.

Colloidal Coomassie staining shows the same pattern like the hot staining procedure.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. Electrophoresis 21 (2000) 1037–1053.

Note: the best silver staining results are obtained when fixing is performed overnight.

Silver staining often produces a pattern different from the pattern achieved with Coomassie blue and other staining procedures like with Sypro® Ruby (Görg *et al.* 2000). Prestaining a gel with Coomassie blue intensifies the signal of silver staining, resulting in improved detection sensitivity. Another advantage is that double staining obviously prevents negative silver staining. When the Coomassie blue staining is fast, it can almost compete with a conventional fixing procedure for silver staining.

1.5.5.3 Detection with Fluorescence

Urwin V, Jackson P. Anal. Biochem 209 (1993) 57–62.

Fluorescence labeling Several fluorescent pre-labeling procedures have been proposed, for instance, the method using monobromobimane according to Urwin and Jackson (1993). However the most successful approach is the DIGE technique, which employs different

charge and size matched fluorescent tags and allows multiplex detection of co-migrated proteins. This method is described in detail in Chapter 1.5.2 *Pre-labeling of proteins for difference gel electrophoresis* on page 68 ff and page 132 ff.

All other gel-staining methods require a fixing step.

Uniquely fluorescent dyes can be scanned while the gels are still in the cassette. Sometimes the work cycle does not allow immediate scanning. In this case it is recommended to place the cassettes into a cold room or refrigerator. The diffusion of spots will be only minor. Although it has been proposed in some publications to open the cassettes and fix the gels with an acidic solution, this procedure is not advised, because this will reduce the strength of the fluorescent signal.

As already mentioned above, in the low molecular weight area the spots of minimal labeled and non-labeled proteins are resolved. When low molecular weight spots have to be picked from the gel, the gel needs to be post-stained with a fluorescent dye in order to pick the spots containing the 97% non-labeled proteins.

Fluorescence staining A number of fluorescence dyes are available. They exhibit a wide dynamic range of about four orders of magnitude, and they are therefore very well suited for quantification of proteins. The most sensitive dyes are Sypro Ruby, RuBPS solution, Flamingo Pink™, and Deep Purple. All these stains can be used in gels as well as on blots. While the first four dyes are chemically synthesized and based on heavy metal atoms, Deep Purple is a natural occurring fluorophor produced by the fungal species *Epicoccum nigrus,* and it is fully biodegradable. Because its attachment to proteins is reversible, it is highly compatible with downstream analysis like mass spectrometry (Coghlan *et al.* 2005).

Coghlan DR, Mackintosh JA, Karuso P. Org Lett 7 (2005) 2401–2404.

Steinberg TH, Agnew BJ, Gee KR, Leung W-Y, Goodman T, Schulenberg B, Hendrickson J, Beechem JM, Haugland RP, Patton WF. Proteomics 3 (2003) 1128–1144.

Specific staining of post-translational modifications Two types of post-translational modified proteins can be visualized by staining the gel or a blot membrane with specific fluorescent dyes: "Pro-Q®-Diamond" and Phos-Tag™ for specific detection of phosphorylated proteins (Steinberg *et al.* 2003), and "Pro-Q®-Emerald" for glycosylated proteins (Hart *et al.* 2003). Because the dyes exhibit different excitation and emission wavelengths, multiplex detection can be performed in a gel or a blot (Wu *et al.* 2005). Pro-Q-Emerald is based on the periodic acid Schiff's reagent procedure according to Zacharius *et al.* (1969), thus wrong positive detections cannot be completely excluded. For specific determinations of glycoproteins blotting and detection with lectins in various glycan kits is still the more reliable way.

Wu J, Lenchik NJ, Pabst MJ, Solomon SS, Shull J, GerlingIC. Electrophoresis 26 (2005) 225–237.

Hart C, Schulenberg B, Steinberg TH, Leung WY, Patton WF. Electrophoresis 24 (2003) 588–598.

Zacharius RM, Zell TE, Morrison JH, Woodlock JJ. Anal Biochem 30 (1969) 148–152.

1.5.5.4 **Mass Spectrometry Compatibility**

Coomassie brilliant blue-stained gels are usually compatible with mass spectrometry analysis, because the dye can be completely removed from the proteins. It is important to use a dye with good quality to avoid contaminants showing up in the mass spectrogram. For hot staining, shown in the two figures above, a very pure Coomassie brilliant blue R-350 dye was used, which is available in tablet form.

They use usually colloidal staining.

A spot, which is visible with Coomassie blue staining, contains enough protein for identification and characterization with mass spectrometry. Therefore many proteomics laboratories use this staining procedure in their routine work.

Shevchenko A, Wilm M, Vorm O. Mann M. Anal Chem 68 (1996) 850–858.

Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH, Dunn MJ. Electrophoresis 21 (2000) 3666–3672.

Silver staining can be modified for mass spectrometry compatibility by omitting glutardialdehyde from the sensitizing solution, and formaldehyde from the silver solution (Shevchenko *et al.* 1996, Yan *et al.* 2000). The detection sensitivity decreases to about one-fifth of the non-modified procedure. The silver–protein complexes are located only on the surface of the gel, the remaining proteins inside the gel layer can be further analyzed.

At the present state of development it is a fact that you are on the safe side with Coomassie blue staining, whereas spot analysis of silver stained spots is still an adventure.

In practice, however, it happens very frequently that silver stained spots show no signals in mass spectrometry. There might be many reasons for this, for instance too long development time resulting in too much contact of proteins with formaldehyde, or the protein amount in the spot is simply too low.

Fluorescent dyes are becoming more important for protein detection. The CyDye fluors used in the DIGE approach do not interfere with mass spectrometry: when minimal dyes are used, the high majority of the proteins are not modified at all; for cysteine labels the molecular mass of the added molecule has to be entered into the search software like it is done for alkylated cysteines. However, it has been reported that heavy metal-containing fluorescent dyes can interfere with mass spectrometry analysis (Laane and Panfilov, 2005). Tannu *et al.* (2006) have directly compared the effect of staining reagents on peptide mass fingerprinting from in-gel trypsin digestions and conclude that the DeepPurple stain can result in increased peptide recovery compared to heavy metal containing dyes.

Laane B, Panfilov O. J Proteome Res 4 (2005) 175–179.

Tannu NS, Sanchez-Brambila G, Kirby P, Andacht TM. Electrophoresis 27 (2006) 3136–3143.

1.6 Image Analysis

The evaluation and comparison of the complex 2-D patterns with the eye is impossible. Therefore, the gel images have to be converted into digital data with a scanner or camera, and analyzed with a computer.

Modern imaging systems such as the Typhoon™ create very wide dynamic range images of 0–100,000 levels of signal resolution. However, the most commonly used file for images is a 16 bit TIFF (tagged image file format), which corresponds to 65,536 levels. For a conversion the image analysis ".gel" format uses a square root algorithm to compress the possible 100,000 levels of an image into the levels available. The square root compression provides higher signal resolution at the low end where changes in signal are more critical. Thus, with a ".gel" file it is possible to differentiate between more subtle differences in low activity samples. Software packages like ImageMaster™ Platinum, DeCyder and ImageQuant™ TL take this conversion into account.

Usually a pixel format of 100 μm is applied for scanning. Higher pixel resolution does not improve image analysis.

> Note: ***If the resolution is too high, the image files will become too big to become processed in a reasonable time and take up a lot of space on the hard disk.***

Preparing the gels for spot picking When the gels should be placed into an automatic spot picker after image analysis, the gels must be fixed to a glass plate with Bind-Silane or to a plastic film support. Two self-adhesive reference markers – available also with fluorescence and radioactive signals – are glued to the bottom of this support. The positions of these markers are roughly predetermined to make it easier for the spot picker camera to find them automatically. The reference markers are scanned together with the spots. Picking after the spot coordinates acquired with image analysis is the most accurate procedure. But it requires gels, which are fixed to glass or film surfaces.

Picking from unbacked gels is described on page 140 in Section 1.7 Spot handling.

1.6.1 Image Acquisition

1.6.1.1 Still CCD camera systems

Chemoluminescence detection on blot membranes requires accumulation of the signal, therefore a fixed CCD camera in a dark cabinet is employed.

For high sensitivity the CCD cameras must be cooled.

Scanners for visible dyes Gels with visible spots have to be scanned in transmission mode. Otherwise quantification of spots would be incorrect. This is possible with high-end, liquid-insulated desktop line scanners. For appropriate image analysis it is important to scan the gels in grayscale mode with at least 16 bit signal depth. Blot membranes are scanned in reflectance mode. It is usually the scanning area, which sets the limit for the 2-D gel sizes. An A3 format scanner costs considerably more than a standard A 4 format instrument.

For the definition of OD see glossary.

The scanner must be calibrated using a gray step tablet. The measured dimensionless intensity is converted into OD values (or absorbance units, AU).

New desktop scanners afford quick scanning with wide linear range from 0 OD to 3.6 OD in transmission mode and with high spatial resolution. In practice the offered resolution of down to 20 μm cannot be applied, because the image file would become too big. Scanning a 20×20 cm gel with 300 dpi takes 30 seconds to 1 minute.

Film- or glass-plate backed gels are placed on the scanning area with the gel surface down, with a thin layer of water in between. Therefore the standard desktop scanners have to be modified by sealing the scanning area against liquid leakage.

For accurate picking of spots according to the data of image analysis the x/y positions need to be absolutely correct in the μm tolerance range. Usually the x/y data have to be calibrated for each scanner with the help of a grid. These calibration data have to be imported into the spot picker computer for each scanner used.

Fluorescence imagers As already mentioned above, the signals of fluorescent dyes exhibit a wide linearity and wide dynamic range, up to four orders of magnitude. This advantage should, of course, be utilized to the full extent. But this is only possible, when the dynamic range of the imager can cope with it. The selection of an adequate imager is therefore important.

Laser scanners The functions for storage phosphor imaging and multicolor fluorescence detection can be combined in one instrument. Lasers with different wavelengths are combined with different filters for the various scanning modes. The detectors are usually very sensitive photomultipliers. The dynamic range of such an instrument, shown in Figure 1.46, extends to five orders of magnitude.

Differentially labeled samples can be analyzed in the following way: with direct exposure both ^{35}S and ^{32}P signals are recorded; with a second exposure through a thin copper foil only ^{32}P labeled proteins are detected.

Radiolabeling provides the most sensitive signals. Storage phosphor screen scanners have a much wider linear dynamic range than X-ray films. The detection is much faster. After the exposure of a dried gel or a blotting membrane, the storage screen is scanned with a HeNe laser at 633 nm. For reuse, the screen is exposed to extra-bright light to erase the image.

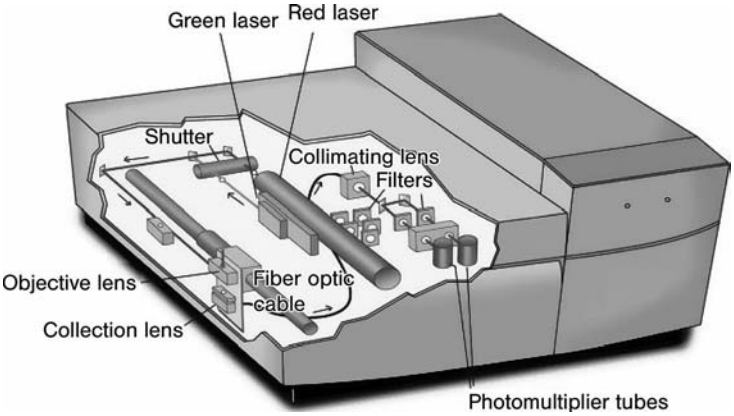


Fig. 1.46: Variable mode laser scanner Typhoon.

Staining or labeling proteins with fluorescent dyes shows similar or even higher sensitivity than silver staining and a much wider linear dynamic range. Modern instruments have a confocal scan head to cancel signals from scattered excitation light, and to reduce fluorescence background coming from glass plates and other supporting material. The laser light excites the fluorescent label or bound fluorescent dye; the emitted light of an offset wavelength is bundled with a collection lens and transported to the detector through a fiber optic cable (see Figure 1.47). Signals emitted from bands or spots, which are excited by stray light, are focused out, they will not hit the "peep hole", and will thus not be conducted by the fiber optic cable.

A second feature of confocal optics is the adjustment of the focusing depth. For instance, when gels are scanned as inside their cassettes, the scan head is adjusted to focus at 3 mm above the platen. For scanning blot membranes it is switched to the platen level.

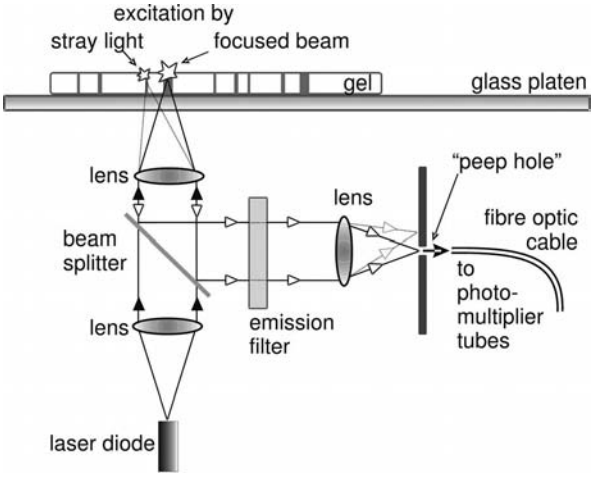


Fig. 1.47: Schematic diagram of the principle of fluorescence detection with confocal optics.

GE Healthcare Handbook: Fluorescence imaging: Principles and Methods GE Healthcare Life Sciences (2002) 63-0035-28.

State of the art instruments are equipped with up to three lasers with wavelengths of, for instance, 532 nm (green), 633 nm (red), and 457 nm and 488 nm (blue). Many aspects of fluorescence imaging are described in detail in the fluorescence imaging handbook by GE Healthcare (2002).

The scanning sensitivity is adjusted by modifying the voltage of the photomultiplier tubes after a quick prescan. In order to facilitate the imaging of DIGE gels in cassettes, hardware accessories and little software tools are available to facilitate scanning and organizing the images for subsequent image analysis. The glass platen is big enough to accommodate two large gel cassettes.

Scanners with confocal optics deliver very exact geometric data, therefore x/y calibration for accurate spot picking is not required.

Instruments of this type are less expensive than laser multifluorescence scanners.

Scanning CCD cameras Scanning CCD cameras with white light source can also provide adequate quantitative and qualitative measurements. Figure 1.48 shows a schematic diagram of such a system. By filtering the white light with an excitation filter monochromatic light is created for selectively exciting just one of the fluorophores. For an even distribution of the light on the sample it is divided by glass fibers and guided into a ring light. A particular low band pass emission filter is used to avoid crosstalk between the sample channels, which are recorded by a CCD camera.

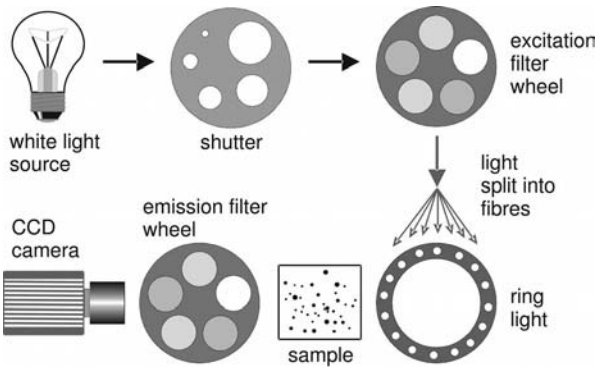


Fig. 1.48: Schematic representation of the mode of functioning of a multi-fluorescence imager based on white light and scanning CCD camera.

The CCD camera should scan and read out on the fly. Conventional systems with CCD cameras, which needed to stop for the readout, have delivered the images in patches, which had to be stitched together with special computer programs. Novel systems use a time delay integration technique, which precisely synchronizes image

acquisition and CCD readout rate. The sensitivity can be adjusted by selecting the optimum scanning velocity. Such new systems are available with a dynamic range of 3.5 orders of magnitude.

Practical hints for imaging DIGE gels Because fluorescence is temperature dependent, the gels should be scanned at room temperature. Gels from the refrigerator or cold room should be allowed to warm to room temperature prior to scanning (Rozanas and Loyland, 2006).

Rozanas CR, Loyland SM. In: Liu BCS, Ehrlich JR, Eds. Tissue Proteomics: Approaches for Pathways, Biomarkers, and Drug Discovery. Humana Press, Totowa, NJ (2006) in press.

It is important to crop the DIGE images with the scanner software tool to exclude the poorly resolved marginal areas and to achieve perfect pattern overlays. The CyDyes have different signal strengths. In order to obtain comparable patterns, the images are normalized with the DeCyder software after the definition of spot and area exclusions parameters has been carried out.

1.6.2 Image Analysis and Evaluation

The evaluation of the spot patterns is the most demanding part of the 2-D electrophoresis technique. The application and control of the image analysis software tools take a lot of time. And manual spot editing, spot splitting and other human interventions are influenced by bias of an individual. Ideally the images would be analyzed and compared fully automatically.

Image analysis of 2-D gels has to deliver various types of information:

- Comparison of 2-D pattern of treated with non-treated samples;
- Interpolation of isoelectric points and molecular sizes;
- Detection of novel, missing, or modified proteins;
- Quantification of protein spots;
- Detection of up- or down-regulated proteins;
- Definition of spot positions for spot cutting;
- Detection and characterization of protein families and pathways;
- Statistical analysis of experimental results;
- Enabling database queries;
- Linkage of 2-D data to mass spectrometry data;
- Integration of the image data with a laboratory workflow system.

The development of software for 2-D electrophoresis gel image analysis is a continuously ongoing process. The functions become more reliable, reproducible and automated from year to year. With the latest developed program it is already possible to compare gels of different sizes, shapes, and even damaged gels. However, irreproducible results cannot be fixed, even not by the most sophisticated software. On the contrary: the highly developed programs recognize inconsistencies between different patterns. Exact and tidy laboratory work is still requested. The software cannot turn bad separation results into good results.

There are two types of 2-D imaging software on the market:

- Cheap to free of charge tools for basic image analysis;
- Professional programs, which evaluate with high reproducibility and offer many valuable functions for image analysis, statistical evaluation, and reporting.

The most critical parts in image analysis are spot detection and background subtraction. Manual spot editing and background definitions are always user-biased.

Furthermore it is very important that the image analysis program does not modify the raw data.

There are two different concepts of 2-D electrophoresis, which require different approaches for image analysis and evaluation:

- One sample per gel separations;
- Multiplex separations like achieved with DIGE.

1.6.2.1 Traditional One Sample Per Gel Analysis

The following brief description is based on the concept of one of the most advanced software packages, which is available under the name ImageMaster Platinum, also known under the previous name "Melanie". The goal is to apply automatic procedures wherever possible, to exclude manual editing and human interference.

This can vary with different sample types, pH gradients, gel size and composition, detection techniques.

Spot detection The most important step is spot detection, where different parameters have to be optimized:

- With a "smooth" factor splitting of spots can be influenced.
- The selection of a certain "minimal area" eliminates spots below the set threshold.

- The definition of "saliency" discriminates between real spots and artifacts (real spots have higher saliency values).

These parameters are defined in a selected enlarged restricted area and then applied on all gels of an experiment. Properly optimized spot detection reduces spot editing close to zero.

Background correction A background subtraction function can easily lead to errors. After many years of experience with spot volume quantification it has been found that by defining the spot boundary at 75% of the peak maximum and calculating the spot volume for only for the values above the boundary, the relative spot volume quantification comes very close to the real situation and ensures a very high reproducibility.

Normalization Normalization takes care of the variation in protein loading and staining. The spot volumes are normalized against the total spot volume of the gel fully automatically without any user interaction.

pI and M_r calibration There are two ways to calibrate the image for pI and M_r annotation (see also pages 98 and 118):

- *1-D calibration*. Calibration curves are derived from IPG pH gradient graphs for pI determination for one dimension and from the positions of co-run molecular weight markers for the second dimension by importing a ladder.
- *2-D calibration*. pI and M_r values are calibrated at the same time by interpolating between known values. These values usually come from mass spectrometry analysis. A number of spots with known protein properties are selected, the pI and M_r information are imported from the respective protein lists belonging to these spots.

Gel viewing For visual inspection the spots they can be viewed in two- and three-dimensional view.

Matching For differential analysis it is important to be able to superimpose the spot patterns. This cannot be done directly, because typically there are local distortions caused by imperfections in gel matrix, variations in gel running conditions, temperature effects, uneven focusing, and polymerization issues. Formerly spot matching tried to find corresponding spots in pairs of gels. However for automatic

For controlling the matching efficiency vectors between the spots of the gel pair are displayed. The directions and lengths of the vectors between the corresponding spots make it easy to detect mismatches.

matching it is more efficient to search for pairs of features, using spot clusters, spot shapes, sizes, and positions together. Even this feature-based matching can sometimes produce mismatches. Therefore it is necessary to control this process by visual inspection, and perform corrections with setting landmarks.

Data analysis 2-D gel patterns are compared to detect qualitative and/or quantitative protein expression changes between individual samples or different populations. As already mentioned in the introduction, the challenge is to find biologically significant changes between different samples against the background of inherent biological variations, induced variations during sample preparation, and gel-to-gel variations.

Populations of replicate gels are compared to each other with so called "match sets". Arbitrary reference gels are no longer employed. The match sets can be organized in various ways; this allows creating different groups of gels.

Several statistical tools and clustering techniques are available to extract results and to discover similarities or significant differences.

Preparing the spot picking list The x/y positions of the selected spots, including the reference markers, are exported into a table. The numbers of the reference markers are automatically named "IR1" and "IR2" (internal reference).

Reporting and exporting of data The results can be printed out in images with or without annotations and tables. The files can also be exported into Excel and other office software.

1.6.2.2 The Analysis of DIGE Gels

This reduces the user bias to a minimum.

DIGE gels are obtained by labeling the proteins in different samples with different, spectrally distinct fluorescent dyes, the CyDye fluors, and running the mixed samples together in the same gel. Because of the multiplex feature and the co-migration of the proteins to the identical spot coordinates, DIGE gels can be analyzed in a completely different way than traditional gels. This analysis runs almost fully automatically.

The main philosophy in the differential approach of the DeCyder software is the assumption that the protein compositions of different samples within an experiment are very similar; that there are only a few changes in protein expression levels, which are significant and should be detected.

The key points for the evaluation of DIGE gels are:

- The co-detection of spots;
- The co-separation of the pooled internal standard in each gel;
- Utilizing the internal standard for in-gel normalization.

Co-detection As already mentioned before, because of the co-migration of the same proteins the spots do not need to be matched within a gel. Thus the sample spot volumes can be directly related to the standard spot volumes, which are originating from protein mixtures of all samples. DeCyder does this automatically by employing a co-detection algorithm. Co-detection means that for the definition and assessment of a spot the information of all three channels is combined. Each particular spot receives the identical spot boundary across all three channels. In this way the relative spot volume calculation becomes very accurate, and splitting of double or multiple spots is more reliable than manual splitting. If co-detection would not be available, the operator of the software, as the supervisor of evaluation, would detect the spot shoulders and perform manual splitting. Figure 1.49 shows a typical situation. With triple co-detection the correct spot boundaries are applied. Single detection would lead to erroneous spot matching.

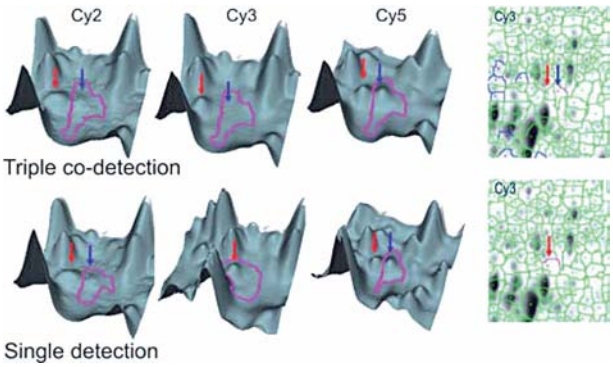


Fig. 1.49: Comparison of spot boundary definition with triple co-detection and single detection. On the left hand side the three-dimensional view of all three channels; on the right hand side the two-dimensional view of the Cy3 channel. From Josef Bülles with kind permission.

With the help of the internal standard the software can automatically normalize the spot volumes.

Linking the images of different gels via the internal standard A 2-D electrophoresis experiment always consists of more than one gel. Therefore the spot volume ratios have also to be compared across different gels. In this case a spot matching procedure has to be carried out, because there are always some gel-to-gel variations, which do not allow direct superimposing of the images. However, in each gel one channel contains the image of the pooled internal standard. Because these standard images originate from the identical sample mix, spot matching is greatly facilitated. Figure 1.50 shows how the spot volume ratios are compared across different gels with the help of the internal standard.

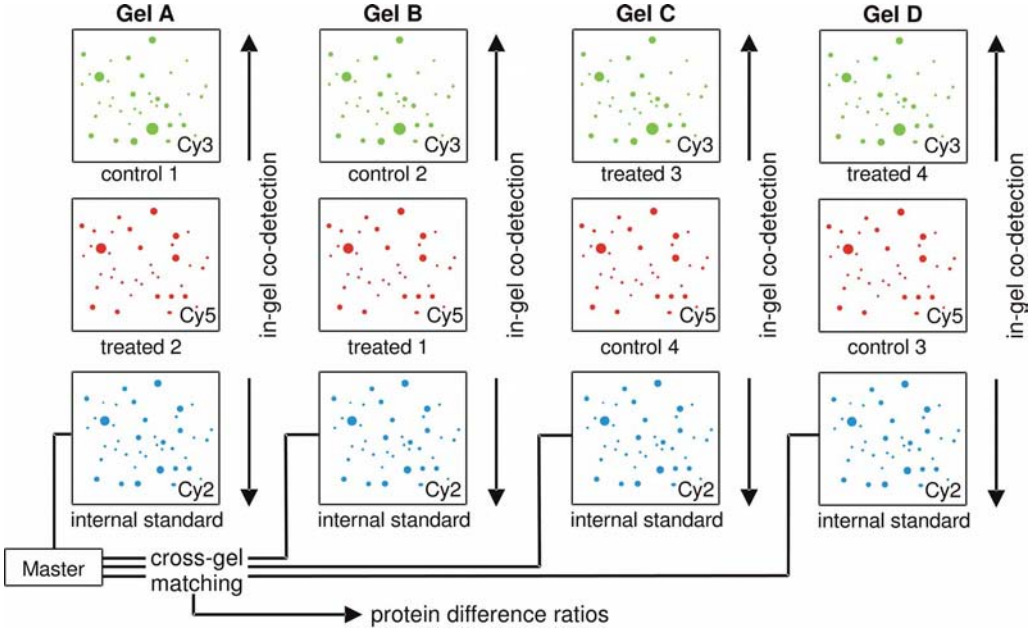


Fig. 1.50: Schematic diagram showing the linking of data from several gels via the internal standard images. Note also the reversed labeling and the planned randomization of sample application.

The unique difference of this approach to other programs is the very high evaluation speed from importing the images to a reliable result.

With a batch processor a high number of images are evaluated fully automatically, which saves hands-on time and keeps user bias to a minimum. The significance of changes and quantitative differences between protein expression levels can easily be checked with several inbuilt statistical tools: Student's *t*-test, one- and two-way ANOVA, and false discovery rate adjustment. Differences in protein expression levels of less than 10% can be detected with over 95% confidence.

Two examples By using the internal standard concept gel-to-gel variations and technical variations of software are considerably decreased. Alban *et al*. (2003) have demonstrated the power of incorporating the pooled internal standard using an experimental model. They generated a model for a time / dose experiment by spiking four different commercial proteins into *E. coli* lysate: eight samples have been prepared with different levels of the spikes. These samples were analyzed with conventional one color 2-D electrophoresis and with 2-D DIGE employing the pooled internal standards. The ability of both methods to measure abundance changes and monitor trends in protein expression were compared. Besides the fact that the conventional method is much slower, is more work intensive and needs more gels, the results were inaccurate and the trends were incorrect when compared to the defined spiking amounts and the results achieved with the DIGE concept using the internal standard.

Alban A, David S, Björkesten L, Andersson C, Sloge E, Lewis S, Currie I. Proteomics 3 (2003) 36–44.

In the paper by Friedman *et al*. (2004) a very good real life example for the usefulness of the internal standard is presented: In this study healthy and tumor tissue of six patients were analyzed with 2-D DIGE. Image analysis and data evaluation of the obtained results were performed two times, with and without taking the internal standard in respect. Without the internal standard ten significant differences between the healthy and the tumor samples were found; but using the internal standard revealed in total 52 significant differences.

Friedman DB, Hill S, Keller JW, Merchant NB, Levy SE, Coffey RJ, Caprioli RM. Proteomics 4 (2004) 793–811.

Statistical evaluation Some valuable statistical aspects on difference gel analysis can be found in a book chapter on DIGE applications in clinical research (Sitek *et al*. 2006).

Sitek B, Scheibe B, Jung K, Schramm A, Stühler K. In: Proteomics in Drug Research (M Hamacher et al. Eds.) Wiley-VCH, Weinheim (2006) pp 33–55.

Extended data analysis Although 2-D DIGE in conjunction with the powerful image analysis program can be applied for rather complex experiments like multifactorial time/dose studies, there is a limitation of the sample number contained in one experiment. Also, when too many samples are analyzed in one experiment, one-time events can be diluted out of the internal standard. For this reason and in order to extract as much information as possible from a series of experiments some additional multivariate statistical tools have been made available:

- Principal component analysis (PCA) reduces the dimensionality of the data, in order to find the underlying sources of variation. It is a useful tool to find initial groupings of the data and detect outliers.
- Different clustering techniques reveal proteins with similar expression profiles to find complex

partners and proteins which function together in certain biological pathways.

- With discriminant analysis proteins are found, which could serve as classifiers as well as diagnostic or prognostic markers. With unsupervised clustering unknown samples can be classified without user bias.

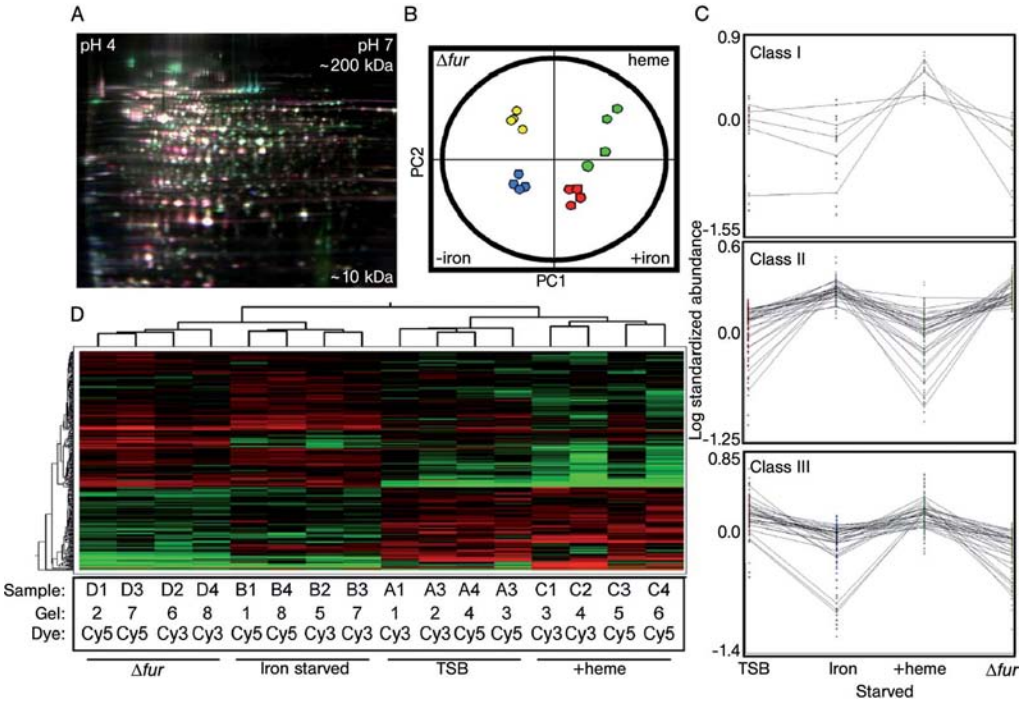


Fig. 1.51: Application of DeCyder™ EDA: (A) False-colored representative gel containing three differentially labeled samples. Cy2-labeled internal standard (blue), Cy3-labeled control No. 1 (green), and Cy5-labeled iron-starved No. 1 (red) are overlaid. (B) Unsupervised PCA properly groups the 16 individual DIGE expression maps differentiated by two principle components (PC1 and PC2) and demonstrates high reproducibility between the replicate samples within each group. (C) Composites of DIGE expression patterns representing the five proteins that increase abundance in the presence of hemin, the 29 Class II protein features negatively affected by Fur and iron, and the 30 Class III protein features positively affected by Fur and iron. (D) Unsupervised hierarchical clustering of 16 individual DIGE expression maps (groups, shown along top) and of individual proteins (shown on the left), with relative expression values for each protein displayed as a heat map using a relative scale ranging from –0.5 (green) to +0.5 (red). From: DOI: 10.1371/journal.ppat.0020087.g001

Friedman *et al.* (2006) have applied these multivariate statistical tools on their study on the influences of *Staphylococcus aureus* on the central metabolism of the host. Figure 1.51 shows combined images of the 2-D electrophoresis result in false color representation, a PCA grouping, K-means cluster, and a heat map of unsupervised hierarchical clustering.

Friedman DB, Stauff DL, Pishchany G, Whitwell CW, Torres VJ, Skaar EP. PLoS Pathog 2 (2006): e87. DOI: 10.1371/journal.ppat.0020087

It is very useful, when the images of the results and the 2-D DIGE experiment can be directly linked. The add-on software package DeCyder EDA is programmed in such a way that the original experimental data are directly available via a hyperlink by clicking on a PCA spot or a band on the heat map.

In this way the results can be traced back very easily.

Spot picking list Because the fluorescent dyes are not visible with the eye, it is particularly useful to pick the spots from 2-D DIGE gels according the x/y data of the image file.

1.6.3 Use of 2-D Electrophoresis Data

Most of the image analysis software includes a web browser to check results of other laboratories in the World Wide Web. Because there is no standardization for sample preparation and for running 2-D electrophoresis, the spot patterns of different laboratories are not easy to compare in practice.

A protein cannot be identified on the basis of its position in the gel alone. Identification is only possible by further analysis of the protein, for instance with mass spectrometry. It is furthermore incorrect to conclude that a protein is up or down regulated, just using the information from growing or shrinking spot intensity. It is also possible that another protein had changed its pI due to phosphorylation or another post-translational modification, and is now sitting on top of the protein observed.

Therefore proteins of interest have to be analyzed further. This is done mainly with mass spectrometry: The gel plugs containing the protein are cut out of the gel slab subsequently to image analysis.

1.7 Spot Handling

Between image analysis and further analysis the gels can be kept in a refrigerator for several weeks. It is also possible to dry the gels for storage and soak them in water before spot cutting.

In high-throughput proteomics it is very important to keep track of each sample and of each protein to be analyzed. Before the proteins

contained in the spots can be analyzed with mass spectrometry a series of steps have to be performed:

- Spot picking;
- Destaining and washing of the gel plug;
- In-gel digestion;
- Peptide extraction;
- Spotting of peptides on MALDI target slides.

During this procedure mix-ups and contaminations have to be prevented to avoid wrong conclusions.

In order to keep control over all these steps and parallel processes, as well as the results, two things are necessary:

- Automation;
- Workflow database.

Therefore a workstation has been developed, which handles all steps automatically. Figure 1.52 shows a schematic drawing of such an integrated system. In the fully automated system a robotic arm transports gels, microtiter plates and target slides between the stations. The workstation is controlled by a laboratory workflow system software. This software can control the entire proteome analysis workflow including sample tracing.

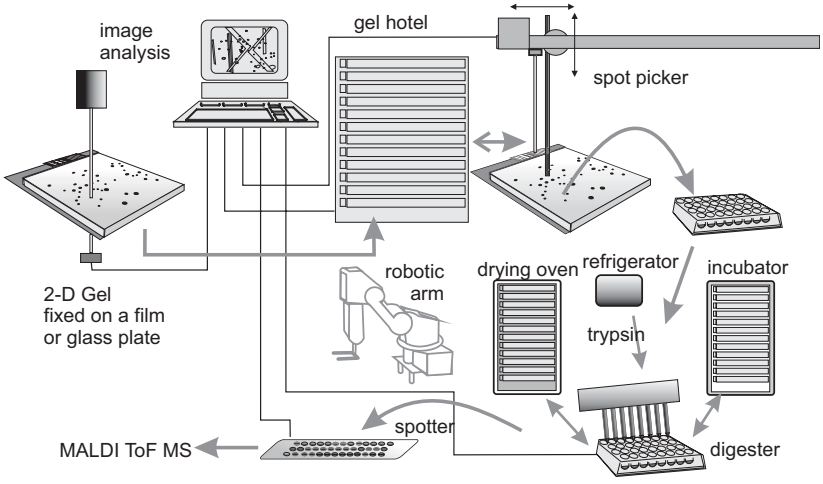


Fig. 1.52: Schematic drawing of an automated workstation for spot picking, in-gel digestion, and spotting peptides on a MALDI target slide.

The gels are identified with the help of barcodes on the support films and the trays. After spot picking the gel is transported back to the gel hotel. Many moves are needed for the digestion step. Spot

picking and spotting are combined to be carried out by the same instrument.

The laboratory workflow system can be extended to include all instruments and software involved in the entire proteomics analysis, from sample preparation to mass spectrometry.

In most of the laboratories the different steps are performed by stand-alone instruments.

1.7.1 Spot Picking

Picking spots from a gel manually is a very cumbersome job. The spots, marked in the printout of the image analysis for further analysis, have to be found in the gel again and transferred to the correct reaction tube or well of a microtiter plate. Sample tracing is not easy and errors can easily occur. Additionally, contaminations with keratin and other stuff must be avoided. This is a typical work for a laboratory robot.

Robotic spot pickers are much more reliable for excising selected spots from the gel slab and transferring them to defined wells of microtiter plates. This automated picking can be controlled with a CCD camera or by transferring the pixel coordinates from image analysis into the machine coordinates of the picker instrument.

The camera in this system has only the function to find the positions of the two reference markers.

For the second technique, the gel has to be immobilized on a rigid support – a glass plate or a plastic film – prior to scanning. Two self-adhesive reference markers, glued onto the support plate or film, are necessary to enable the machine to recalculate the x/y positions of the proteins from the imported picking list. The two markers are automatically recognized by the camera and used as calculation points for the spot positions. This procedure provides a very high picking efficiency and accuracy, because it utilizes the high resolution and sensitivity of the scanning device. Furthermore, using the image analysis data, also fluorescent, non-stained and radioactive labeled spots can be excised.

Note: ***For this approach it is necessary and very important that the image analysis software does not modify the raw data.***

Figure 1.53 shows the assembly of a robotic spot picker working according this concept. The gel is placed into a liquid-containing tray to prevent drying during the picking process. The glass plate or film support is fixed at the bottom of the tray.

A picking file has to be created to assign the spot position to the respective microtiter plate well.

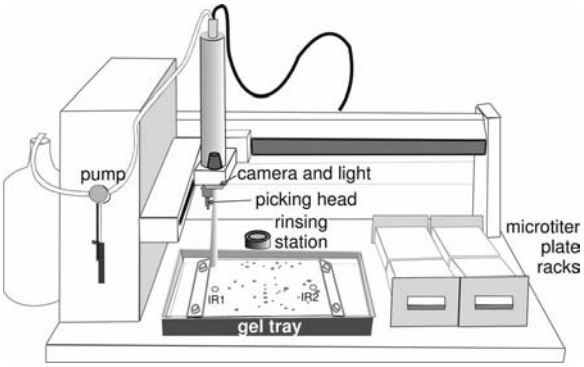


Fig. 1.53: Schematic drawing of an automatic spot picker, which cuts the spots according to an imported picking list and transfers the gel plugs to microtiter plates.

The picking head is coated with a hydrophobic layer to prevent gel plugs sticking to it and protein cross-contamination. The gel plugs are cut out by punching down to the backing plate or film, followed by two side movements to shear the plug off the backing. Between each spot removal the cutting head cleans itself in a rinsing station to avoid cross contamination of proteins.

Such an instrument can also be operated in a semi-automated way in order to cut out a few spots with eye control. In this case the camera is also employed to find the spot to be removed from the gel.

1.7.1.1 Picking from Non-backed Gels

GE Healthcare User Manual: Ettan Spot Picker Nonbacked Gel Kit. GE Healthcare Life Sciences (2003) 11-0002-69.

In practice it can happen that such an automated spot picker should also be used for picking from non-backed gels. In order to prevent shrinking or swelling of the gel it has to be stabilized during scanning and spot picking. This can be done with a little accessory kit and Cellophane® film following a procedure, which has been developed by Burghardt Scheibe: A cellophane sheet is soaked in water and clamped into a set of drying frames. The upper cellophane side is dried with lint-free tissue paper and the reference markers are stuck to the cellophane. The frame-cellophane assembly is turned upside down to form a tray. The gel slab is soaked in water to swell as much as possible and placed onto the cellophane. The gel is allowed to dry on the cellophane for about 15 minutes. Then the gel is scanned, the image is evaluated, and a picking list is created. When the gel–cellophane assembly is covered with a glass plate, the gel will not dry and the assembly can be stored for several hours. A dark plastic plate is placed into the spot picker instead of the tray as shown in Figure 1.54. The camera is recalibrated, because of the lower picking level.

The frame with the gel is placed on the plastic plate and the spots are picked like from a film- or glass-supported gel. In order to prevent curling up of the gel edges, every half hour a few milliliters of water need to be pipetted on the gel. A more detailed instruction can be found in the GE Healthcare User Manual.

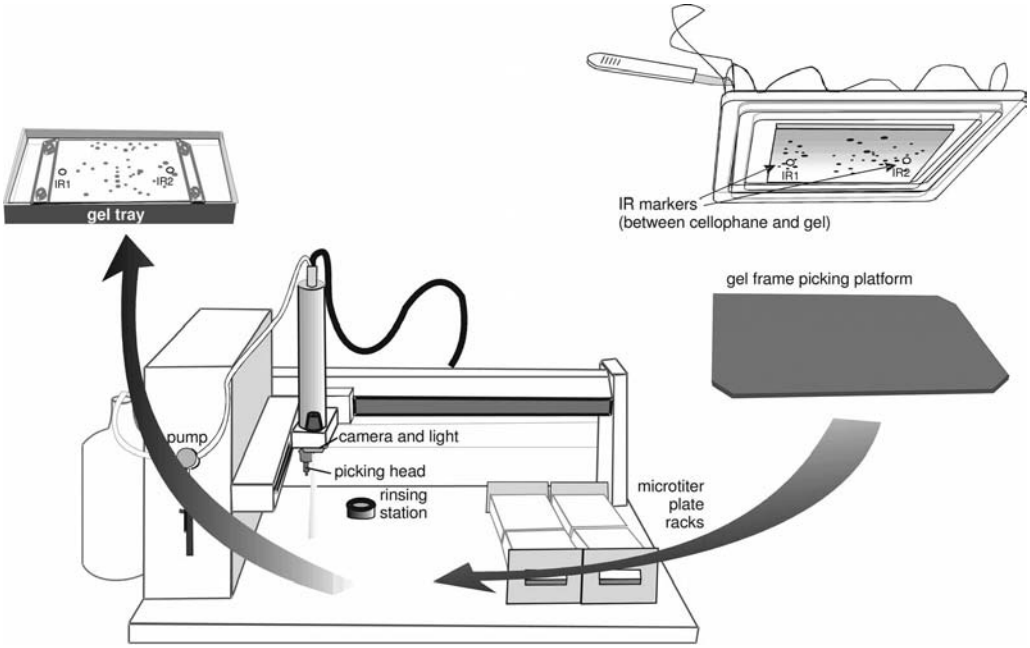


Fig. 1.54: Schematic drawing how to pick from unbacked gels with an automatic spot picker.

1.7.2 Protein Cleavage

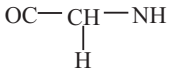
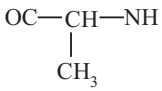
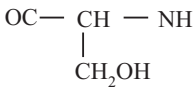
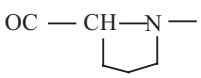
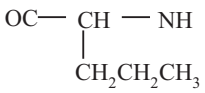
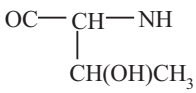
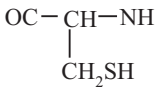
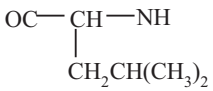
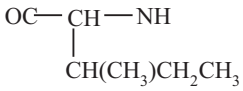
As the mass spectrometers become ever more sensitive, delivering the sample of interest to the mass spectrometer in a manner which takes advantage of the innate high sensitivity of the mass spectrometer will become increasingly crucial.

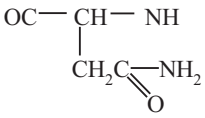
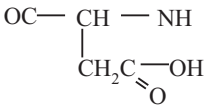
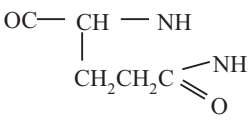
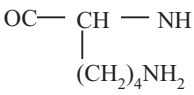
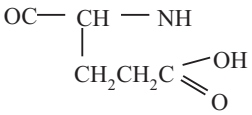
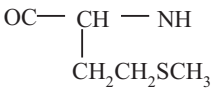
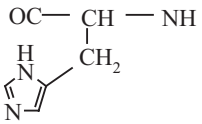
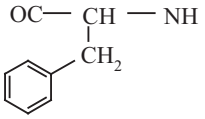
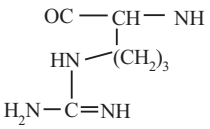
The most commonly used strategies for protein identification by mass spectrometry require the protein of interest to be cleaved, proteolytically or chemically, into its constituent peptides (assembly of amino acids, see Table 1.7). In the pathway highlighted in this book protein cleavage is performed by proteolysis after the protein has been separated by two-dimensional gel electrophoresis. This has been successfully applied to a wide range of proteome projects such as the

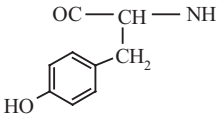
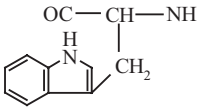
Langen H. Takács B, Evers S, Berndt P, Lahm HW, Wipf B, Gray C, Fountoulakis M. Electrophoresis 21 (2000) 411–429.

analysis of the *Haemophilus influenzae* proteome (Langen *et al.* 2000). Other methods have described the use of proteolysis without separation of the proteins by 2-D electrophoresis (see Section 2).

Tab. 1.7: 6 Twenty common amino acids detailing structure, molecular weights, symbols and common modifications.

Residue (3 & 1 letter symbols)	Residue structure (R = side-chain)	Average mass	Monoisotopic mass	Common modifications (and nominal molecular weight additions)
Glycine (Gly & G)		57.05	57.02146	
Alanine (Ala & A)		71.08	71.03711	
Serine (Ser & S)		87.08	87.03203	Phosphorylation (80) Glysosylation[a]
Proline (Pro & P)		97.12	97.05276	
Valine (Val & V)		99.13	99.06841	
Threonine (Thr & T)		101.11	101.04768	Phosphorylation (80) Glysosylation (*O*-linked sugars)
Cysteine (Cys & C)		103.15	103.00919	Carbamidomethyl[b] (57) *S*-propionamide[c] (71) *S*-pyridylethyl[d] (105)
Leucine (Leu & L)		113.16	113.08406	
Isoleucine (Ile & L)		113.16	113.08406	

Residue (3 & 1 letter symbols)	Residue structure (R = side-chain)	Average mass	Monoisotopic mass	Common modifications (and nominal molecular weight additions)
Asparagine (Asn & N)		114.10	114.04293	N-linked glycosylation[e] (Sequon Asn-X-Ser/Thr)
Aspartic acid (Asp & D)		115.09	115.02694	Esterification, methyl ester[f] (14)
Glutamine (Gln & Q)		128.13	128.05858	
Lysine (Lys & K)		128.17	128.09496	Acetylation[g] (42)
Homo-arginine (42)				
Glutamic acid (Glu & E)		129.16	129.04259	Esterification, methyl ester[f] (14)
Methionine (Met & M)		131.20	131.04049	Oxidation[h] (16)
Histidine (His & H)		137.14	137.05891	
Phenylalanine (Phe & F)		147.18	147.06841	
Arginine (Arg & R)		156.19	156.10111	

Residue (3 & 1 letter symbols)	Residue structure (R = side-chain)	Average mass	Monoisotopic mass	Common modifications (and nominal molecular weight additions)
Tyrosine (Tyr & Y)		163.18	163.06333	Phosphorylation (80)
Tryptophan (Trp & W)		186.21	186.07931	Oxidation (16)

Key:

a *O*-linked glycosylation – a range of sugars can link to Ser/Thr, including pentoses ($C_5H_{10}O_5$), hexoses ($C_6H_{12}O_6$) and *N*-acetylhexosamines

b Modification of cysteine residue with iodacetamide

c Modification of cysteine residue with acrylamide monomer

d Modification of cysteine residue with 4-vinylpyridine

e *N*-linked glycosylation – a pentasaccharide core linked to the Asn residue, with a variety of structures extending from this core

f Also esterification of the C-terminal carboxyl group of each peptide

g Also acetylation of the α-amino group at the n-terminus of each peptide

h Oxidation of methionine to the sulfoxide and the sulfone

1.7.2.1 Protein Cleavage – Proteolysis

Proteolysis has become the routine method of protein cleavage used in proteomics with a range of enzymes available (see Table 1.8). Proteolysis offers several practical advantages; including high specificity, minimal side reactions and good cleavage efficiency. Important practical considerations for protein digestion include the digestion buffer and its pH, the enzyme:substrate ratio, temperature and time.

Restricting the basic residues to the c-terminus enables efficient peptide fragmentation during an MS/MS product ion experiment, see Section 3.3.4.

Of all the enzymes available, the most commonly used enzyme is trypsin. Trypsin has well defined specificity, yields tryptic peptides of an appropriate size for efficient MS analysis and locates the basic residues at the terminus of the peptide. Though trypsin is commonly used there may be occasions where digestion with an alternative enzyme will be advantageous, specifically post-translational studies such as phosphorylation analysis. The specific site of phosphorylation may not reside on a tryptic fragment that is appropriate size for efficient MS analysis.

Patterson S, Aebersold R. Electrophoresis 16 (1995) 1791–1814.

Tab. 1.8: Enzymes commonly used in proteomics

Method of protein cleavage	Site of cleavage	Exception	pH range
Trypsin	C-terminus of R-X, K-X	If X = P	7–9
Endoproteinase Glu-C (V8-DE)	C-terminus of E-X, D-X	If X = P	4–8
Chymotrypsin	C-terminus of F, Y, W, L, I, V, M	If X = P	7.5–8.5
Endoproteinase Lys-C	C-terminus of lysine, K-X	If X = P	8.5–8.8
Arg-C	C-terminus of arginine, R-X	If X = P	7.5–8.5
Elastase	Not very specific. C-terminal side of G, A, S, V, L, I		8–8.5
Pepsin	C-terminus of F, L, E		2–4
Pronase	Pronase is a mixture of endo- and exo-proteinases. It cleaves almost any peptide bond.		7–8, dependent on proteases present

The simplest digestion would be an in-solution digestion. However, optimal electroelution of a protein from the polyacrylamide gel matrix into solution for digestion is difficult and highly variable from protein to protein (Aebersold and Patterson 1995). The electrotransfer (blotting) of the protein to a membrane (of which there were many types) with subsequent digestion on the membrane surface (Aebersold *et al*. 1987; Pappin *et al*. 1995).

Pappin DJC, Rahman D, Hansen HF, Jeffery W, Sutton CW. Methods in protein structure analysis (1995) 161–173.

Aebersold R, Leavitt J, Saavedra RA, Hood LE, Kent BS. Proc Natl Acad Sci USA 84 (1987) 6970–6974.

A further development of the blotting technology was described by Bienvenut *et al*. (1999). In this method the proteins are blotted through a membrane of immobilised trypsin, onto a support membrane where the constituent peptides are trapped and scanned directly by MALDI peptide mass fingerprinting. This technology is ultimately envisaged as the basis of a clinical scanner (Binz *et al*. 1999; Bienvenut *et al*. 1999; Schleuder *et al*. 1999).

Bienvenut WV, Sanchez JC, Karmime A, Rouge V, Rose K, Binz PA, Hochstrasser DF. Anal Chem 71(1999) 4800–4807.

Despite these methods the in-gel digestion methodology has become routine for proteins separated by 2-D electrophoresis. Wilm and co-workers (1996), and Shevchenko *et al*. (1996a) reported the in-gel digestion of proteins from Coomassie Blue and silver stained gels which was compatible with protein identification by mass spectrometry.

Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, Mann M. Nature 379 (1996) 466–469.

Shevchenko A, Wilm M, Vorm O, Mann M. Anal Chem 68 (1996) 850–858.

The basic theory is summarised in the following paragraphs, with some novel developments reported. Following visualization of the gel, the protein spots of interest can be excised from the gel manually or automatically with commercially available gel spot pickers and be subsequently destained. The destaining of the protein spot is particu-

75 mM ammonium bicarbonate in 40% ethanol is an effective CBB destain.

larly important. For instance, Coomassie brilliant blue binds to proteins via ionic and hydrophobic interactions. The ionic interactions between the sulfonic acid group of the CBB and the basic residues of proteins (arginine and lysine residues) affect the trypsin digestion efficiency, and the presence of CBB in the final sample can hamper MS performance. This destaining step also allows the removal of unwanted detergents such as SDS from the digestion procedure and the MS analysis (in which detergents ionize very efficiently).

The presence of glutardialdehyde in many silver stain procedures precluded the stained protein from subsequent analysis, as the glutardialdehyde reacted with free amino groups in the protein. However, Shevchenko *et al.* (1996) demonstrated that this component could be omitted from the staining procedure and still realize high sensitive visualization and successful MS analysis; Yan *et al.* (2000) also published a silver stain method compatible with mass spectrometry.

The residual silver ions from the silver stain procedure can also be removed using the method reported by Gharahdaghi *et al.* (1999).

At this stage many methods have reported the reduction and alkylation of cysteine residues contained within the protein embedded in the gel piece (Rosenfield *et al.* 1992; Moritz *et al.* 1996; Shevchenko *et al.* 1996b; Gevaert and Vandekerckhove 2000). The derivatization comprises two steps: reduction of the disulfide bonds and alkylation of the subsequent thiol side chain of the cysteine. This step is included to improve the detection of cysteine containing peptides and hence improve the potential protein coverage. However, the cysteine thiol group can become modified as it passes through the PAGE gel with free acrylamide monomer. Sechi and Chait (1998) noted that the methods referred to above do not generally label the cysteine residues post electrophoresis with the same mass addition that occurs within the gel, hence potentially producing a heterogeneous derivatistion of cysteine residues making protein identification complicated, as the cysteine residues have effectively been labelled with different reagents.

However, the 2D-electrophoresis method described in this book includes a quantitative reduction and alkylation derivatization step of the cysteine residues prior to the second dimension SDS-PAGE. If the procedure is repeated post-electrophoresis and prior to digestion, then the same reagent should be used.

Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler WH, Dunn MJ. Electrophoresis 21 (2000) 3666–3672.

Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM. Electrophoresis 20 (1999) 601–605.

Additionally, the negative zinc/imadazol stain is compatible with mass spectrometric analysis.

Rosenfield J, Capdevielle J, Guillemot JC, Ferrara P. Anal Biochem 203 (1992) 173–179.

Shevchenko A, Jensen ON, Podtelejnikov A, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H, Mann M. Proc Natl Acad Sci USA 93 (1996) 1440–1445.

Moritz RL, Eddes JS, Reid GE, Simpson RJ. Electrophoresis 17 (1996) 907–917.

Gevaert K, Vandekerckhove J. Electrophoresis 21 (2000) 1145–1154.

Sechi S, Chait BT. Anal Chem 70 (1998) 5150–5158.

The cysteine residues become modified with acrylamide monomer to form the β-propionamide derivative, a mass difference of 71 Da. The modification of the cysteine with iodoacetamide (a mass difference of 57 Da) and 4-vinylpyridine, a mass difference of 105 is generally performed (see Table 1.7).

The in-gel digestion of the embedded protein can be performed either manually or automatically. Staudenmann *et al.* (1998) reported that the digestion of proteins above 10 pmol was routine, giving few problems. However, once the concentration dropped below the 5 pmol level the number and yield of the peptides drops significantly, suggesting sample loss onto vessel surfaces and digestion efficiency as potential reasons for the disparity.

Staudenmann W, Dainese-Hatt P, Hoving S, Lehman A, Kertesz M, James P. Electrophoresis 19 (1998) 901–908.

Some major considerations involved with in-gel digestion that ultimately affect MS performance may include:

- Digestion efficiency:
  - Substrate-enzyme kinetics;
  - Delivering the enyme to the protein whilst minimizing autolysis.
- Extraction efficiency of peptides from the polyacrylamide gel matrix after digestion;
- Sample handling;
- Losses during the digestion procedure, for example onto the walls of vessels;
- Presence of detergents or staining agent;
- Minimizing contamination with other proteins, such as keratin.

Before addition of the enzyme to the gel plug, the gel plug (containing the embedded protein) is dehydrated using successive washes of acetonitrile and subsequent dried under vacuum. The dehydration of the gel plug to a completely dry dust-like material will enable efficient uptake of the enzyme when the gel plug is rehydrated. A sufficient volume of the enzyme in the digestion buffer (volatile buffer at the optimum pH for enzyme activity) is applied to rehydrate the gel plug. The enzyme has to passively diffuse through the gel matrix and digest the protein before autolysis has a significant effect. The rehydration step of the digestion procedure can be performed on ice (Shevchenko *et al.* 1996) presumably to minimize autolysis of the trypsin whilst it diffuses through the gel during the rehydration step. Additionally, the modified form of the enzyme can be purchased to minimize autolysis.

Shevchenko A, Jensen ON, Podtelejnikov A, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H, Mann M. Proc Natl Acad Sci USA 93 (1996) 1440–1445.

Upon completion of the digest reaction, between 3 and 24 hours, the resultant digested peptides need to be extracted from the gel matrix. To enhance peptide recovery, the inclusion of detergents such as SDS, Tween 20, Triton X-100 or NP-40 in the extraction buffer has been reported. However, the presence of detergent was detrimental to MS analysis, both for matrix-assisted laser desorption ionization and electrospray ionization (see Section 3.1). Instead, high peptide recovery can be obtained from the gel matrix by passive diffusion using

acidic and organic solvents, namely solutions of trifluoroacetic acid and acetonitrile.

Once extracted the peptides have to be delivered to the mass spectrometer in a form, which enables optimal MS performance. As described in Section 3.1 though MALDI and ESI MS are highly applicable to the analysis of biomolecules they are considerably different techniques, requiring different sample preparation approaches. MALDI involves the analysis of the sample from a remote, solid surface after co-crystallization with the matrix, whilst ESI is a liquid inlet system requiring the sample to be presented in the liquid state. Thus ESI is effectively coupled with separation techniques such as capillary electrophoresis and particularly high performance liquid chromatography (HPLC).

Unfortunately, the extracted peptides tend to be in a large volume after extraction (>50 μL) and this is not ideal for MALDI or ESI analysis, especially if the sample is low in abundance. Hence the sample needs to be concentrated. However, extraction of the digested peptides also extracts unwanted material from the gel which may hamper MS ionization and reduce MS performance.. Simply concentrating the gel extracts is insufficient, as both the contaminants and analyte will be concentrated. Also, concentrating the sample by drying in vacuum or by lyophilization can be problematic because of irrecoverable sample loss on the walls of the digestion vessel; this is particularly relevant for high sensitivity analysis.

An efficient approach to removing the salts, buffers and unwanted contaminants from the digestion process is to use reversed phase chromatography (RP-HPLC) off line prior to MALDI MS. In such a method using a RP 300 μm × 15 cm column, the peptides of interest can generally be eluted into two or three fractions in a concentrated volume (~4–5 μL) for subsequent MALDI MS analysis. In addition, the eluate, if eluted with the correct solvent, is ideally suited for ESI MS analysis.

A quicker solution for both MALDI and ESI analysis can be achieved by binding the digested peptides to small amounts of reversed phase resin packed into gel-loader pipette tips in a microscale purification. This not only has the desired effect of desalting, but also concentrates the sample in a suitable volume for the MALDI analysis (Kussmann *et al.* 1997) Similarly, this approach can be applied to on-target desalting, a method described by Gevaert *et al.* (1998).

Stensballe and Jensen (2001) described a methodology where the digestion and MALDI analysis was performed on the MALDI target, minimizing sample manipulation and losses.

Alternatively a small aliquot (<0.5 μL) can be spotted directly from the digestion mixture onto a pre-formed layer of matrix (α-cyano-4-

Kussmann M, Nordhoff E, Rahbek-Nielsen H, Haebel S, Rossel-Larsen M, Jakobsen L, Gobom J, Mirgorodskaya E, Kroll Kristensen A, Palm L, Roepstorff P. J Mass Spectrom 32 (1997) 593–601.

Gevaert K, De Mol H, Sklyarova T, Houthaeye T, Vandekerckhove J. Electrophoresis 19 (1998) 909–917.

Stensballe A, Jensen ON. Proteomics 1 (2001) 955–966.

Vorm O, Roepstorff P, Mann M. Anal Chem 66 (1994) 3281–3287.

hydroxy-cinnamic acid) and nitrocellulose. After the sample has dried, the salts from the digestion reaction can be effectively removed by washing with 0.1%TFA (Vorm *et al*. 1994; Jensen *et al*. 1996).

Jensen ON, Podtelejnikov A, Mann M. Rapid Commun Mass Spectrom 10 (1996) 1371–1378.

1.7.2.2 **Protein Cleavage – Chemical Methods**

Chemical methods of protein cleavage are complementary to proteolysis, though not used as commonly. They are often used for specific applications where no enzyme is available or proteolysis is not appropriate, such as cyanogen bromide cleavage of insoluble or membrane proteins. In this instance, a chemical fragmentation in an organic solvent capable of solubilizing the proteins is an attractive alternative (Wasburn *et al*. 2001). Cyanogen bromide cleaves specifically at methionine residues yielding relatively large peptides. Similarly large peptides can be expected if cleavage at tryptophan residues is performed with 2(2-nitrophenylsulfonyl-3-indolenine) (BNPS-skatole).

Washburn MP, Wolters D, Yates JR. Nat Biotechnol 19 (2001) 242–247.

Acid hydrolysis of proteins with a mild formic acid solution has been reported to be an effective method. The formic acid solution is a good solvent for a whole range of proteins, and yields a specific cleavage at aspartyl residues.

Li A, Sowder RC, Henderson LE, Moore SP, Garfinkel DJ and Fisher RJ. Anal Chem 73 (2001) 5395–5402.

See Table 1.9 for further examples of chemical methods of protein cleavage

Tab. 1.9: Chemical reagents for protein cleavage

Chemical reagent	Site of cleavage
Cyanogen bromide	C-terminus of methionine
Acid hydrolysis	Cleavage at aspartyl residues
Hydroxylamine	Cleavage of asparagine–glycine bonds
BNPS-skatole	Cleavage at tryptophan

2
Liquid Chromatography Techniques

This chapter gives inspiration, hints and ideas on how to tackle the challenges in proteomics by applying the tools of liquid chromatography (LC), a complementary approach to the already well established gel-based workflow.

In order to overcome some of the existing limitations of 2D electrophoresis the need for complementary approaches becomes apparent.

Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR III. Nature Biotech 17 (1999) 676–682.

It is obvious that the capabilities of liquid chromatography are investigated (Link *et al.* 1999). There is evidence that LC-based methods can close some of the gaps and deliver supplementary information if we state that 2D electrophoresis is the established standard method. However, we have always to keep in mind that protein separation is not just a tool by itself rather than an important intermediate step towards protein identification and characterization by mass spectrometry.

2.1
Basic Principles of Important Liquid Chromatography Techniques

Proteins are separated using chromatography techniques according to differences in their specific properties, as shown in Table 2.1. The following chapter is based on information for protein purification and adapted to the requirements of protein pre-fractionation in proteome analysis.

GE Healthacre "Protein Separations' Handbook Collection"

Tab. 2.1: Separation principles in protein chromatography.

Property	Technique
Charge	Ion exchange chromatography, chromatofocusing
Size	Gel filtration
Hydrophobicity	Reversed phase chromatography, hydrophobic interaction chromatography
Biorecognition (ligand specificity)	Affinity chromatography

Originally developed for the purpose of protein purification, these methods are also of paramount importance to pre-fractionate and de-complexify crude samples in proteome analysis.

Knowing that there exist numerous methods for protein separation, the following chapters cover only the most relevant LC techniques for protein pre-fractionation.

Besides personal preferences for one or the other chromatography technique there are more objective selection criteria available (see Table 2.2). It is not only important to look at the performance of a single technique, but also it is even more important to plan and consider how to best interconnect the methods in order to bypass avoidable intermediate steps that bare the risk of sample losses that lead to an unwanted reduction in protein recovery.

Tab. 2.2: Selection criteria of LC methods for protein pre-fractionation in proteomics.

	IEX	RPC	AC	GF
Enrichment	High	High	Very high	Dilution
Loadability	Very high	Medium	Very high	Low
Peak capacity	Medium	High	N/A	Low
Desalting capability	N/A	Yes	N/A	Yes
Raw sample compatibility	Very good	Limited	Excellent	Good
Running costs (column/ eluents)	High/low	Moderate/ high	Low/low to excessive/ high	High/low
Volatile buffer	No	Yes	No	Possible

Ion exchange chromatography is the first choice.

Unless there are meaningful affinity columns or media available (e.g. high-abundancy protein depletion for serum, selective glyco- or phospho-protein enrichment) ion exchange chromatography is the first choice to apply the initial sample.

Columns for protein separations are somewhat cost intensive, but with a favorable life time if handled with care.

No matter what sample concentration, volume or amount, IEX works perfectly at any scale. Columns in all different dimensions packed with the same identical material are available to enable seamless up- or down-scalability. Diluted samples are efficiently concentrated and separated with high resolution and reasonable peak capacity. The method can easily be performed under native or denaturing and/or reducing conditions, depending on the requirements of the sample.

> ***In proteome analysis, the preservation of biological activity could be of minor importance, as long as recovery is high.***

Due to its triple functionality RPC concentrates diluted samples and efficiently releases samples from salt, urea or other additives prior to

separating the proteins at highest possible resolution and is therefore an excellent consecutive step for all salt containing fractions. Furthermore, due to the use of volatile solvents RPC fractions can directly be evaporated or lyophilized, prior to tryptic digestion, in the same vessel or microtiter plate without unnecessary sample transfer steps.

Affinity chromatography is one of the most selective and specific separation techniques. Numerous ligands, both commercial and self-made, are available to isolate or remove a variety of proteins and protein classes. Dealing with this subject in detail would go beyond the aim of this book. Some dedicated proteomics applications, as the enrichment of phospho- and glyco-proteins or peptides as well as the depletion of high-abundant plasma proteins are described below.

Gel filtration is probably the most simple to perform LC technique. But, due to its low loadability, low resolution and peak capacity, in combination with its diluting rather than concentrating character it is not one of the first methods to consider in proteome analysis. Nevertheless, under special conditions it might be worth to consider.

2.1.1 Ion Exchange Chromatography

IEX is used from micro-scale separations and analysis through to fractionation of micrograms, milligrams, grams of sample and more.

Ion exchange chromatography (IEX) for the separation of proteins was introduced in the 1960s and continues to play a major role in the separation of proteins. Today, IEX is one of the most frequently used techniques for the separation of proteins and peptides offering high resolution and loading capacity. The technique is capable of separating molecular species that have only minor differences in their charge properties, for example two proteins differing by one charged amino acid. These features make IEX well suited for concentration, enrichment, isolation and fractionation.

This chapter provides a general introduction to the theoretical principles that underlie every ion exchange separation. An understanding of these principles will enable the separation power of ion exchange chromatography to be fully appreciated.

IEX separates molecules on the basis of differences in their net surface charge.

Practical aspects of performing IEX separations related to proteome analysis are covered in Chapter 2.3.

2.1.1.1 Charge on Proteins

The charge on proteins arises from some of the amino acid side-chains, as well as the carboxyl and amino termini, bound ions, and some prosthetic groups.

The charge on amino acid side-chains depends on the pH of the solution and the pK_A of the side-chains. It is also affected by the microenvironment around a side-chain. We assume the following:

pK_A values for ionizable groups on the protein and that the side-chains will have the same pK_A values regardless of their environment within the protein (Table 2.3). We also assume that the separation is based on the total net charge of the protein. Therefore, a protein with a charge of –15 will bind more tightly to an anion exchanger than a protein with a charge of –10.

Tab. 2.3: pK_A values of charged amino acids, C- and N-terminus.

Group	pK_A
Acids	
Carboxyl terminus	3.1
Aspartate	4.4
Glutamate	4.4
Cysteine	8.5
Tyrosine	10.0
Bases	
Amino terminus	8.0
Lysine	10.0
Arginine	12.0
Histidine	6.5

The charge on these proteins can be calculated. In brief, when the pH is less than the pK_A of a group, the protonated form of the group predominates. This leaves the acidic side-chains with a charge approaching 0 and the basic side-chains with a charge approaching a limiting value of +1. Conversely, when the pH is greater than the pK_A of a group, the deprotonated form predominates, giving acidic side-chains a charge approaching –1 and basic side-chains a charge approaching 0. The charge on the protein is the sum of the charges on the individual amino acid side-chains.

Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge, charge density and surface charge distribution. The charged groups within a molecule that contribute to the net surface charge possess different pK_A values depending on their structure and chemical microenvironment.

Since all molecules with ionizable groups can be titrated, their net surface charge is highly pH dependent. In the case of proteins, which are built up of many different amino acids, containing weak acidic and basic groups.

Each protein has its own unique net charge versus pH relationship which can be visualized as a titration curve. Their net surface charge

will change gradually as the pH of the environment changes as proteins are amphoteric. The titration curve reflects how the overall net charge of the protein changes according to the pH of the surroundings. Figure 2.1 illustrates some theoretical protein titration curves (these curves can be generated using an isoelectric focusing gel, but in practice titration curves are rarely used).

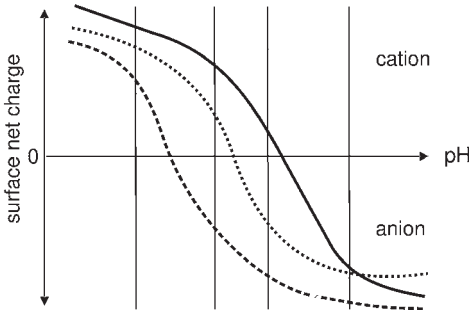


Fig. 2.1: Theoretical protein titration curves, showing how net surface charge varies with pH.

IEX chromatography takes advantage of the fact that the relationship between net surface charge and pH is unique for a specific protein. In an IEX separation reversible interactions between charged molecules and oppositely charged IEX media are controlled in order to favor binding or elution of specific molecules and achieve separation.

A protein that has no net charge at a pH equivalent to its isoelectric point (pI) will not interact with a charged medium.

However, at a pH above its isoelectric point, a protein will bind to a positively charged medium (anion exchanger) and, at a pH below its pI to a negatively charged medium (cation exchanger). In addition to the ion exchange interaction, other types of binding may occur. These effects are mainly due to van der Waals forces and non-polar interactions, which occurs frequently with hydrophobic proteins and peptides.

2.1.1.2 **Steps in an IEX Separation**

An IEX column comprises a matrix of ideally spherical particles substituted with ionic groups that are negatively (cationic) or positively (anionic) charged (see Figure 2.2).

The matrix is usually porous to give a high internal surface area. In small- and micro-scale separations also non-porous media are used.

The medium is filled into a column to form a packed bed. The bed is then equilibrated with buffer which fills the pores of the matrix and the space in between the particles.

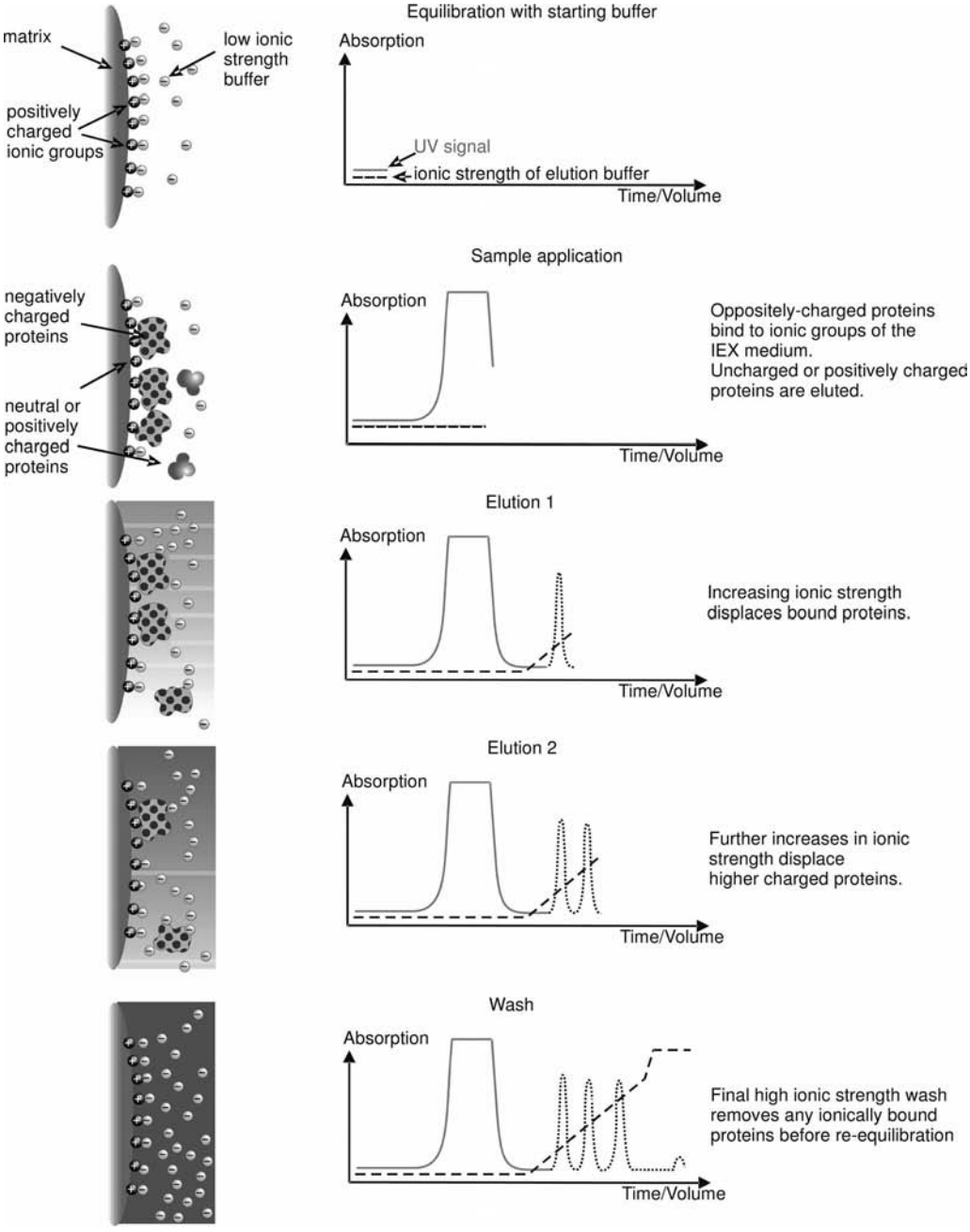


Fig. 2.2: Principles of an anion exchange separation.

The pH and ionic strength as well as the other composition of the equilibration buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the column. The proteins which bind are effectively concentrated onto the column while proteins that do not have the correct surface charge pass through the column with the flow of buffer, eluting during or just after sample application.

Note: ***The condition of the sample is very important in order to achieve the most effective high-resolution separations and make the most of the high loading capacity. Ideally, samples should be in the same condition as the starting buffer.***

When the entire sample has been loaded and the column washed so that all non-binding proteins have passed through the column (i.e. the UV signal has returned to baseline), conditions are altered in order to elute the bound proteins.

Most frequently, proteins are eluted by increasing the ionic strength (salt concentration) of the buffer.

As ionic strength increases, the salt ions (typically Na^+ or Cl^-) compete with the bound proteins for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases. Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last.

The higher the net charge of the protein, the higher the ionic strength that is needed for elution.

By controlling changes in ionic strength using different forms of gradient, proteins are eluted differentially in a purified, concentrated form. A wash step with very high ionic strength buffer removes most tightly bound proteins and impurities at the end of an elution. The column is then re-equilibrated in start buffer before applying another sample in the next run.

2.1.1.3 **Selectivity**

Good selectivity (the degree of separation between peaks) is a more important factor than high efficiency in determining resolution (see Figure 2.3) and depends not only on the nature and number of the functional groups on the matrix, but also on the experimental conditions, such as pH (influencing the protein charge), ionic strength and elution conditions. It is the ease and predictability with which these experimental conditions can be manipulated, when using a suitably designed chromatography medium, which gives IEX the potential of extremely high resolution.

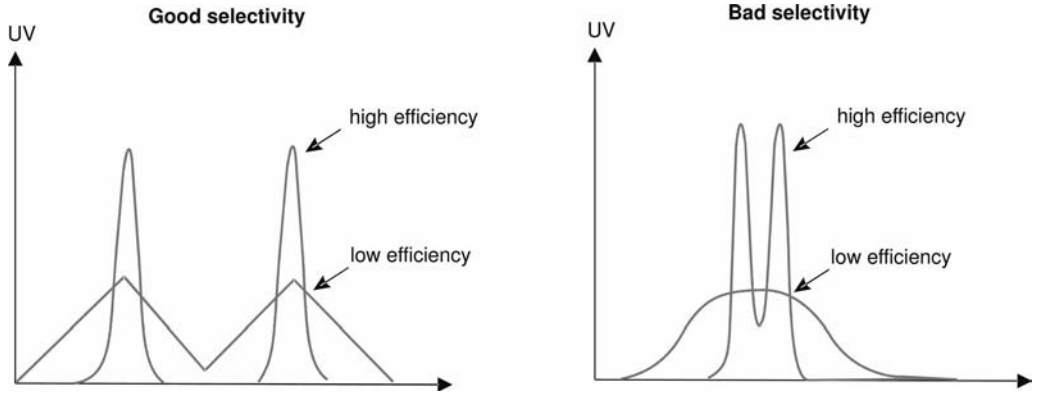


Fig. 2.3: Effect of selectivity and efficiency on resolution.
From GE Healthcare handbook: "Hydrophobic Interaction and Reversed Phase Chromatography. Principles and Methods".

2.1.1.4 **Selectivity and pH**

In protein separation good selectivity is achieved by performing IEX separations at pH values carefully selected to maximize the differences in net charge of the components of interest. Optimum selectivity can be expected at a pH where there is maximum separation between the titration curves for the individual proteins (i.e. the difference in net charges between the species is greatest) and when using an ion exchanger with a charge opposite to the charge of the proteins at the particular pH. The order in which proteins are eluted cannot always be predicted with absolute certainty since a titration curve (produced in practice by measuring electrophoretic mobility in a gel) reflects the total net charge on a protein and IEX chromatography depends on the net charge on the surface of the protein.

A pH interval of plus/minus 1.5 pH unit apart from the physiological pH seems to be reasonable.

For the purpose of protein pre-fractionation where in principle all proteins must be isolated, which in practice is impossible, a pH is selected with regard to protein stability and recovery for all, or at least the majority of the proteins.

2.1.1.5 **Selectivity and Elution**

The figures below illustrate the most common forms of IEX separation in which proteins are eluted by increasing the ionic strength of a buffer (typically with NaCl) using linear gradient or step elution. The UV absorbance and conductivity traces show the elution of protein peaks and the changes in salt concentration, respectively, during elution.

Buffer volumes used during sample application, elution, washing and re-equilibration are expressed in column volumes, for example 5 CV=5 mL for a column with a 1 mL bed volume.

Using column volumes to describe a separation profile facilitates method development and transfer of methods to columns of different dimensions when scaling-up or scaling-down.

Gradient elution is used when starting with an unknown sample (as many components as possible are bound to the column and eluted differentially to see a total protein profile) and for high-resolution separation that are required in protein pre-fractionation.

2.1.1.6 **Components of Ion Exchange Media**

Chromatography media for ion exchange are made from porous or non-porous matrices, chosen for their physical stability, their chemical resistance to stringent cleaning conditions and their low level of non-specific interaction. The matrices are substituted with functional groups that determine the charge of the medium.

Matrix

- High porosity offers a large surface area covered by charged groups and so ensures a high binding capacity. High porosity is also an advantage when separating large proteins. Non-porous matrices are preferable for extremely high-resolution separations when diffusion effects must be kept to a minimum.
- An inert matrix minimizes non-specific interactions with sample components.
- High physical stability ensures that the volume of the packed medium remains constant despite extreme changes in salt concentration or pH, thus improving reproducibility and avoiding the need to repack columns.
- High physical stability and uniformity of particle size facilitate high flow rates, particularly during cleaning or re-equilibration steps, to improve throughput and productivity.
- High chemical stability ensures that the matrix can be cleaned using stringent cleaning solutions if required.
- Modern IEX media use polymer-based matrices to fulfill not only the requirements for high binding capacity, chemical and physical stability,

but to generate media with suitable particle sizes for a range of applications (Table 2.4).

Tab. 2.4: Ion exchange matrices.

Product	Form	Mean particle size
MiniBeads™	Polystyrene/divinyl benzene	3 µm
MonoBeads™	Polystyrene/divinyl benzene	10 µm
SOURCE™ 15	Polystyrene/divinyl benzene	15 µm

MiniBeads is a matrix made from polystyrene, with divinyl benzene as cross-linker, to produce highly spherical (monodispersed), very small (3 µm), non-porous particles that facilitate micro-preparative or analytical separations when extremely high resolution is more important than high binding capacity or high flow rates.

The most suitable matrix can be selected according to the degree of resolution, binding capacity and flow rates desired for the separation.

MonoBeads and SOURCE are matrices made from polystyrene with divinyl benzene to produce highly spherical (monodispersed), small (10–15 µm), porous particles

Functional groups The functional groups substituted onto a chromatographic matrix (Table 2.5) determine the charge of an IEX medium i.e. a positively charged anion exchanger or a negatively charged cation exchanger.

Tab. 2.5: Functional groups used on ion exchangers.

Anion exchanger	Functional group
Quaternary ammonium (Q), strong	$-O-CH_2N^+(CH_3)_3$
Diethylaminoethyl (DEAE), weak	$-O-CH_2CH_2N^+H(CH_2CH_3)_2$

Cation exchanger	Functional group
Sulfopropyl (SP), strong	$-O-CH_2CHOHCH_2OCH_2CH_2CH_2SO_3^-$
Methyl sulfonate (S), strong	$-O-CH_2CHOHCH_2OCH_2CHOHCH_2SO_3^-$
Carboxymethyl (CM), weak	$-O-CH_2COO^-$

The terms strong and weak do not refer to the strength with which the functional groups bind the proteins.

The terms strong and weak refer to the extent that the ionization state of the functional groups varies with pH.

Strong ion exchangers show no variation in ion exchange capacity with change in pH. These exchangers do not take up or lose protons with changing pH and so have no buffering capacity, remaining fully

charged over a broad pH range. Strong ion exchangers include Q (anionic), S and SP (cationic).

There are several advantages to working with strong ion exchangers:

- Development and optimization of separations is fast and easy since the charge characteristics of the medium do not change with pH.
- The mechanism of interaction is simple since there are no intermediate forms of charge interaction.
- Sample loading (binding) capacity is maintained at high or low pH since there is no loss of charge from the ion exchanger.

The majority of proteins have isoelectric points within the range 5.5 to 7.5, i.e. the first choice would be to start with a strong anion exchanger at physiological or slightly basic pH. Tris with a pK of 8.3 is ideal for buffers with a pH between 7.2 and 9.0.

2.1.1.7 Binding Capacity and Recovery

The capacity of an IEX medium is a quantitative measure of its ability to take up counter-ions (proteins or other charged molecules). The total ionic capacity is the number of charged functional groups per mL medium, a fixed parameter of each medium. Of more practical relevance is the actual amount of protein which can bind to an IEX medium, under defined experimental conditions. This is referred to as the available capacity of a medium for a specific protein. If the defined conditions include the flow rate at which the medium was operated, the amount bound is referred to as the dynamic capacity for the medium. The available and dynamic capacities depend upon the properties of the protein, the IEX medium and the experimental conditions.

In practice, in order to achieve best possible performance, a protein load of 10 to 20% of the dynamic capacity is recommended.

The capacity of an IEX medium will vary according to the molecular size of the specific protein (which affects its ability to enter all the pores of the matrix) and its charge/pH relationship (the protein must carry the correct net charge at a sufficient surface density at the chosen pH). With earlier ion exchange media, larger proteins had limited access to the functional groups, significantly reducing the binding capacity. Nowadays, state-of-the-art macroporous polymer-based ion exchange matrices have exclusion limits for globular proteins in excess of 1×10^6 and are therefore suitable for the majority of biomolecule separations. Binding capacities will still vary according to the

molecular size of the proteins. For example, a matrix with a high degree of small pores will exhibit a higher binding capacity for smaller molecules. Experimental conditions such as pH, ionic strength, counter-ion, flow rate and temperature should all be considered when comparing binding capacities of different IEX medium. Modern IEX media show very low levels of non-specific adsorption so that sample recovery under suitable separation conditions is very high, typically between 90–100%.

2.1.2 Reversed Phase Chromatography

GE Handbook "Reversed Phase Chromatography, Principles and Methods"

GE Handbook "Hydrophobic Interaction and Reversed Phase Chromatography, Principles and Methods"

The following section on reversed phase chromatography (RPC) is based on information material included in the GE Handbook "Reversed Phase Chromatography" and modified to the requirements of protein pre-fraction in proteome analysis.

Unless precipitation occurs, denaturation is not a problem when using RPC to separate proteins or peptides for primary structure determination and proteome analysis.

In the presence of non-polar solvents proteins are more likely to lose activity than peptides. The interaction of proteins or polypeptides with a hydrophobic surface in the presence of organic solvents generally leads to some loss of tertiary structure, often giving rise to different conformational states that may interact differently with an RPC medium. However, denaturation and consequent loss of activity can be minimized by returning the protein to conditions that favor the native structure, as demonstrated by the widespread use of RPC for large-scale purification of recombinant and synthetic proteins and peptides, such as insulin and growth hormone.

Adsorption chromatography depends on the chemical interactions between solute molecules and specifically designed ligands chemically grafted to a chromatography matrix. Over the years, many different types of ligands have been immobilized to chromatography supports for biomolecule separation, exploiting a variety of biochemical properties ranging from electric charge to biological affinity. An important addition to the range of adsorption techniques for preparative chromatography of proteins has been reversed phase chromatography in which the binding of mobile phase solute to an immobilized n-alkyl hydrocarbon or aromatic ligand occurs via hydrophobic interaction.

Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character, such as proteins and peptides, can be separated by reversed phase chromatography with excellent resolution and recovery. In addition, the use of ion pairing modifiers in the mobile phase allows reversed phase chromatography of charged solutes such as hydrophilic peptides. Preparative reversed phase chromatography has found applica-

tions ranging from micropurification of protein fragments for sequencing by mass spectrometry to process scale purification of recombinant protein products.

2.1.2.1 Theory and Mechanism of Reversed Phase Chromatography

The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic ligand, i.e. the stationary phase.

The actual nature of the hydrophobic binding interaction itself is a matter of a heated debate, but the conventional wisdom assumes the binding interaction to be the result of a favorable entropy effect. The initial mobile phase binding conditions used in reversed phase chromatography are primarily aqueous which indicates a high degree of organized water structure surrounding both the solute molecule and the immobilized ligand (see Figure 2.4). As solute binds to the immobilized hydrophobic ligand, the hydrophobic area exposed to the solvent is minimized. Therefore, the degree of organized water structure is diminished with a corresponding favorable increase in system entropy. In this way, it is advantageous from an energy point of view for the hydrophobic moieties, i.e. solute and ligand, to associate.

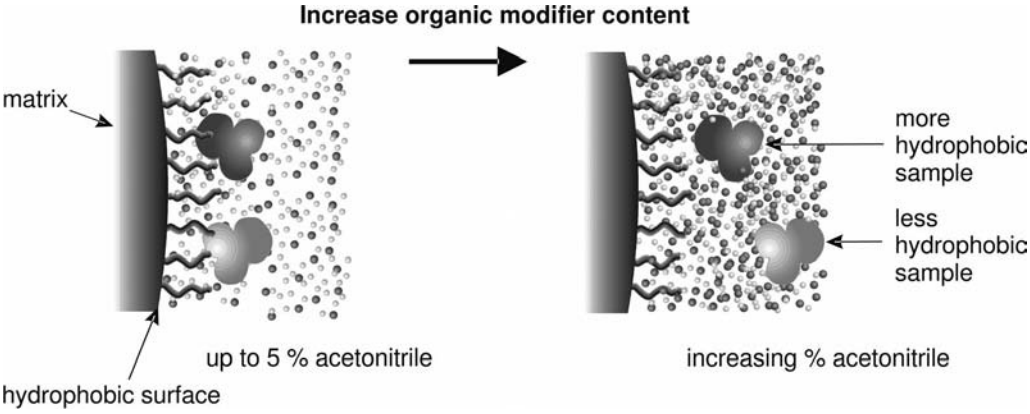


Fig. 2.4: Interaction of a solute with a typical reversed phase medium. Water adjacent to hydrophobic regions is postulated to be more highly ordered than the bulk water.

Reversed phase chromatography is an adsorptive process by experimental design, which relies on a partitioning mechanism for small peptides, in combination with an "on/off" adsorption effect for intact bigger proteins to accomplish the separation. The solute molecules partition (i.e. an equilibrium is established) between the mobile phase and the stationary phase. The distribution of the solute between the two phases depends on the binding properties of the medium, the hydrophobicity of the solute and the composition of the

mobile phase. Initially, experimental conditions are designed to favor adsorption of the solute from the mobile phase to the stationary phase. Subsequently, the mobile phase composition is modified to favor desorption of the solute from the stationary phase back into the mobile phase. In this case, adsorption is considered the extreme equilibrium state where the distribution of solute molecules is essentially 100% in the stationary phase. Conversely, desorption is an extreme equilibrium state where the solute is essentially 100% distributed in the mobile phase.

Reversed phase chromatography of proteins generally uses gradient elution instead of isocratic elution.

While proteins strongly adsorb to the surface of a reversed phase matrix under aqueous conditions, they desorb from the matrix within a very narrow window of organic modifier concentration. Along with these high-molecular-weight proteins with their unique adsorption properties, the typical biological sample usually contains a broad mixture of proteins with a correspondingly diverse range of adsorption affinities. The only practical method for reversed phase separation of complex biological samples, therefore, is gradient elution. In summary, separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase. The majority of reversed phase separation experiments are performed in several fundamental steps.

The first step in the chromatographic process is to equilibrate the column packed with the reversed phase medium under suitable initial mobile phase conditions of pH, ionic strength and polarity (mobile phase hydrophobicity). The polarity of the mobile phase is controlled by adding organic modifiers such as acetonitrile. Ion-pairing agents, such as trifluoroacetic acid (TFA) or formic acid (FA), are used in the majority of all protein and peptide separations with RPC. The polarity of the initial mobile phase (usually referred to as mobile phase A) must be low enough to dissolve the partially hydrophobic solute yet high enough to ensure binding of the solute to the reversed phase chromatographic column.

In the second step, the sample containing the solutes to be separated is applied. Ideally, the sample is dissolved in the same mobile phase used to equilibrate the stationary phase. The sample is applied to the column at a flow rate where optimum binding will occur.

Once the sample is applied, the chromatographic column is washed further with mobile phase A in order to remove any unbound and unwanted solute molecules. Bound solutes are next desorbed from the reversed phase medium by adjusting the polarity of the mobile phase so that the bound solute molecules will sequentially desorb and elute from the column.

In reversed phase chromatography this usually involves decreasing the polarity of the mobile phase by increasing the percentage of

organic modifier in the mobile phase. This is accomplished by maintaining a high concentration of organic modifier in the final mobile phase (mobile phase B). Generally, the pH of the initial and final mobile phase solutions remains the same.

The gradual decrease in mobile phase polarity (increasing mobile phase hydrophobicity) is achieved by an increasing linear gradient from 100% initial mobile phase A containing little or no organic modifier to 100% (or less) mobile phase B containing a higher concentration of organic modifier. The bound solutes desorb from the reversed phase medium according to their individual hydrophobicities.

The fourth step in the process involves removing substances not previously desorbed. This is generally accomplished by changing mobile phase B to near 100% organic modifier in order to ensure complete removal of all bound substances prior to re-using the column.

The fifth step is re-equilibration of the chromatographic medium from 100% mobile phase B back to the initial mobile phase conditions.

Separation in reversed phase chromatography is due to the different binding properties of the solutes present in the sample as a result of the differences in their hydrophobic properties.

The degree of solute molecule binding to the reversed phase medium can be controlled by manipulating the hydrophobic properties of the initial mobile phase. Although the hydrophobicity of a solute molecule is difficult to quantitate, the separation of solutes that vary only slightly in their hydrophobic properties is readily achieved. Because of its excellent resolving power, reversed phase chromatography is an indispensable technique for the high-performance separation of complex biological samples.

Typically, a reversed phase separation is initially achieved using a broad range gradient from 100% mobile phase A to 100% mobile phase B. The amount of organic modifier in both the initial and final mobile phases can also vary greatly.

> ***For peptide separations routine percentages of organic modifier are 5% or less in mobile phase A and 80% or more in mobile phase B. For intact protein separations starting with 25% organic modifier in mobile phase A and 75% or more in mobile phase B can be generally recommended. In most cases, acetonitrile is the organic modifier of choice.***

The technique of reversed phase chromatography allows great flexibility in separation conditions so that the researcher can choose to bind the solute of interest, allowing the contaminants to pass unretarded through the column, or to bind the contaminants, allowing the desired solute to pass freely. Generally, it is more appropriate to bind

the solute of interest because the desorbed solute elutes from the chromatographic medium in a concentrated state. Additionally, since binding under the initial mobile phase conditions is complete, the starting concentration of desired solute in the sample solution is not critical allowing dilute samples to be applied to the column. Ionic binding may sometimes occur due to ionically charged impurities immobilized on the reversed phase chromatographic medium, especially for non- or insufficiently end-capped silica-based media.

The combinations of hydrophobic and ionic binding effects are referred to as mixed-mode retention behavior. Ionic interactions can be minimized by judiciously selecting mobile phase conditions and by choosing reversed phase media with end-capping or based on polymers which are commercially produced with high batch-to-batch reproducibility and stringent quality control methods.

2.1.2.2 **RPC of Peptides, Polypeptides and Proteins**

The separation of peptides involves continuous partitioning between the mobile and stationary phase. Polypeptides and proteins, however, are too large to partition into the hydrophobic layer; instead, they adsorb to the hydrophobic phase after entering the column and remain adsorbed until the concentration of the mobile phase reaches the critical concentration necessary to cause desorption. They then adsorb and interact only slightly with the stationary phase as they elute down the column.

> ***Desorption takes only place within a very narrow window of organic modifier concentration. This results in complete retention until the critical mobile phase concentration is reached and sudden desorption of the protein takes place. This sudden desorption produces sharp peaks.***

The "hydrophobic foot" of a polypeptide or protein which is responsible for the separation, is very sensitive to molecular confirmation. It can be observed that proteins with intact tertiary structure elute earlier than expected compared to denatured proteins because the "hydrophobic foot" is involved in the interaction, while the rest of the protein is in contact with the mobile phase. After desorption very little interaction takes place between the protein and the reversed phase surface and subsequent interactions have little effect on the separation, i.e. column can be kept short, as column length has little effect on the separation. Short columns in combination with shallow gradients can be used very effectively to separate similar proteins. Because proteins diffuse slowly, RPC results in broader peaks than obtained with peptides.

Column dimensions The adsorption/desorption of proteins responsible for their separation takes place almost entirely near the top of the column. Therefore, column length does not significantly affect separation and resolution of proteins. Consequently, short columns of ≤5 cm in length are often used for protein separation.

Mobile phase considerations

Since decades, all in all acetonitrile as organic modifier and TFA as ion-pairing reagent has proven to be best compromise for reversed phase chromatography of peptides and proteins. For direct coupling of LC to mass spectrometry acetic or formic acid are giving better overall results due to significant higher ionization efficiency.

Propanol-2 is often used for large or very hydrophobic proteins. The major drawback of propanol-2 is its high viscosity, especially in mixtures with water. To reduce the viscosity of propanol-2, while maintaining its hydrophobic characteristics, it is recommended using mixture of 50:50 with acetonitrile. Adding 1 to 3% propanol-2 to acetonitrile has shown to increase protein recovery in some cases.

Propanol-2 is the best solvent for retaining biological activity. Ethanol and methanol are slightly worse. Acetonitrile causes the greatest loss of biological activity, however, for protein pre-fractionation of proteomic samples this is of no importance, as long as the primary structure of the molecule remains unaffected and the recovery is sufficient.

In rare cases trifluoroacetic acid (TFA) can be replaced by pentafluoropropionic acid (PFPA) or heptafluorobutyric acid (HFBA). However, the effect is minimal to non-visible.

There is no good reason to deviate from TFA as the ion-pairing reagent and invest time and money to evaluate exotic reagents. RPC coupled to MS or MS/MS may be seen as an exception and it is worth trying other ion pairing reagents to obtain a better performance in MS.

2.1.3 Affinity Chromatography

Affinity chromatography (AC) separates proteins on the basis of a reversible interaction between a protein or a group of proteins and a specific ligand coupled to a chromatography matrix, as illustrated and described in full detail in the GE Handbook "Affinity Chromatrography, Principles and Methods".

The technique offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest. Enrichment can be in the order of several thousand-fold and recoveries of active material are generally very high.

GE Handbook "Affinity Chromatography, Principles and Methods" (2002) 18-1022-29

Affinity chromatography is unique in separation technology since it is the only technique that enables the purification of a biomolecule on the basis of its biological function or individual chemical structure. Purification that would otherwise be time-consuming, difficult or even impossible using other techniques can often be easily achieved with affinity chromatography. The technique can be used to separate active proteins from denatured or functionally different forms, to isolate, concentrate and enrich pure substances present at low concentration in large volumes of crude sample and also to remove specific contaminants, such as the depletion of high-abundant proteins from plasma or serum, prior to proteome analysis.

2.1.3.1 **Affinity Chromatography in Brief**

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein or a group of proteins and a specific ligand coupled to a chromatography matrix.

The technique is ideal for concentration or enrichment steps as well as for a depletion or elimination in a separation protocol and can be used whenever a suitable ligand is available for the protein(s) of interest. With high selectivity, hence high resolution, and high capacity for the protein(s) of interest, purification levels in the order of several thousand-fold with high recovery of active material are achievable.

Biological interactions between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Figure 2.5 describes the mode of action of affinity chromatography. In a single step, affinity purification can offer immense time-saving over less selective multi-step procedures. The concentrating effect enables large volumes to be processed. Target molecules can be separated from complex biological mixtures, native forms can be separated from denatured forms of the same substance and small amounts of biological material can be purified from high levels of contaminating substances.

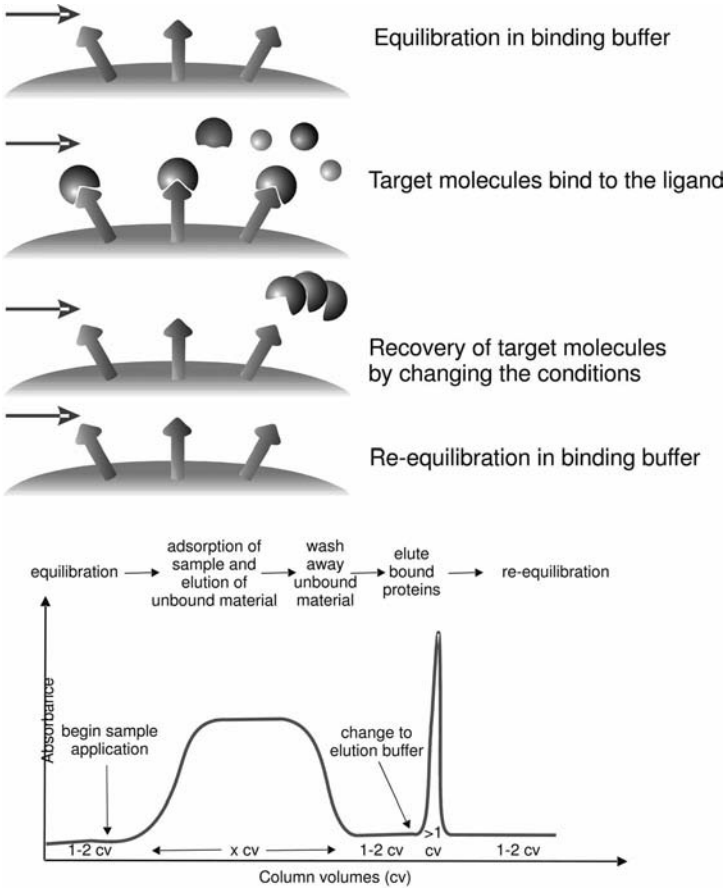


Fig. 2.5: Schematic representation of the mode of operation of affinity chromatography and a typical affinity chromatogram.

Successful affinity purification requires a biospecific ligand that can be covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be removed in an active form. Any component can be used as a ligand to purify its respective binding partner. Some typical biological interactions, frequently used in affinity chromatography for proteomics, are listed below:

- Antibody–antigen: for efficient IgG and albumin depletion from plasma or serum samples;
- Lectins: selective glycoprotein enrichment;

The high selectivity of affinity chromatography enables many separations to be achieved in a single, simple step.

- Metal ions: selective enrichment of phosphorylated peptides and proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces (IMAC).

2.1.3.2 Immuno-affinity Chromatography

Immuno-affinity chromatography utilizes antigens or antibodies as ligands (sometimes referred to as adsorbents, immunoadsorbents or immunosorbents) to create highly selective media for the depletion of e.g. the most high-abundant plasma proteins. Antibodies, immobilized to a column are also extremely useful as ligands and can be used as baits for the isolation of special proteins.

Especially for the depletion of albumin, immuno-affinity columns are superior compared to immobilized Cibacron blue, which is less selective and also binds other classes of proteins rather than albumin. Albumin, as a transporter protein, also has a strong affinity to the proteins to be transported. It is assumed that these proteins remain bound to albumin and will at least partially get lost during the depletion step.

Gundry RL, Fu Q, Jelinek CA, Van Eyk JE, Cotter RJ. Proteomics Clin Appl 1 (2007) 73–88.

Recently, a new discipline called "Albuminomics" was founded, which deals with the analysis of proteins and peptides that are bound to albumin.

The availability of commercial products is growing constantly. User of these products for the depletion of high-abundancy proteins generally state a good performance but at the same time criticize high purchase and running costs as well as too low a capacity.

Occasionally a leakage of the immobilized antibodies is reported, which results in high amounts of peptides leading to identification of immunoglobulins after MS analysis and affecting the analysis of low-abundant proteins.

2.1.3.3 Lectin Affinity Chromatography

Glycoproteins and polysaccharides react reversibly, via specific sugar residues, with a group of proteins known as lectins (see Table 2.6). As ligands for separation columns, lectins are used to isolate and separate glycoproteins, as well as other glycosylated molecules. Glycoproteins bound to the lectin column are resolved by using a gradient of ionic strength or of a competitive binding substance.

Tab. 2.6: Specificity of selected lectins.

Lectin	Specificity
Mannose/glucose binding lectins	
Con A, *Canavalia ensiformis*	Branched mannoses, carbohydrates with terminal mannose or glucose (aMan > aGlc > GlcNAc).
Lentil lectin, *Lens culinaris*	Branched mannoses with fucose linked a(1,6) to N-acetyl-glucosamine, (aMan > aGlc > GlcNAc).
N-acetylglucosamine binding lectins	
Wheat germ lectin, *Triticum vulgare*	Chitobiose core of N-linked oligosaccharides, [GlcNAc(b1,4GlcNAc)1–2> bGlcNac].

Yang Z, Hancock WS, Chew TR, Bonilla L. Proteomics 5 (2005) 3353–3366.

In addition to the phosphoproteins, glycoproteins represent another important group of proteins for the study of post-translational modifications. Although the basics and theory of using lectins to selectively isolate glycoproteins is known since decades, its application proteome analysis is still underdeveloped. The study of post-translational modifications in glycoproteins is hampered by the lack of well established methods. Recently a paper was presented that applied a multilectin affinity column for the enrichment of plasma and serum proteins coupled prior to digestion and LC-MS.

2.1.3.4 Immobilized Metal Affinity Chromatography

Immobilized metal affinity chromatography (IMAC is a special kind of affinity chromatography that is not based on biospecific recognition. A metal-chelating group [e.g. iminodiacetic acid, nitrilotriacetic acid (NTA)] is immobilized to the stationary phase of the column. Originally used for the purification of proteins and peptides with exposed amino acids, His, Cys, Trp, and/or with affinity for metal ions, IMAC today is an indisputable tool for the purification of recombinant poly-His fusion proteins.

Recently IMAC has been applied successfully in proteomics for the isolation of phosphorylated peptides and proteins in order to study the nature of post-translational modifications (PTM).

Proteins and peptides that have an affinity for metal ions can be separated using immobilized metal affinity chromatography. The metals are immobilized onto a chromatographic medium by chelation. Certain amino acids, e.g. histidine and cysteine, form complexes with the chelated metals around neutral pH (pH 6–8) and it is primarily the histidine content of a protein which is responsible for its binding to a chelated metal. Metal chelate affinity chromatography is excellent for purifying recombinant $(His)_6$ fusion proteins as well as many natural proteins. Before use the column is loaded with a solution of divalent metal ions such as Ni^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+} or Fe^{2+}. The binding reaction between the ligand and the target protein

Dubrovska A, Souchelnytskyi S. Proteomics 5 (2005) 4678–4683.

Phosphoprotein Purification Handbook, Qiagen, 2002

Morrice N. In: The Encyclopedia of Mass Spectrometry, Volume 8, Hyphenated Methods in The Encycopedia of Mass Spectrometry, (Niessen WMA, Ed), Elsevier, Amsterdam, Netherlands (2006) 930–939.

is based on coordination chemistry (chelate) and therefore pH dependent. Bound sample is, most commonly, eluted by reducing the pH and increasing the ionic strength of the buffer or by including EDTA or imidazole in the buffer.

The study of post-translational modifications is complicated by the lack of appropriate methods for a group-selective enrichment of intact phosphoproteins and -peptides. Today, IMAC can be applied successfully for their isolation at high recovery. Furthermore, this technique is both compatible with 2D electrophoresis and liquid chromatography as well as MS and can be applied to cell cultures, tissues and other samples.

Any continuative general recommendations as binding conditions, like metal ion selection, and elution conditions cannot be given here, because it would go beyond the aim of this book. These have to be customized depending on the nature and origin of the sample during the method-development phase.

In practice immobilized NTA is reported to be superior over other groups.

2.1.4 Gel Filtration

Gel filtration is the simplest and mildest of all the chromatography techniques and separates molecules on the basis of differences in size.

For more than forty years since the introduction of Sephadex™, gel filtration (GF) has played a key role in the purification of enzymes, polysaccharides, nucleic acids, proteins and other biological macromolecules.

This technique is also known as size exclusion chromatography (SEC) or gel permeation chromatography (GPC).

The technique can be applied in two distinct ways:

- Group separations: the components of a sample are separated into two major groups according to size range. A group separation can be used to remove high- or low-molecular-weight contaminants (such as phenol red from culture fluids) or to desalt and exchange buffers after tryptic digestion.
- High-resolution fractionation of proteins: the components of a sample are separated according to differences in their molecular size. High-resolution fractionation can be used to isolate one or more components or to separate monomers from complexes or randomly formed aggregates.

Gel filtration separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. Unlike ion exchange or affinity chromatography, molecules should not bind to the chromatography medium. Consequently, a significant

advantage of gel filtration is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation.

Gel filtration is well suited for proteins that may be sensitive to changes in pH, concentration of metal ions or co-factors and harsh environmental conditions. Separations can be performed in the presence of essential ions or cofactors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37 °C or in the cold room according to the requirements of the experiment. Purified proteins can be collected in any chosen buffer.

Gelfitration is not the first choice of technique in protein pre-fractionation as resolution and loading capacity is limited and the initial sample is diluted significantly.

To perform a separation, gel filtration medium is packed into a column to form a packed bed. The medium is a porous matrix in the form of spherical particles that have been chosen for their chemical and physical stability, and inertness (lack of reactivity and adsorptive properties). The packed bed is equilibrated with buffer, which fills the pores of the matrix and the space in between the particles. The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in equilibrium with the liquid outside the particles, referred to as the mobile phase. It should be noted that samples are eluted isocratically, i.e. there is no need to use different buffers during the separation. However, a wash step using the running buffer is usually included at the end of a separation to facilitate the removal of any molecules that may have been retained on the column and to prepare the column for a new run.

Figure 2.6 shows the most common terms used to describe the separation and Figure 2.7 illustrates the separation process of gel filtration.

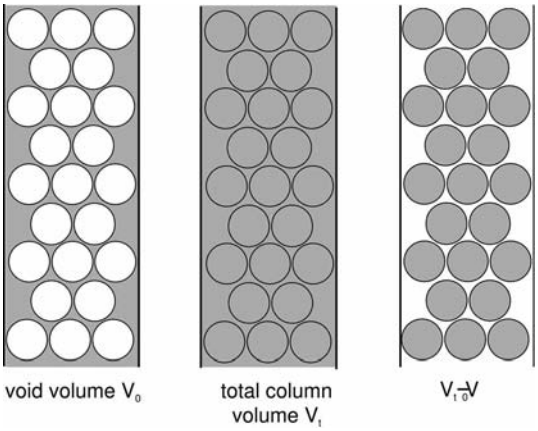


Fig. 2.6: Common terms in gel filtration. V_e: elution volume is measured from the chromatogram and relates to the molecular size of the molecule; V_o: void volume is the elution volume of molecules that are excluded from the gel filtration medium because they are larger than the largest pores in the matrix and pass straight through the packed bed; V_t: total column volume is equivalent to the volume of the packed bed (also referred to as CV).

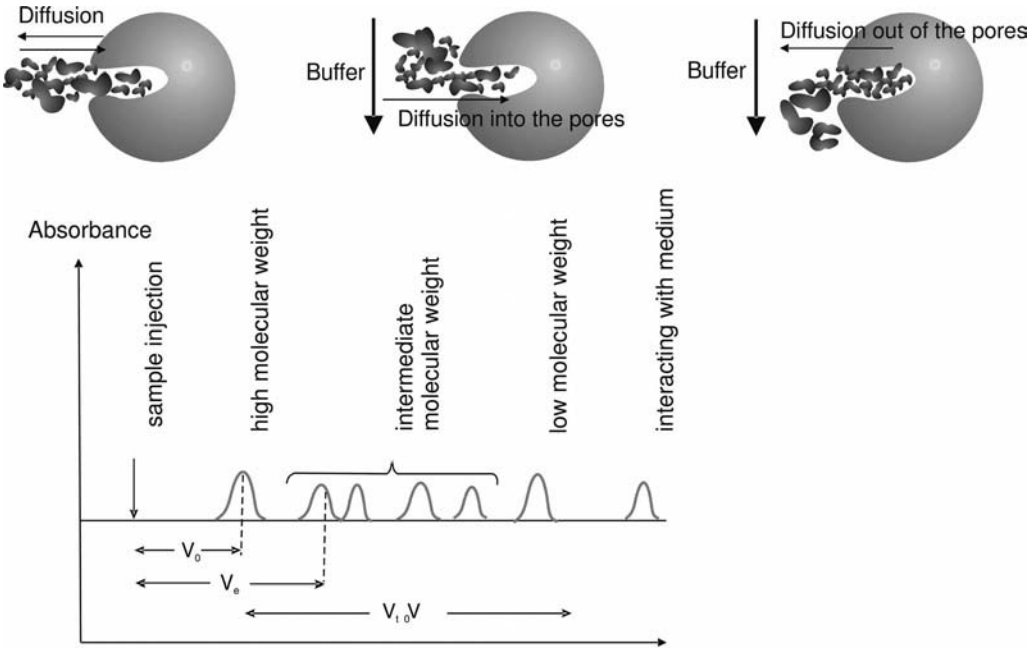


Fig. 2.7: Separation process of gel filtration.

2.2 Strategic Approach and General Applicability

It is undisputable that proteomics would not be possible without the major achievements in mass spectrometry during the last decade. Nevertheless, it is unfair to call the high-performance separation techniques further up-stream in the workflow as just sample preparation. The separation performance prior to MS has a very significant impact to the overall success rate in proteome analysis.

A remarkable simulation by computer modeling, performed by Eriksson and Fenyo (2007), disclosed some of the secrets of successful proteome analysis. Factors influencing the success rate of proteome analysis (listed in order of importance):

- Initial sample amount;
- Number of proteins in mixture (ideally ~30 to <300);
- Power of protein and peptide separation;
- Mass spectrometric dynamic range.

Eriksson J, Fenyo D. Nature Biotech 25 (2007) 651–655.

Surprisingly, the three most important success factors are not related to sensitivity, resolution and dynamic range of the MS instrumenta-

tion. Reducing sample complexity, loading more sample amount – if available – and better separation performance on protein and peptide level are the most crucial elements in the workflow. Implementing all these factors can ideally be achieved with state-of-the-art separation tools like e.g. liquid chromatography alone, but also in combination with electrophoretic techniques.

Since the mid nineties of the last century, especially in North America, LC methods like two-dimensional liquid chromatography (2DLC), an intelligent combination of ion exchange (IEX) and reversed phase chromatography (RPC), also described as MudPIT began to catch more and more attention, while the majority of European scientists continued to rely more or less exclusively on 2DE. Today the majority of scientists worldwide take advantage from LC-based methods in order to obtain more complete information complementary to 2DE. It was just logical and consequent that researchers started to compare 2DE with 2DLC.

2D or not 2D? Is that the question?

2D or not 2D? Is that the question? In fact it is not, as it is comparing apples and oranges; because 2DE separates intact proteins, whereas 2DLC separates peptides (digested proteins).

The picture below (Figure 2.8) clearly illustrates where the strengths of the various separation techniques –electrophoresis and liquid chromatography – are.

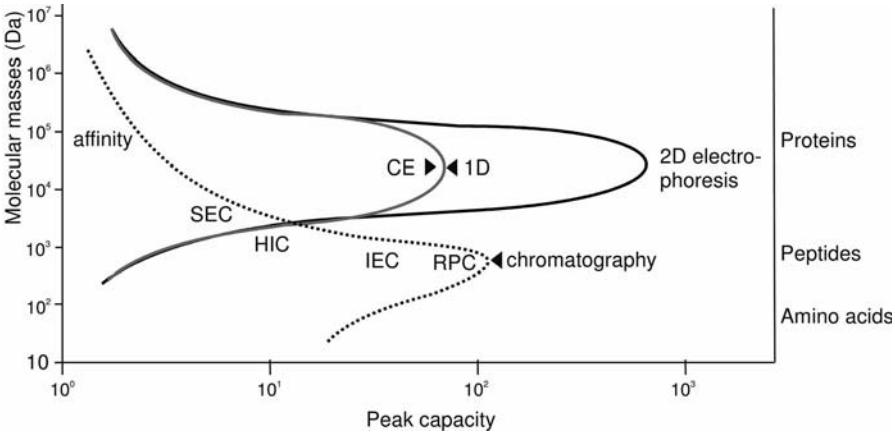


Fig. 2.8: Separation capacity of different methods depending on the molecular mass of the analytes. Modified from Lottspeich and Engels (2007).

Lottspeich F, Engels JW (Eds.) Bioanalytik, 2. Auflage, Elsevier, Spektrum Akademischer Verlag (2006)

Occasionally, there have been attempts to use LC-based methods for the separation at protein level in order to replace 2DE as well. However, until today, protein chromatography techniques cannot compete with 2DE in terms of resolution, peak capacity, and last but not least,

the visualization of the separation, post translational modifications and protein isoforms included.

However, for sample preparation purposes, such as depletion of high-abundant proteins, concentration of low-abundant proteins, group-specific enrichment, or any other type of sample de-complexification or pre-fractionation, LC is the method of choice, even for sample preparation prior to subsequent electrophoretic separations. It is obvious that reversed phase chromatography and ion exchange chromatography – or the combinations of both – are the methods of choice for the separation of digested proteins: peptides.

2.3 Liquid Chromatography Techniques and Applications in Proteome Analysis

2.3.1 Peptide Separation

For more than 20 years reversed phase chromatography (RPC) has been the most frequently used technique for peptide separations; playing a key role in protein identification and characterization. During the zenith of Edman sequencing, a technique called peptide mapping routinely used chromatography to aid protein identification and characterization. However, the development of two powerful mass spectrometry (MS) ionization techniques and subsequent instrument evolution (see Chapter 3), meant mass spectrometry and not Edman Sequencing became the key tool for proteomics applications. Along side this MS evolution, chromatography did not stand still either. The miniaturization of LC and the availability of robust, commercially available columns and instrumentation enabled peptide separation to be performed at a very high sensitivity (<100 fmol with UV detection). The reduction in column internal diameter (i.d.) to sub 100 μm specifications and the availability of nano LC systems, which could be coupled with nano electrospray sources, have been the main drivers towards higher sensitivity (see Table 2.7). This level of performance enabled the detection of much smaller amounts (<<1 fmol) of sample.

Peptide separation on-line to tandem mass spectrometry equipped with electrospray for peptide sequence analysis is routine, but application and sample dependent. The incorporation of a chromatography step has been key to achieving this and the commonly used peptide chromatographic technique is a single dimension of RPC. In recent years it has also been possible to combine an RPC peptide separation with MALDI ToF (see Section 3.1.1 on pages 220 f).

Tab. 2.7: Relationship between column i.d. and sensitivity.

Scale	Column i.d. (mm)	Typical flow rate (μL/min)	Theoretical gain in sensitivity
Analytical	4.6	1,000	1
Narrow	2.1	200	5
Micro	1	40	21
Capillary	0.3	4	237
Nano	0.075	0.2	3,322

Ideally, such a simple sample could be analyzed with nanospray alone.

If the sample of interest is rather simple, e.g. a tryptic digest of a gel spot, ideally containing a single or a few proteins, successful protein identification with a very high sequence coverage can be routinely achieved with a single dimension of RPC separation prior to MS.

A high-speed MS analyzer is beneficial.

However, as a sample becomes more complex the required resolution of the RPC separation prior to tandem mass spectrometry sequence analysis also needs to increase. In this instance, a single RPC separation prior to MS analysis may still generate a significant number of protein identifications, but the sequence coverage may drop considerably (for instance an immunoaffinity pull down experiment, followed by digestion of the 1DE gel bands – see introduction and figure 4). The question becomes, does the MS have sufficient time to analyze the peptides (i.e. perform sequence analysis) as they co-elute from the column? One possibility to address the co-elution is to employ extra high-resolution RPC with very long columns, where the system pressure approaches 10,000 psi (700 bar; 6,895 kPa). In one such publication, Shen *et al.* (2001) demonstrated that a single high-resolution RPC separation prior to tandem mass spectrometry with an FT-ICR instrument, yielded the identification of over 1,000 yeast proteins. Instead of following the approach with very long columns, which has never got a wide acceptance, one could use shorter columns packed with very small particles (1–2 μm) instead. Such a strategy, marketed as UPLC, is more promising and is currently gaining some popularity. Both, columns and dedicated instrumentation, are commercially available.

Shen Y, Zhao R, Belov ME. Anal Chem 73 (2001) 1766–1775.

Alternatively, the resolution of an RPC step can be enhanced by simply increasing the length of the RPC gradient within certain limits (0.25% acetonitrile/min increase should be the flattest gradient).

This is particularly important for the analysis of peptides deriving from low-abundant proteins.

If the sample complexity exceeds a certain level, such as with a sample deriving from of a whole cell lysate digest, the ability to efficiently separate the mixture into manageable fractions with one dimension only becomes effectively impossible. This is critical as the ability of the MS to analyze the co-eluting peptides will be seriously impaired; not only will the sequence coverage of identified proteins be reduced, but insufficient peptides will be detected and analyzed to deliver unambiguous protein identifications, significantly reducing

the number of proteins identified. Consequently, a second dimension of peptide separation is required to increase the resolution of the given sample, effectively giving the MS more time to analyze the peptides as they elute from the column.

An example of how varying the LC conditions can impact on the MS performance is given in Figure 2.9. The figure represents how the sequence coverage obtained for a particular protein in a mixture of six proteins can vary according to the LC configuration used. The summary of this exercise is that increasing the resolution of the chromatographic separation, aids the MS performance, which ultimately translates to increased sequence coverage obtained for a particular protein.

The appropriate chromatographic separation reduces sample complexity, which resultantly has a positive impact on protein identification success rates i.e. more proteins can be identified.

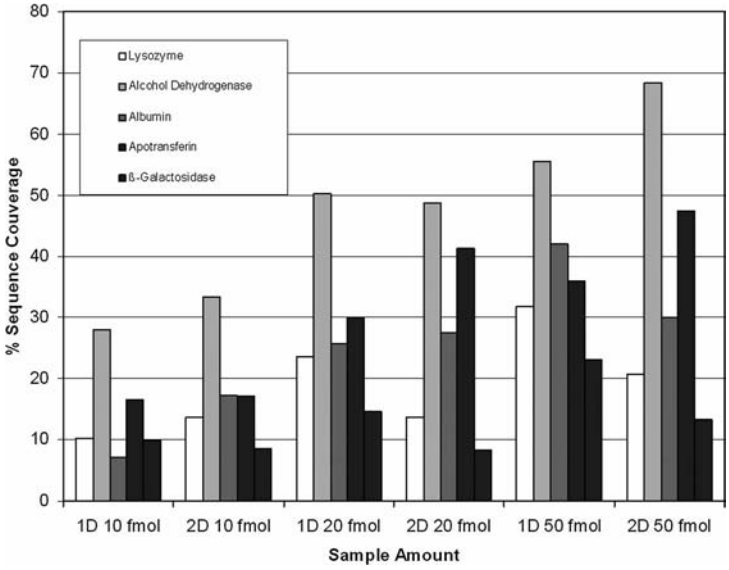


Fig. 2.9: Graph representing how the sequence coverage obtained for a particular protein in a mixture of six proteins can vary according to the LC configuration used. Courtesy of M. Berg, M-Scan.

The commonly used additional separation dimension is ion exchange chromatography (IEX), specifically strong cation exchange (SCX) and the coupling together of these two techniques is routinely known as 2DLC (IEX/RPC). The technique utilizes a peptide's unique physical properties of charge and hydrophobicity to enhance separation. The

following rule of thumb can be used to calculate the required degree of separation power:

- 1DLC (RPC) (1–50 proteins) 50–2,500 peptides
- 2DLC (IEX/RPC) (10–500 proteins) 500–25,000 peptides

2.3.2 2DLC Peptide Separation

Peptide 2DLC is the key component of MudPIT (Multidimensional Protein Identification Technology), a strategy for protein identification whereby an non-separated complex protein mixture is digested prior to LC-MS/MS analysis. The technique was pioneered for proteomics applications by Yates and co-workers (Link *et al.*, 1999). The group initially described the combination of IEX and RPC media sequentially packed into a pulled capillary column on-line to an MS equipped with ESI (see Figure 2.10). The chromatography proceeded in steps, each step consisting of an increasing, specific salt concentration, which eluted peptides off the strong cation exchange media onto the RPC compartment. In one reported example 1,484 proteins were identified in a single experiment (Washburn *et al.* 2001).

Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR 3rd. Nat Biotechnol. 7 (1999) 676–682.

Washburn MP, Wolters D, Yates JR 3rd. Nat Biotechnol. 3 (2001) 242–247

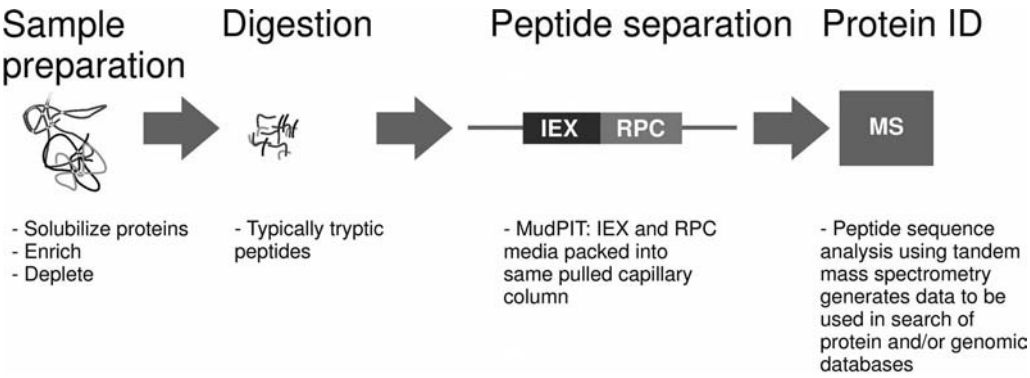


Fig. 2.10: A schematic of the MudPIT technique reported by Link *et al.* for the analysis of complex mixtures.

The technique is a non-gel based approach, and it has often been argued that it can be used as a replacement for 2DE. In reality, the technique is a powerful complementary tool to the 2DE approach as they both allow the detection of different subsets of the proteome.

A key advantage of the technique being once the sample was loaded onto the column, no manual sample handling was required. Essential method developments included identifying salt containing buffers

that were compatible with MS analysis, typical IEX buffers such as sodium chloride and potassium chloride are not. Ammonium chloride, which is volatile, was identified as a compatible buffer.

Following its introduction, the technique has evolved and a number of commercial systems have been developed, the key difference being that separate SCX and RPC columns are used. Still, the technique is relatively straightforward (see Figure 2.11). The complex protein sample is denatured, (cysteine residues) reduced and alkylated and the proteins digested, typically, with trypsin. The peptide mixture is subsequently acidified and loaded onto the SCX column. As in a typical IEX separation, positively charged peptides bind to the IEX column and the uncharged and negatively charged peptides pass in the flow through and bind to a reversed phase trap column. The peptides are subsequently eluted onto a nano (75 μm i.d.) RPC column, separated and eluted into a tandem mass spectrometer for peptide sequence analysis. Subsequently, the salt concentration is increased, either in a series of steps or via a salt gradient (see below), displacing further peptides from the IEX column onto the RP trap column and then onto the nano RPC column for elution into the MS. Each RPC separation generates multiple protein identifications in the MS analysis.

Two common variations have evolved (and supported commercially), each with its own benefits and drawbacks (see Figure 2.11), which are discussed below.

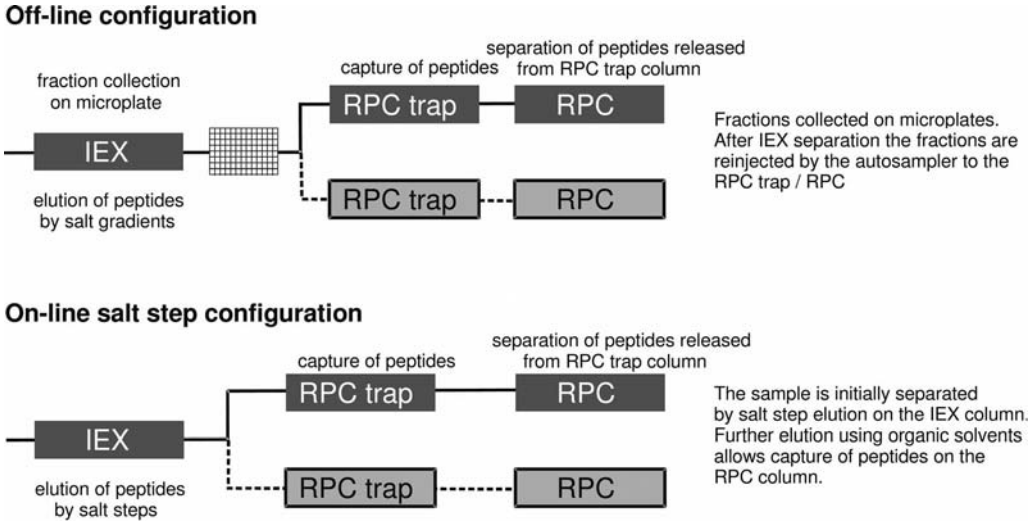


Fig. 2.11: Schematics of the two common methods for 2DLC separation of complex peptide mixtures prior to MS analysis. From GE Healthcare application note: Comparison of methods for two-dimensional liquid chromatography of peptides using Ettan microLC System. (2003) 18-1170-93.

2.3.2.1 On-line 2DLC with Salt Plugs

This configuration offers convenient, easy-to-use, and automated analysis of moderately complex protein mixtures. On-line salt-plug injection employs a combination of SCX in the first-dimension separation with RPC in the second dimension. Elution from the SCX column is performed using discrete salt plugs of increasing salt concentration. Eluted proteins are bound to the RPC trap column (while efficient desalting and concentration takes place) and subsequently separated on the second-dimension RPC column using a nanoflow gradient elution with organic solvent. Unfortunately, optimal SCX separation of peptides requires a certain concentration of acetonitrile in the buffer (as high as 30%). However this level of acetonitrile is incompatible with the RPC second dimension as the more hydrophilic peptides will not bind to the column (see Figure 2.12). As a compromise, the acetonitrile concentration in SCX is reduced to 5%, which is more compatible – by no means perfect – not for SCX nor for the subsequent RPC step. One major drawback of this method is often that the same peptide(s) elute in subsequent fractions, which generates redundant data in the MS as the same peptide is analyzed repeatedly. This observation has been made and reported by numerous laboratories.

- Advantages:
  - Fully automated operation;
  - On-line desalting and sample concentration;
  - Potentially greater sensitivity due to reduced sample loss.
- Disadvantages:
  - Sub-optimal running conditions for both SCX and RPC;
  - Binding behavior of peptides to SCX column unpredictable as strongly influenced by unspecific side effects;
  - Loss of small, hydrophilic peptides as they do not bind to the RPC trap column;
  - Distribution (smearing) of one and the same peptide to several runs;
  - Difficult to consolidate data;
  - Need to perform always a combined analysis of all runs to get a picture from a particular protein.

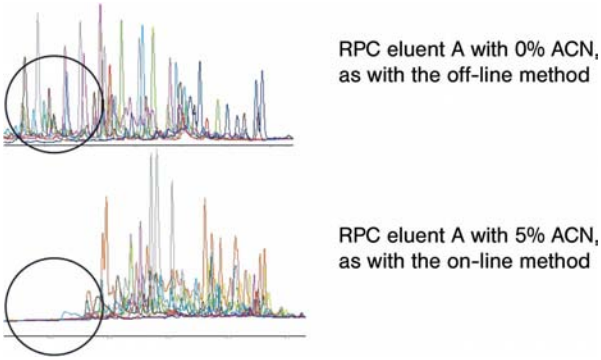


Fig. 2.12: The effect of acetonitrile used in the IEX gradient for retention and recovery of peptides in 2DLC analysis. Note that hydrophilic peptides get lost if starting with 5% acetonitril in eluent A, because they will not be bound by the RPC trap column. From GE Healthcare application note: Comparison of methods for two-dimensional liquid chromatography of peptides using Ettan microLC System. (2003) 18-1170-93.

2.3.2.2 **Off-line 2DLC**

This semi-automated off-line method gives the best overall performance in terms of protein ID and sequence coverage. This method allows greater flexibility in terms of total amount of sample loaded, column dimension, and flow rate in the first-dimension SCX step. Separation under optimal conditions for each dimension with no losses of small hydrophilic peptides. Fractions are collected after SCX prior to the RPC step. The off-line SCX/RPC method enables greater freedom to choose selected fractions of interest for further analysis by MS/MS. The key advantages of this configuration are that the SCX and RPC separations can be optimized individually, not compromised and there is minimal overlap of peptides between fractions which results in less redundant data.

- Advantages:
  - Best conditions for maximum resolution and peak capacity at both dimensions;
  - Free choice of buffer system and sample volume can be adapted to RPC;
  - Higher resolution compared to salt step or dual-gradient elution;
  - Fast method despite being an off-line method, SCX is a single, continuous run;
  - Minimal overlap of peptides between fractions;

  - Store SCX fraction for re-analysis;
  - Higher sample load on the SCX dimension, allows for higher sensitivity.
  - When the first dimension SCX is finished, priority fractions can be selected (e.g. through visual inspection of the UV trace) and moved forward to LC-MS
- Disadvantages:
  - Not fully automated, requires an acetonitrile evaporation (e.g. SpeedVac®) or dilution step prior to the injection on to the second dimension RPC trap column.

A comparison between high-throughput 1D RPC and the two common 2D-LC variations has been performed in the analysis of rat spermatozoa. The rat spermatozoa proteome is estimated, from the number of spots observed in 2D-PAGE analysis, to consist of about 2,000 proteins.

Chromatograms from the three configurations are shown in Figure 2.13.

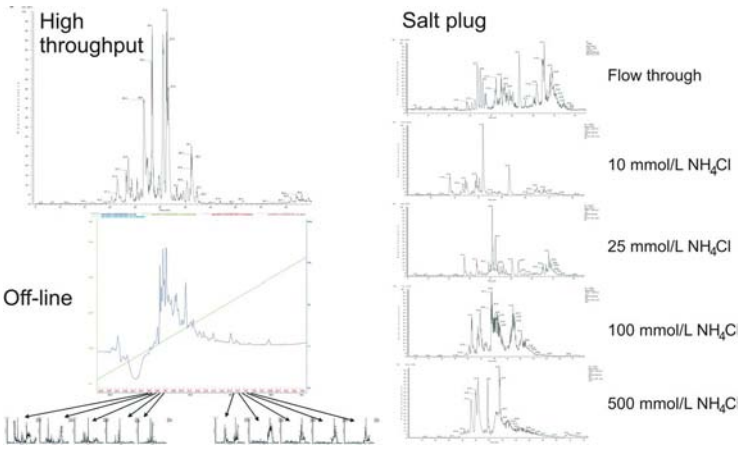


Fig. 2.13: Chromatograms obtained for the analysis of rat spermatozoa using high-throughput 1D RPC, on-line 2DLC, and off-line 2DLC. Courtesy of GE Healthcare.

By adding an orthogonal separation technique (typically IEX) to RPC, the separation power of the method increases substantially. One way to describe separation power is to use peak capacity.

Orthogonal means the combination of consecutive separation techniques according to different independent physicochemical properties of proteins / peptides.

Peak capacity for a one-dimensional separation is defined as:

$$PC = (t_{last} - t_{first})/w_{1/2}$$

where t_{last} is the retention time of the last eluting peak, t_{first} is the retention time of the first eluting peak, and $w_{1/2}$ is the width of a typical peak at half height.

The theoretical peak capacity for an orthogonal, multi-dimensional separation can be calculated as follows:

$$PC_{total} = PC_{column\ a} \times PC_{column\ b} \times PC_{column\ c} \ldots \times PC_{column\ z}$$

The peak capacity of an LC run in the experiments described in Section 2.3.2.2 (see Figure 2.13) is approximately 70, assuming a separation window of 30 min and an average peak width of 30 s. Consequently, the peak capacity for on-line salt-step 2DLC is five times higher than for 1DLC, i.e. 350. As shown in Table 2.8, the number of identified proteins increased by approximately 25% when using on-line salt-step 2DLC, compared with high-throughput 1DLC. The increase in identifications was relatively small considering the high peak capacity. This is mainly due to overlapping peptides between successive salt steps.

Tab. 2.8: The number of proteins identified from the rat spermatozoa identified using the three different chromatographic methods.

LC configuration	Proteins identified from soluble sample*	Proteins identified from insoluble sample*
High-throughput 1DLC	113	69
On-line salt step 2DLC	142	91
Off-line 2DLC	457	233

* = X!Tandem search algorithm, expectation value 0.05

For the on-line salt step method, many of the peptides were found in more than one salt step. A more prominent increase in protein identifications, more than 200%, was shown when using off-line 2DLC compared with on-line salt-step 2DLC. The theoretical increase, calculated from the increase in peak capacity, is about 350%.

The superiority of off-line 2DLC is driven by two factors:

- Optimal SCX conditions are used, including a linear salt gradient for elution and 20 to 30% organic modifier in the mobile phase to suppress unspecific interactions.
- A narrow-bore SCX column (2.1 mm i.d.) is used in the first dimension. Therefore, at least 40 times more sample can be injected.

The off-line 2DLC method features superior productivity in terms of number of protein identifications

At first glance the off-line configuration seems to be time consuming as it is not fully automated. The total analysis time for the three different configurations, not including data analysis and sample preparation, is 60 min for high-throughput 1DLC, 300 min for on-line salt-step 2DLC, and 2000 min for off-line 2DLC. However, the productivity, expressed as time/positive identification ratio is by far superior for the off-line approach.

In total, a large number of proteins, approximately 600, were identified in rat spermatozoa using the off-line configuration. The types of proteins that were identified are shown in Figure 2.14. Soluble proteases were found in higher number in the soluble sample compared with the insoluble sample, whereas the opposite was true for redox and structural proteins, which are often hydrophobic/membrane proteins. Table 2.8 shows the top score proteins when the off-line 2DLC configuration was used.

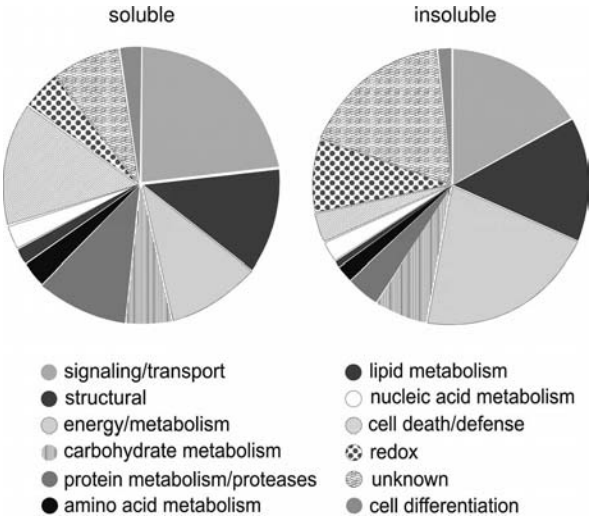


Fig. 2.14: Functional classification of proteins identified by the 2DLC analysis of rat spermatozoa. Courtesy of GE Healthcare.

In summary, the rat spermatozoa proteome was analyzed using the three preprogrammed LC configurations: high-throughput 1DLC, on-line salt-step 2DLC and off-line 2DLC. The combination of a large sample load, optimal SCX conditions, and sensitive nanoscale LC-MS/MS using the off-line 2DLC–MS/MS approach resulted in nearly 600 identified proteins.

For in-depth proteome profiling of a complex sample, the off-line 2DLC–MS/MS configuration is recommended. If the sample is less complex, and analysis time and automation are issues, 1DLC with lengthened gradient time or on-line salt-step 2DLC is recommended.

Type of instrumentation Compared to the general HPLC supply the number of vendors offering dedicated nanoLC systems is more clearly arranged. In the majority of cases a nanoLC system is used exclusively front-end to a nano electrospray mass spectrometer or, with increasing popularity, to a spotter which collects fractions for MALDI MS analysis on a MALDI target. In the past, flow rates in the range of a few hundred nano-liters per minute, typically 200 nL/min for a 75 μm I.D. column, could only be generated with the help of a flow splitter. Still today, most of the commercially available nano LC systems are using this somewhat outmoded operation principle. Once such systems are well run-in they offer a very acceptable degree of reliability and performance. However, troubleshooting on a splitter based system can be a time-consuming challenge.

Today, splitter-less systems are entering the market.

Elimination of flow-splitting overcomes the inaccuracies and plumbing problems caused by splitter-based systems. Consequent miniaturization leads to the complete absence of check valves and the reduction of moveable parts to the absolute minimum and makes nanoLC more rugged and reliable. The spread of splitter-less systems has already begun and will continue. Such state-of-the-art instruments are very likely to dominate the market in the near future.

During the purchase process of a nanoLC system it is recommended to define the current and future needs carefully as it prevents from frustrations and from spending too much money for features and extras that are not needed in real life.

Quite frequently, UV detection appears on the wish list, however, it is very rarely used in practice as it contributes to additional and unwanted dead volumes and delay times and results in broader peaks. As nanoLC systems in proteomics are normally working at extreme sensitivity, UV detection will hardly detect any peak of interest as it far too insensitive to generate a useful UV chromatogram.

In practice the most suitable nanoLC system consists of an autosampler to allow unattended operation of a larger set of samples and a valve arrangement that provides the use of two alternating trap columns in order to optimize the time per analysis and productivity. Besides a gradient pump that generates the nano flow an additional pump that facilitates the transfer of the sample from the autosampler

to the trap column is strongly recommended. Real-time flow and gradient monitoring is absolutely essential.

All nanoLC systems are small in flow rate, but some of them are large in weight and footprint. A big and heavy cabinet is not necessarily a quality feature, rather than concealing outdated technology. From time to time, e.g. for troubleshooting or maintenance, the nanoLC system has to be disconnected from the mass spec and moved away. In such cases a small light-weight with a low balance point is desired.

Last, but not least, the nanoLC system of choice should be capable of communicating with the control software of all major MS systems around, in order to maintain a smooth and seamless single-point control of the entire nanoLC-MS system.

COFRADIC™ In this "COmbined FRActional DIagonal Chromatography" (COFRADIC™) approach, according to Gevaert *et al.* (2003), the complex peptide mixture of a tryptic digest of the protein sample is first separated in reversed phase chromatographic run and collected in fractions. In every fraction a subset of peptides is modified by the use of a specific reaction, for instance by oxidizing the methionine-containing peptides with a H_2O_2 solution. The modified peptides show altered properties, when they are rerun in the same chromatographic system, and elute at a different time than the unmodified peptides. Only the modified peptides are further analyzed by mass spectrometry.

Gevaert K, Goethals M, Martens L, van Damme J, Staes A, Thomas, GR, Vandekerckhove J. Nature Biotechnology 21 (2003) 566–569.

Diagonal means using the same physico-chemical property of a protein / peptide for separation on the same media with a modification step of the analyte in between (in contrast to orthogonal).

2.3.3 Affinity Chromatography and LC-MS/MS

Increasing numbers of studies demonstrate that proteins involved in cell mechanisms rarely act on their own, but in complexes of two or more proteins. The yeast two-hybrid technology has enabled the detection of interactions between two proteins (Fields and Song, 1989) and the technology has been applied to the comprehensive analysis of *Saccharomyces cerevisiae* (Uetz *et al.* 2000; Ito *et al.* 2001). Additional method developments have enabled the efficient affinity based isolation of multiple interactions and subsequent analysis of the protein interactions by mass spectrometry (Neubauer *et al.* 1998; Rigaut *et al.* 1999; Husi *et al.* 2000).

Fields S, Song O. Nature 340 (1989) 245–246; Uetz et al. Nature 403 (2000) 623–627.

Ito et al. Proc Natl Acad Sci USA. 98 (2001) 459–474.

Neubauer G, King A, Rappsilber J, Calvio C, Watson M, Ajuh P, Sleeman J, Lamond A, Mann M. Nature Genet 20 (1998) 46–50.

Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. Nature Biotechnol 17 (1999) 1030–1032.

Husi H, Ward M, Choudhary JS, Blackstock WP, Grant SGN. Nature Neurosci 3 (2000) 661–669.

Gavin et al. Nature 415 (2002) 141–147.

Ho et al. Nature 415 (2002) 180–183.

Two publications have combined the affinity isolation approach with on-line LC-MS/MS for the systematic identification of protein complexes in *Saccharomyces cerevisiae* (Gavin *et al.* 2002; Ho *et al.* 2002). Both groups employed a similar approach (see Figure 2.15). Firstly, affinity tags were bound to a wide variety of proteins. Secondly, the DNA encoding the affinity tagged proteins was introduced into yeast cells. Thirdly the affinity tag, and subsequently the protein and those complexed to it, were isolated by affinity chromatography. Finally, the eluted proteins were separated by 1D SDS PAGE, digested with trypsin and analyzed by LC-MS/MS. The former publication identified 232 distinct multiprotein complexes, whilst the latter identified over 3600 associated proteins under the given experimental conditions.

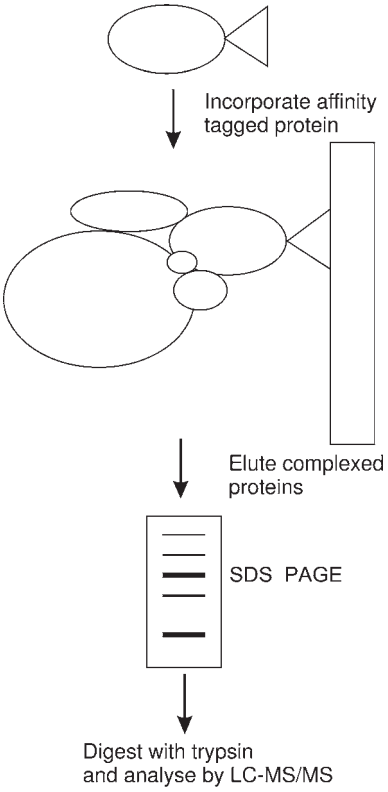


Fig. 2.15: Workflow for the affinity interaction, LC-MS/MS approach to proteomics.

Graumann J, Dunipace LA, Seol JH, McDonald WH, Yates JR, Wold BJ, Deshaies RJ. Mol Cell Proteomics. 3 (2004) 226–237.

The combination of MudPIT technology with tandem affinity purification (TAP) has been used to study protein interactions in yeast (Graumann *et al.*, 2004). The proteins identified by the TAP-MudPIT

technique were involved in 102 previously known and 279 potential physical interactions.

For some applications, it will be true that a 2DLC peptide separation is still insufficient to provide the resolution necessary for the MS analysis. At this level of complexity, a tier of protein level separation is needed in addition to the peptide separation; this tier if often referred to as protein pre-fractionation.

For slightly complex samples with a total number of a few thousand of proteins proteomics investigations can start directly at the digested protein – peptide – level. However, if sample complexity exceeds a certain level, it is essential to start the separation on the basis of intact proteins and not at peptide level alone.

2.3.4 Protein Pre-fractionation

If sample complexity exceeds a certain level, it is essential to start the separation at intact protein level.

Proteomics studies of complex samples such as plasma/serum or cell tissue require an intelligent protein pre-fractionation.

■ ***From recent studies we know that the complexity of a resulting peptide mixture after protein digestion can be much higher (ten-fold) than expected, due to miscleavages, truncations, etc.***

Presumably, after tryptic digestion of an average protein the peptide mixture does not only contain 60 but 600 peptides. For reasons of comprehensive planning it might be useful to make a rough calculation in order to match sample amount, complexity and realistically expected recovery with the general performance and sensitivity of the entire workflow, including the MS system in use.

Random or specific protein and peptide losses during separation, unavoidable intermediate steps and digestion have to be considered, too. For that reason it is essential to start the separation on the basis of whole proteins [protein pre-fractionation (PPF)] and not straight at peptide level. If sample complexity exceeds a certain level, 2DLC (IEX/RPC) on peptide level is no longer sufficient. It becomes necessary to add more dimensions at protein level prior to digestion.

This is very much in line with the statement of a famous pioneer in proteomics who believes that the challenge in proteomics it is not the mass spec rather than the chemistry and the separations.

A kind of resurgence of LC approaches is happening right now.

■ Note: ***Starting at protein level offers the advantage to correlate an identified peptide with the intact protein with all its post-translational modifications or at least with the fraction where it has been collected.***

The first step in protein pre-fractionation can still be regarded as sample preparation for the purpose of eliminating irrelevant proteins such as albumin and immuno-globulins and other high-abundancy proteins in plasma or serum where affinity chromatography is the method of choice. The flow-through fraction is then subject to intelligent multidimensional protein pre-fractionation and sample de-complexification, while the remaining proteins on the column will be stored for other investigations or simply discarded during column equilibration and cleaning.

Post-translational modifications are perhaps the largest frontier for proteomics.

And that is going to require development of new technologies as well as the refinement of already existing tools, like e.g. affinity chromatography. It can be efficiently used to simplify protein samples by enriching subsets of the proteome such as phosphorylated proteins by IMAC, glycoproteins on lectin columns, just to mention the most frequently used applications. In this case the retained proteins are of main interest and the flow-through fraction is less important but might be stored for continuative investigations.

Samples that do not contain any major contaminating proteins can be processed directly. For reasons of simplicity and clarity the following explanations are covering two-dimensional separations only, which in practice will be relevant in most cases.

■ Note: ***Too many steps can result in an unacceptable loss of proteins. In this case less can be more. As much separation as necessary, but as little as possible. Sticking to the "keep it simple" rule can be a good advice and a consideration for success.***

See also Figure 6 on page 9.

Even with the most sophisticated strategy, and the best analysis techniques, it will – for the foreseeable future – not be possible to isolate all proteins in a complex proteomics sample.

Sample complexity and dynamic range

In order to identify and characterize proteins present at very low concentrations, it is inevitably to start with large sample amounts and/or volumes in order to assure their presence in sufficient concentration. One of the most exiting samples is probably blood. Dealing just with complex samples is already difficult enough; however, if the composition of a sample is similar to plasma, the challenges are increasing exponentially. About 90% of the amount of plasma proteins is albumin and IgG and just 25 high-abundant proteins represent 99% of the total amount. The remaining thousands (or millions?) of proteins are distributed over a huge concentration range (see Figure 2.16).

Plasma is the largest and deepest version of the human proteome: Largest, i.e. most proteins (estimates range from >30,000 to >10,000,000). Deepest, i.e. widest dynamic range (>10^9). See paper by Anderson and Anderson (2002).

Anderson NL, Anderson NG. Mol Cell Proteomics 1 (2002) 845–867.

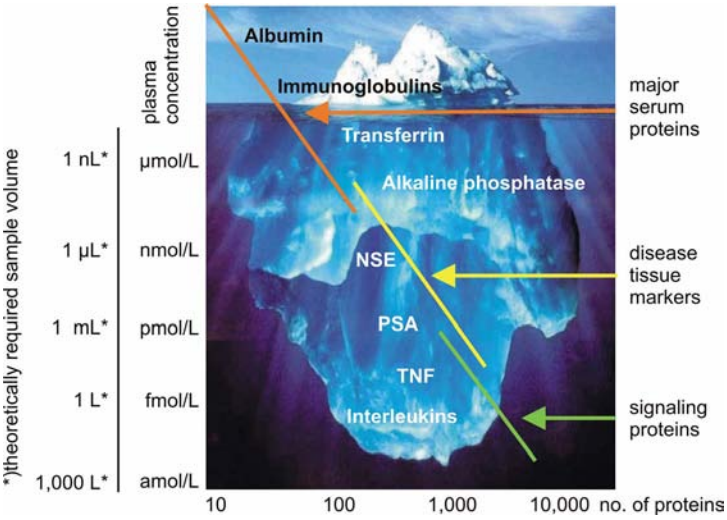


Fig. 2.16: The analogy with an iceberg illustrates the challenges in proteomics in terms of sensitivity and dynamic range. In order to detect a biomarker present at a concentration of 1 fmol/L, theoretically a sample volume of 1L is required, if the LOD of the MS equipment is 1fmol.

Currently there is research ongoing for developing strategies on how to tackle this demanding dynamic range and sensitivity challenge.

Key to this task is to get rid of as many of the low-interest high-abundant proteins as possible (tip of the iceberg), without losing too many of the free low-abundant proteins and those bound to carrier proteins like e.g. albumin. High-abundant protein affinity depletion followed by a 2DLC (IEX, RPC) strategy on protein level as described further down seems to be the most promising approach. As much as possible a sample load combined with high-recovery/low-loss separation steps and a high-sensitivity mass spectrometer are essential prerequisites for the successful deciphering of at least parts of the human plasma proteome.

A simple calculation may help to highlight the current limits and limitations. Let us assume that a state-of-the-art mass spectrometer can routinely operate at the low femtomole level. If you start with 1 milliliter of sample you theoretically can come to the 10^{-12} mmol/L

range in the iceberg picture, providing a 100% recovery, which is very unlikely, since complex separations cannot be performed quantitatively. Although this is a question of simple mathematics, it is not always appreciated. A possible solution might be to apply more sample, if possible or available, or improve techniques, methods, and skills in order to attack the atto- or even zeptomolar range. Such results are published these days and achievable by the top research labs of the world.

Theoretically, applying 1 L of serum or a zeptomolar sensitivity mass spec, or better both, would provide the desired performance of a 12 to 15 orders of magnitude dynamic range.

LC-MS manufactures claim that the combination of nanoLC and nanoESI at peptide level covers a dynamic range of 10^4 (see Figure 2.17). A typical peak volume at this scale is ideally in the range of 100 nL. Closing the gap with the desired dynamic range is only possible if more sample can be acquired at the very beginning and concentrated in an intelligently designed workflow with high recovery. The more we (can) load, the more we see.

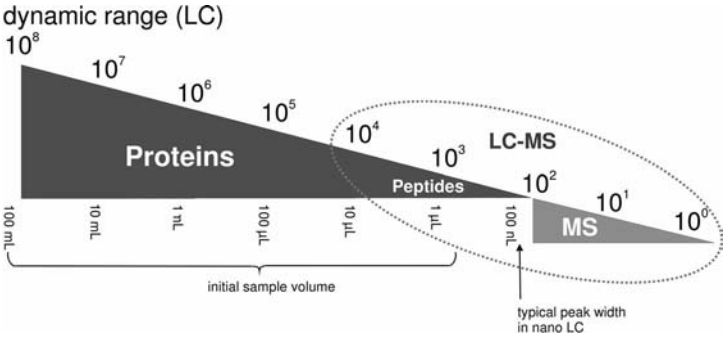


Fig. 2.17: Expanding the overall dynamic range by increasing the initial sample load.

The HUPO organization currently promotes a series of different initiatives to work on the proteomes of plasma (discussed above), brain, liver, and more are expected to follow soon. For all these complex samples in principle – with specific and dedicated sample preparation – very similar strategies can be applied. A schematic general workflow is shown in Figure 2.18.

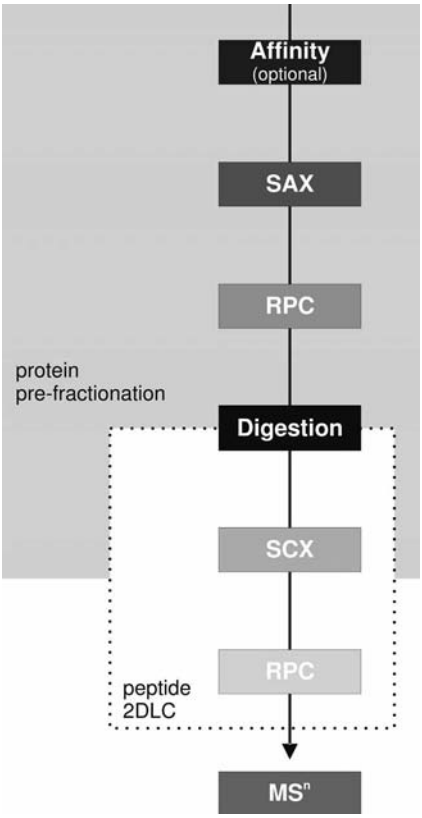


Fig. 2.18: Schematic flow diagram showing the separation steps on protein and peptide level.

Peak capacities and separation performance in protein pre-fractionation

Today, even with the most sophisticated tools and intelligent separation strategies it is impossible to achieve high purity for each protein in a proteomics sample.

Human plasma consists of at least some 100,000 proteins, post translational modifications, splice variants and isoforms not yet included. A pre-fractionation into 1,000 fractions still results in more than 100 proteins per fraction in average, but each single fraction probably simple enough to be identified and characterized after digestion by peptide 2DLC-MS. Literature reports of capacities for just 1D RPC of 200 to 500 peaks; however, in practice under normal lab conditions

one can be happy if 10% of the theoretical values will be achieved. Table 2.9 shows expected peak capacities for real samples in practice.

Tab. 2.9: Expected peak capacities on intact protein level under practical laboratory conditions.

Affinity chromatography	>2
Ion exchange chromatography	~20
Gel filtration	~10
Reversed phase chromatography	~50

2.4 Practical Considerations and Application of LC-based Protein Pre-fractionation

In contrast to the well established electrophoresis techniques, the application of liquid chromatography in proteomics is a – surprisingly – rather young discipline. Fortunately, especially for the widely used separation of tryptic peptides, both for one- and two-dimensional separations, most researchers worldwide rely more or less on the same recipe of running conditions with a very few minor – individual – modifications only.

Apffel A. In Simpson RJ, Ed. Purifying proteins for proteomics: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2003) pp. 75–100.

Dixon SP, Pitfield ID, Perrett D. Biomed Chromatogr 207 (2006) 508–529.

In protein pre-fractionation – a very promising approach still in its infancy – we have not yet achieved any kind of standardization or standard operation procedures (SOP). Based on the well founded and long lasting history with chromatography in general and protein purification in particular, it was not too difficult to come up with a range of suitable approaches and technical solutions as described by Apffel (2003) and Dixon *et al.* (2006).

Although the purpose of protein purification is somewhat different compared to protein pre-fractionation (PPF), as shown in Table 2.10, by far most of the well established rules and basic considerations are applicable for this new strategy in proteomics, too. Although the same equipment, columns and expertise is used, the objective of each approach is different.

Tab. 2.10: The differences between protein purification and pre-fractionation.

Protein purification ≠ protein pre-fractionation	
The aim of **protein purification** is to obtain a single fraction with a single pure protein for continuative studies.	The **protein pre-fractionation** procedure in proteomics results in very many fractions with each still containing tens to hundreds of proteins
During the purification process the number of target protein containing fractions decreases.	During the pre-fractionation process the number of fractions increases exponentially.
The fractions containing contaminant proteins are discarded.	All fractions are of the same high value.

Knowing that within the next future a lot of new and probably even more successful approaches will enter the scene, this section will only cover one protein pre-fractionation workflow as a typical example. This universal two-dimensional workflow combines ion-exchange with reversed phase chromatography (see Figure 2.19). With some individual modifications only, it can be used for a variety of samples.

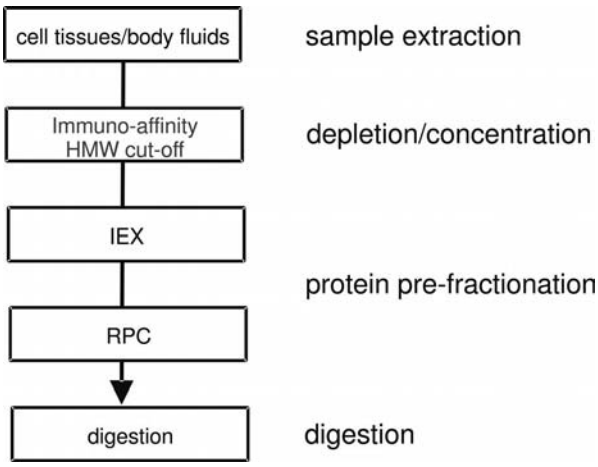


Fig. 2.19: Schematics of the protein part of the LC-based proteome analysis workflow.

2.4.1 Sample Extraction and Preparation

In principle exactly the same methods for sample preparation as described in Section 1.5.1 can be applied. As liquid chromatography deals with UV detection the purity requirements for the reagents can be significantly higher compared to electrophoresis.

■ Note: ***Very hydrophobic proteins, e.g. membrane proteins, are not solubilized with this type of buffers and are thus not accessible with the methods described below.***

2.4.1.1 Plasma, Serum, CSF, and other Body Fluids

A special sample preparation step for the removal of the highest-abundant proteins is required in most of the cases. Such a depletion step can be performed by applying immuno-affinity techniques. Despite its unique selectivity immuno-affinity chromatography does not meet the requirements for loading capacity or economy. Just a few microliters of plasma can be loaded and the running costs are high. In addition, caused by unavoidable leaking effects, small amounts of antibodies can be released into the elution buffer that might result in ambiguous protein identification further downstream in the workflow.

Liotta LA, Ferrari M, Petricoin E. Clinical proteomics: written in blood. Nature 425 (2003) 905.

Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Mol Cell Proteomics 2 (2003) 1096–1103.

Recently, a hypothesis is under discussion that claims that the low-molecular-weight region of the blood proteome, which is a mixture of small intact proteins plus fragments of the large proteins, represents all classes of proteins. As stated by Liotta *et al.* (2003) and Tirumalai *et al.* (2003) this fraction is expected to be the ideal source for biomarker discovery.

Tanaka Y, Akiyama H, Kuroda1 T, Jung G, Tanahashi K, Sugaya H, Utsumi J, Kawasaki H, Hirano H. Proteomics 6 (2006) 4845–4855.

Such an approach, described by Tanaka *et al.* (2006), suggests to cut-off the high-molecular-weight and concentrate the low-molecular-weight proteins by dedicated membrane or hollow-fiber devices and to continue the analysis with the low-molecular-weight fraction only.

Wagner K, Miliotis T, Marko-Varga G, Bischoff R, Unger KK. Anal Chem 74 (2002) 809–820.

Another remarkable paper by Wagner *et al.* (2002) describes the application of protein mapping of biological samples of human hemofiltrate for the analysis of proteins and peptides with a molecular weight below 20 kDa.

Once the sample has been extracted and prepared in a suitable way, a two-dimensional protein pre-fractionation – ion-exchange chromatography in combination with reversed phase chromatography – will follow.

Before starting with the most valuable and difficult sample it is strongly recommended to learn and establish the new method in the laboratory by using a well known simple standard sample first. This period can take or save several weeks and months but is a basic prerequisite for the application of each new technique.

2.4.2 Experimental Setup

2.4.2.1 Scale of Operation

As mentioned earlier in Section 2.2 it is advantageous to start with a higher initial amount of protein in order to extend the overall dynamic range, enable access to lower-abundant proteins and last but not least to compensate for the inevitable losses in each of the individual steps in the entire workflow until the sample finally enters the mass spectrometer.

Protein pre-fractionation as described here permits the separation of initial sample loads between ≈ 100 µg and ≈ 100 mg of protein. Protein pre-fractionation at an even higher, industrial scale has been reported.

Rose K, et al. Proteomics 2004, 4, 2125–2150.

2.4.2.2 Type of Instrumentation

In principle any kind of biocompatible HPLC system equipped with a fraction collector can be used to perform protein pre-fractionation. However, for the efficient separation of proteins and peptides it is very often necessary to use buffers with high salt concentrations at very acidic or basic pH that are extremely corrosive to stainless steel components which are commonly used in conventional HPLC equipment. Besides contaminating the sample with corrosion products that are released from the metal surfaces it may also result in a modifications of the proteins and finally in increased maintenance and replacement costs for the instrumentation.

Conventional HPLC systems are intended for small molecule analytical rather than bio molecule preparative use.

It is recommended to use a dedicated biocompatible, high-performance bio molecule separation system. Such systems have a completely bio-inert flow path and can withstand the even most aggressive buffers, solvents and high salt concentrations (like 8 mol/L urea).

For convenience and ease-of-use the fraction collector should be compatible with all different kind of vessels including microtiter plates.

ÄKTA™ and Ettan™ LC have proven to be suitable for protein pre-fractionation and can be recommended without any restriction or limitation.

2.4.2.3 Choice of Chromatography Column

The number of HPLC columns on the market is huge and unmanageable. It is recommended to use columns that have proven to perform well for protein separations. Except for RPC it is essential that the columns are made of biocompatible material such as glass, PEEK® or other suitable plastic material.

Packing material that is based on silica is not recommended as it is unstable at pH above 7.5. In order to avoid cross-contamination from one sample to another the column must survive a cleaning-in-place (CIP) process, i.e. the column must tolerate wash steps with 1 mol/L NaOH or HCl. A column packed with porous polymer based material is recommended.

Good batch-to-batch reproducibility and long-term availability is best with big and well established manufactures.

Non-porous (NPS) columns show superior resolution but offer only low loading capacity. Besides a good separation performance protein recovery is a key feature.

2.4.2.4 Monitoring Each Separation Step, Monitoring Each Dimension

K. Marcus, et al. "A novel approach towards differential proteomics with multidimensional intact protein prefractionation of brain samples" Poster at the HUPO 4th Annual World Congress Munich (2005).

In general, but especially during the method development process it is beneficial to monitor progress and performance of each protein pre-fractionation step by analyzing each, or representative fractions by SDS electrophoresis, and – if appropriate or necessary – optimize the procedure, as outlined in the poster of K. Marcus *et al.* (2005).

2.4.3 Ion Exchange Chromatography and Protein Pre-fractionation

An ideal technique to deal with samples of crude protein extracts – small to large amounts or volumes, native, denaturing or reducing conditions – is ion exchange chromatography. This technique can be operated at any scale, from micro to macro scale. Due to its adsorptive mode diluted protein solutions can be concentrated and separated with high resolution in less than an hour. Its ease of use and its logi-

cal, directly adaptable theory made IEX the most widely used technique for protein separations.

In order to benefit from the concentration effect all proteins should bind to the column. In theory this can be achieved by loading the sample at very low pH (e.g. glycine/HCl buffer) to a cation, or at very high pH (e.g. piperidine/HCl buffer) to an anion exchange column. However, these extreme pH conditions are not compatible with protein stability. Physiological pH or slightly basic conditions (Tris/HCl buffer) are more favorable conditions for high protein recovery.

Experience from several decades indicates that anion exchange chromatography is the first choice for the separation of intact proteins.

In cases, where AIEX does not give satisfying results, or in the tandem IEX approach, see below in Figure 2.20, the application of CIEX should be considered.

In general anion-exchange-chromatography is the first choice for the separation of intact proteins.

Recently, another technique based on charge, or more precisely, on isoelectric point (IEP, or pI) had its renaissance in the area of protein pre-fractionation: chromatofocusing (CF). CF can be regarded as the LC analogue to isoelectric focusing in electrophoresis, however, without achieving its separation performance. Although the theory is very convincing, in practice this technique is suffering from a very restricted pH range, protein precipitation at the isoelectric point, high running costs and the necessity to remove the pH gradient creating ampholytic reagents (Polybuffer) after the separation. It is quite unlikely that CF will have a major breakthrough in the near future.

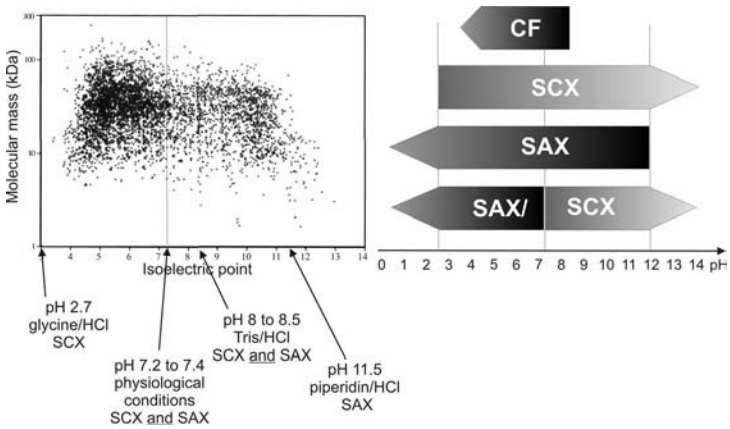


Fig. 2.20: Potential operating area for charge- and pI-based protein separation (theoretical spot map modified from Link *et al.* 1997).

Link AJ, Robison K, Church GM. Electrophoresis 18 (1997) 1259–1313.

2.4.3.1 IEX under Denaturing Conditions

Wiegand G, Parbel A, Seifert MHJ, Holak TA, Reuter W. Eur J Biochem 269 (2002) 5046–5055.

As the most efficient sample extraction takes place under denaturing conditions it is a natural choice to maintain these conditions also during ion-exchange chromatography. Inter and intra protein interactions are widely suppressed and solubility of proteins is improved. The addition of protein inhibitors reduces protease activities and maintains the integrity of the sample. Low percentages of organic modifier (e.g. 6% propanol-2) or the presence of DTT might be advantageous (Wiegand *et al.* 2002). Ion exchange chromatography (IEX) is the natural choice for the first dimension in protein pre-fractionation. Figure 2.21 illustrates the schematic flow path for the IEX separation.

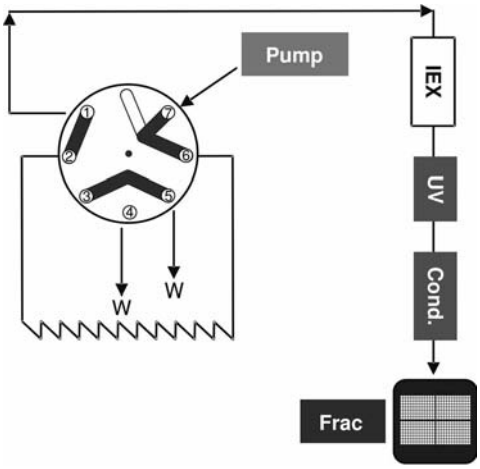


Fig. 2.21: IEX (first dimension) flow path with single sample injection.

Even small IEX columns have a significant loading capacity for high protein amounts in the range of 5–10 mg/mL for best possible performance. The volume loading capacity in principle is unlimited, i.e. even large volumes of 100 mL and more of diluted sample can be applied.

Applications of high sample volumes, of samples with high protein concentrations or samples with high viscosity have to be handled with care.

IEX is a retentive technique where proteins can be effectively concentrated.

During sample introduction it is recommended to considerably reduce the flow rate in order not to exceed the pressure limit of the column.

The adsorbed proteins are eluted from the column with an ascending salt gradient and collected in a fraction collector. A fraction size

approximately three times the base peak width of a typical protein peak is a good initial setting.

Try the following setup, before wasting time with buffer development or column testing.

Columns

RESOURCE™ Q 1 mL (strong anion exchanger, SAX); *Cost effective, try this first.*
or Mono Q™ 5/5, slightly better resolution; *The industry standard.*
or Mini Q™ 4.6/5. *Highest possible resolution for small sample amounts.*

Buffer composition

A: 20 mmol/l Tris, 8 mol/L urea, 6% (v/v) propanol-2, pH 8.5 (HCl).

B: 20 mmol/L Tris, 8 mol/L urea, 6% (v/v) propanol-2, 1 mol/L NaCl, pH 8.5 (HCl). *Dissolving urea in water takes a long time; a heated magnetic stirrer may help to accelerate this job.*

Degas both buffers prior to use.

As it is recommended to use denaturing conditions for protein extraction, it is natural and good practice that the consecutive step also takes place in the presence of chaotropic agents. In contrast to electrophoresis thiourea is not suitable in chromatography, because it makes UV detection troublesome. The use of propanol-2 as an additive is intended to eliminate unspecific interactions between stationary phase and hydrophobic proteins and maintains its solubility. *No filtering through membranes due to the risk of introducing PEG into the sample.*
Prepare fresh at least once a week.

The flow-through fraction, before the gradient starts, contains (theoretically) the basic proteins with a pI > 8.5. Although they do not bind to the column they are not lost and will be transferred as all the other fractions to the next dimension.

2.4.3.2 The Tandem IEX Approach

Depending on the origin and composition of the sample the distribution between acidic, neutral and basic proteins can differ considerably. In order to obtain a preferably even pI distribution of proteins in the different fractions it might be beneficial to apply the tandem arrangement of anion- and cation-exchanger. At a given pH, e.g. 8.5, negatively charged proteins, anions, bind to the anion-exchanger, while the other proteins will pass the column with no interaction. While the adsorbed proteins will be concentrated the other proteins in the flow-through fraction are even more diluted than before sample application. By connecting an additional cation-exchanger after the anion-exchanger all proteins, also the positively charged cations will be adsorbed. Only the species with a net charge of zero, i.e. with a pI equal or near the pH of the buffer, will not bind to either column. *With this approach most of the proteins will be adsorbed and concentrated, and the number of proteins in the flow-through fraction will be significantly reduced.*

The flow path, as outlined below, has been developed on Ettan LC controlled by UNICORN® software.

In principle every suitable HPLC system, preferably bio-inert, that is capable to control two additional pressure- or motor-actuated six-port Rheodyne®- or Valco®-valves, can be used. Figure 2.22 illustrates the schematic flow path.

In general, there is no need for an autosampler for the first dimension, as in IEX only one sample can be run at a time (a third INV valve will be needed if the autosampler is skipped). It is more important to cover a wide dynamic range of injection volumes and to be capable of introducing higher volumes (≤150 mL) of diluted protein extracts.

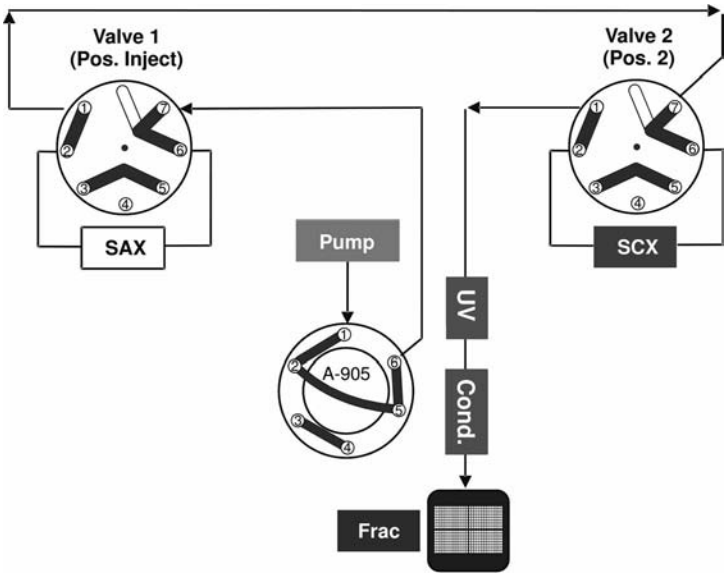


Fig. 2.22: Tandem IEX (first dimension).
Step 1: Sample injection – loading on stacked SAX/SCX columns.

During sample injection both IEX columns are connected in series. In principle the order of columns does not matter. However, as the tandem column approach is derived from the single column SAX layout, the SCX column is installed after the SAX column. As it is not possible, or at least not so easy to compose a buffer that is equally good for both modes, it has proven to be acceptable to take the same buffer as for the SAX column, knowing that it is sub-optimal for the SCX column. After a sufficient long wash period, until both UV and conductivity have leveled out, both columns will be separated from each other by the switching of valve 2 (see Figure 2.23).

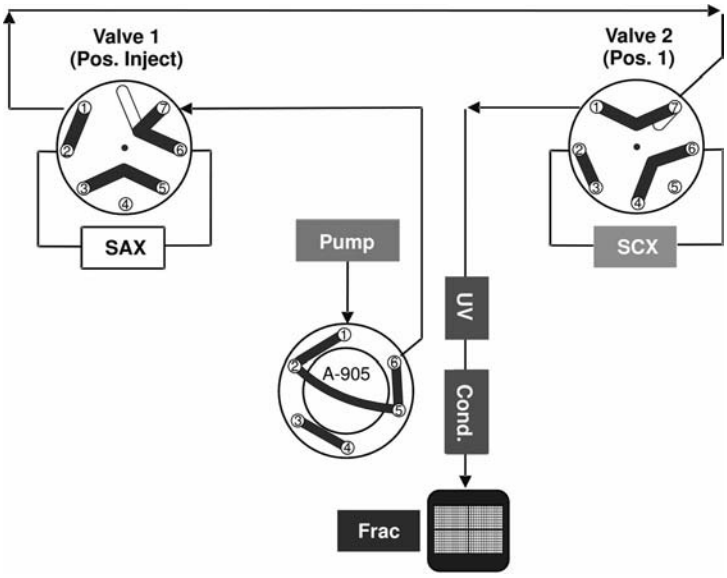


Fig. 2.23: Tandem IEX (1st dimension). Step 2: SAX gradient elution.

Now, only the SAX column is in the flow path, while the SCX column is "parking" in a stand-by position.

Best separation at a minimum of band broadening is achieved at comparably low flow rate and a shallow gradient.

In order to achieve a good separation and a minimum of band broadening at a comparably low flow rate and a shallow salt gradient will be applied and the eluting proteins are collected preferably in micro-titer plates, which are directly compatible with the autosampler for the consecutive 2nd dimension RPC separation. After a full cycle, including washing, regeneration and re-equilibration, until both UV and conductivity have leveled out again, both valves switch and connect the SCX column in the flow path, while the SAX column remains in a parking position (see Figure 2.24).

Now, the SCX column is in the flow path, while the SAX column is idling in a stand-by position. The same gradient as for the SAX column will be applied and the eluting proteins are collected in the fraction collector. When the cycle is completed, before another sample can be loaded, it is recommended to remove remaining proteins and contaminants from the system and the columns by washing according to the data sheet supplied with the columns. After washing out the cleaning reagents quantitatively the system is ready for the next sample. Figure 2.25 shows two typical chromatograms.

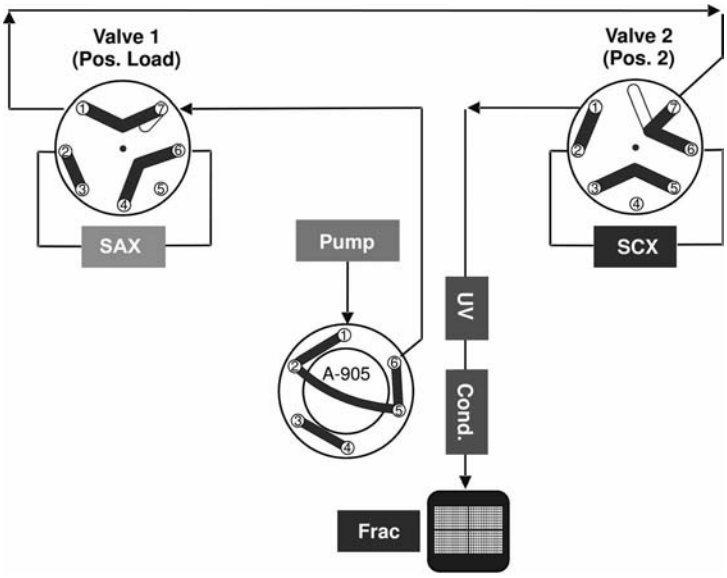


Fig. 2.24: Tandem IEX (1st dimension). Step 3: SCX gradient elution.

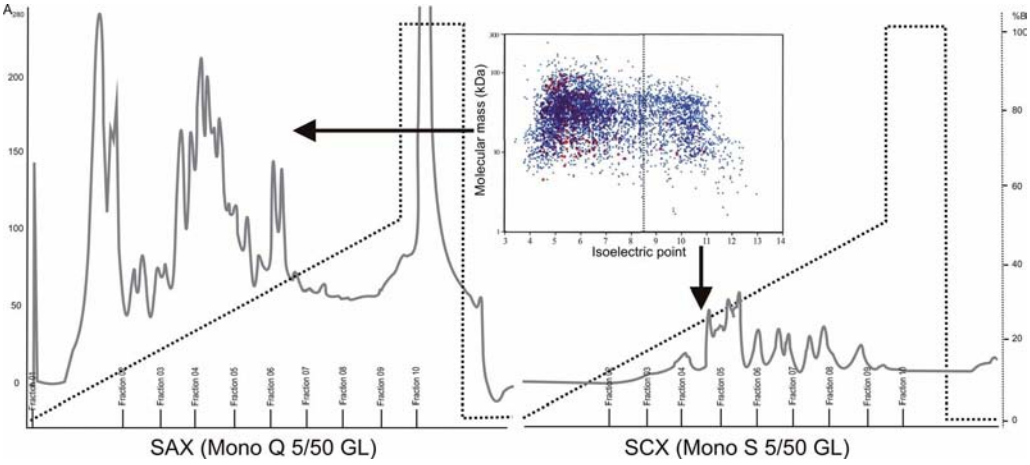


Fig. 2.25: Elution profile of the SAX and SCX column, using a 10 mg sample of *E. coli* in 8 mol/L urea. Please note the good representation of the expected theoretical ratio between acidic and basic proteins in the two chromatograms.

In order to cope with the desired productivity it is convenient to have one system dedicated to the first dimension IEX and another system with for the 2nd dimension RPC separation. Both systems can easily be controlled from the same computer.

2.4.4
Reversed Phase Chromatography and Protein Pre-fractionation

Fundamental information on this topic can be found in a chapter by Carr and Moritz in the laboratory manual edited by Richard Simpson (2003).

Carr CD, Moritz RL. in Simpson RJ, Ed. Purifying proteins for proteomics: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2003) pp 179–208.

Reversed phase chromatography is the ideal subsequent step for any technique that release samples with a high salt concentration, such as the fractions that have been processed by ion exchange chromatography before. The early eluting peaks (flow-through) contain no or very little salt, whereas the late eluting peaks contain typically up to 500 mmol/L NaCl (or more) and all together huge amounts of urea.

An RPC column deals with such samples in a perfect dual way, the combination of desalting and high-performance separation.

All the very hydrophilic, inorganic buffer constituents as well as the urea do not interact with the stationary phase and pass through the column, while the proteins of interest bind to the column – especially under aqueous, acidic conditions – and get concentrated again in a sharp narrow zone on the top of the column. In contrast to the separation mechanism of peptides on an RPC column, which is believed to be a mixed mode between partition and adsorption phenomena, proteins behave more in a "digital" on/off mode, where desorption just takes place in a very narrow range of organic modifier present. This observation implies that column length does not really matter.

In practice there is no significant difference in resolution between a 150 and a 50 mm column.

For protein pre-fractionation it is recommended to select the shortest column available.

In addition to shorter separation times also the washing and re-equilibration periods can be minimized in order to achieve a better overall throughput. Furthermore, the exposure time of proteins with the stationary phase is shorter, which also yields in a better recovery.

In general, the life time of RPC columns in protein pre-fractionation is fairly high. This might be due to the fact that the sample has already passed the IEX column(s) in the first dimension where unwanted contaminations and impurities have been removed.

The installation of a so called trap column on top of the separation column can further extend the life time and robustness of the whole setup.

Ideally, the trap column is packed with exactly the same RPC media as the separation column and has the same inner diameter (i.d.), but can be much shorter (≤ 5 mm). Figure 2.26 shows the flow path of a system that can process all the fractions from a previous IEX or tandem run in a fully automated and unattended way. After RP desalting and separation the fractions are collected preferably in microtiter plates, ready for lyophilization or evaporation and tryptic digestion.

In principle every suitable HPLC system, preferably bio-inert or bio-compatible, that is equipped with an autosampler, fraction collector and capable to control two additional pressure- or motor-actuated six-port Rheodyne- or Valco-valves can be used. The detailed flow

path diagrams as outlined below have been operated on Ettan LC controlled by UNICORN.

During step 1 the pump is pumping acidic eluent A that contains no organic modifier through the sample loop of the autosampler (A-905) to the trap column. The proteins bind to the top of the column while salts and urea pass through the column via the conductivity monitor to waste (W). The conductivity signal confirms to the operator whether the desalting process was successful (or not). In this constellation the RPC separation column is by-passed.

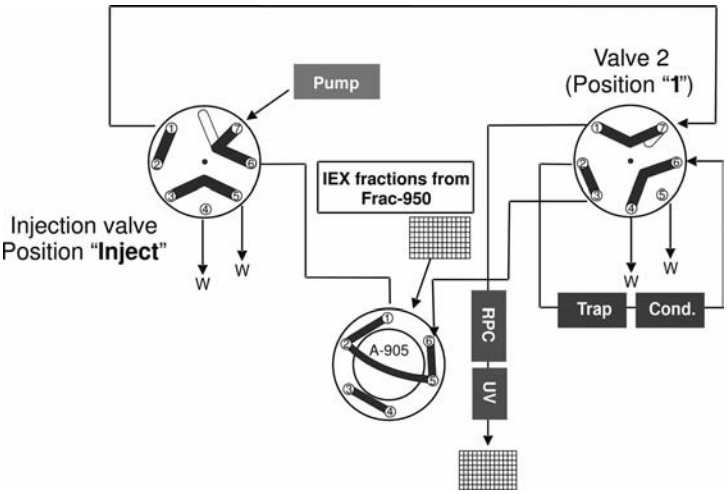


Fig. 2.26: RPC (2nd dimension). Step 1: Sample loading and desalting.

During step 2 (see Figure 2.27) both valves switch to the next position and after a short, but sufficient wash period the gradient elution starts in order to desorb the proteins. Now the trap column is flown through in reverse direction, i.e. the proteins are transported on the shortest possible way – with a minimum of band broadening – to the connected separation column followed by the UV monitor and the fraction collector. In order to minimize the gradient delay volume and to get shortest possible cycle times from sample injection to sample injection, the whole autosampler with the sample loop is by-passed.

After washing and re-equilibration the system switches back to position 1 and the next cycle can start again and again until all the IEX fractions have been desalted, separated by RPC and collected (typical examples are shown in Figure 2.28.

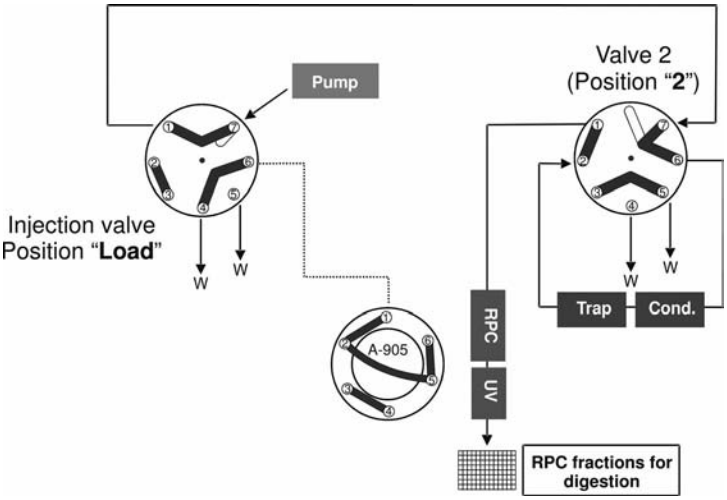


Fig. 2.27: RPC (2nd dimension). Step 2: Sample elution – reversed flow.

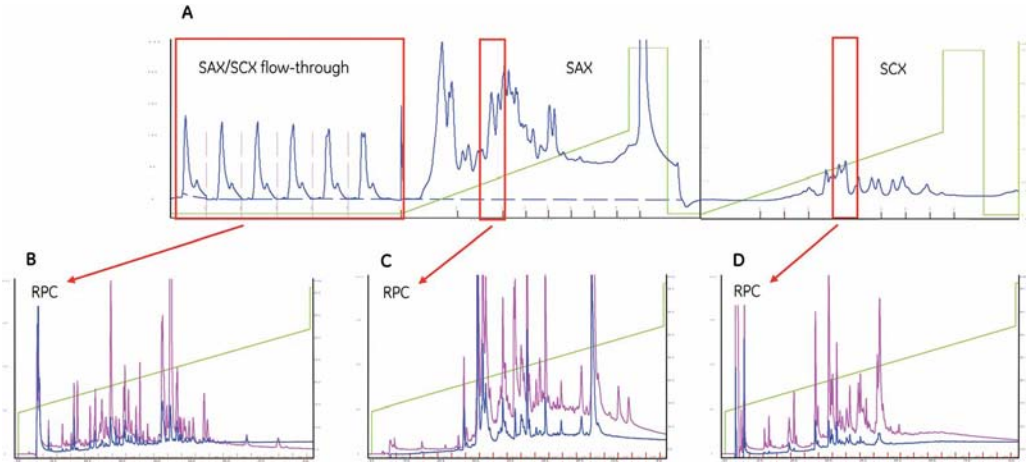


Fig. 2.28: **A**: Stitched SAX/SCX chromatograms.
B: Typical RPC chromatogram of the SAX/SCX flow-through fraction. The flow-through fraction is a pool from six consecutive injections of a total of 10 mg of *E. coli* lysate.
C: typical RPC chromatogram of SAX fraction no. 3, same conditions as **B**.
D: typical RPC chromatogram of SCX fraction no. 5, same conditions as **B**.
RPC columns: BioBasic4, 1×100 mm, trap column, 1×10 mm, flow rate 40 µL/min, gradient 25%B to 75%B in 84 min (blue: 280 nm; magenta: 214 nm).

Please note the excellent peak shape and resolution of this intact protein RPC separation. From: Hoepker HR, Renlund S, Edblad N, Wadensten H, Mascher E, Åström, Fenyö D. Protein pre-fractionation: Passing phase or forward-looking approach? HUPO 4th Annual World Congress, Munich (2005).

In protein pre-fractionation recovery should have a higher priority than resolution.

Although high-performance columns and media should be preferred, an acceptable compromise between resolution and recovery should be envisaged.

There is some evidence that polymer based resins, especially macro-porous resins, offer superior recovery – compared with wide-pore silica based stationary phases – in combination with a fairly good separation power and resolution. Furthermore, polymer-based columns are resistant to very extreme pH values, a prerequisite for efficient column cleaning in order to avoid unwanted memory effects caused by sticky contaminations between the different samples.

For micro-scale protein separations monolytic columns might be a promising alternative.

Recently, very impressive protein separations on monolytic columns were published. Peak shapes are extremely narrow and run times are short. Up to now there are no long term data about lifetimes and recoveries available; loadability normally is (too) low for the application of protein pre-fractionation, where a reasonable loading capacity is required.

Columns The following columns have been used successfully and can be recommended:

Cost-effective, try this combination first.

- RESOURCE RPC 1 ml (desalting/trap column);
- RESOURCE RPC 6 ml (separation column);

Same selectivity as RESOURCE RPC, resolution comparable to silica-based material.

- SOURCE 5RPC, 4.6 , with custom-packed trap columns;

Excellent resolution, suspect to reduced recovery.

- BioBasic® 4, available in 1.0, 2.1 and 4.6 mm i.d., including trap columns.

If, no stationary phase with exactly the same selectivity is available for custom-packed trap columns, it might be beneficial to have a trap column with slightly higher retention characteristics.

Although, not specifically tested for the purpose of protein pre-fractionation columns from other manufacturers like GRACE-Vydac, Agilent, Merck, Dionex, etc. are also suitable.

In the column market, especially for RPC, the offering is overwhelming. If in doubt what product to buy, or before spending/wasting valuable time for column testing it is recommended to screen the literature; or – even better – ask an experienced colleague in your neighborhood for good advice.

Prepare fresh at least once a week.

Eluent composition

These eluents will provide a smooth and stable baseline even at low wavelengths (210 to 220 nm) and high sensitivity.

A: 0.065% (v/v) TFA, sequential grade, in water.

B: 0.050% (v/v) TFA, sequential grade, in 84% (v/v) acetonitrile and 16% water.

Note: ***84% acetonitrile in water is an azeotropic mixture, i.e. if this mixture evaporates, e.g. in summer in a non-air conditioned laboratory, the composition of the mixture remains widely unchanged (Handbook of Chemistry and Physics 1997).***

Handbook of Chemistry and Physics, 78th edition, Lide DR (Ed. in chief) CRC press, Boca Raton, Florida (1997).

Column dimensions and loading capacity With complex samples such as proteins from cell cultures, tissues or even plasma it is impossible to separate all the proteins. However, it is essential that individual proteins are not spread over too many fractions. Keeping the overlap small is of paramount importance, i.e. it is required to perform the chromatography under high-resolution conditions in order to avoid peak broadening and distribution of proteins over too many fractions. According to a simulation it can be achieved that under ideal conditions an individual protein is present in not more than three adjoining fractions.

Serious manufactures give figures for the practical loading capacity which often is just 10% of the theoretical capacity.

In order to be able to publish large numbers, many column manufacturers specify loading capacities as dynamic loading capacity for a single standard protein. Under such conditions the column is completely filled with protein. However, such data are not at all useful for high-performance separations, where overloading must be avoided.

The higher the molecular mass of a protein, the lower the capacity.

For example, although dynamic capacity is very high (e.g. 10 mg/ml for albumin to 50 mg/ml for insulin) these figures are of limited use for high-resolution conditions. As a rule of thumb, loading capacity under high-resolution conditions is at least ten times lower than the dynamic capacity.

When increasing amounts of protein are loaded on an RPC column, the peak width remains nearly constant. After a certain point peak width and asymmetry start to increase and retention times become shorter. The loading limit of a column is normally defined as the maximum amount of sample that can be chromatographed with not more than 10% increase in peak width. This point is called the "overload point". In practice the overload point will vary for different samples. If the peaks are distributed over the effective elution range of the gradient, more sample material can be injected, and each peak may have a similar loading capacity compared to standard proteins.

While the effective elution range of the gradient reaches for proteins between 25 to 75% of organic modifier only, the effective peak capacity per column is finite.

Carried forward to the application area of protein pre-fractionation the following practical loading capacities, as shown in Table 2.11, can be expected. It can be assumed that the capacity for multi-peak samples is higher.

Tab. 2.11: Examples of loading capacities for different columns diameters.

Column i.d.	Practical loading capacity
6.4 mm	200–400 µg
4.6 mm	100–200 µg
2.1 mm	20–40 µg
1.0 mm	4–8 µg

GraceVydac, The Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC, 3rd edn (2002).

Because – for protein RPC – the loading capacity under high-resolution conditions is not a function of the column volume, it is necessary to increase the column diameter, if more loading capacity is required.

2.4.5 Fraction Size and Number of Fractions

Ideally a biological sample is pre-fractionated in such a way that each fraction contains only one protein and that each protein is present in one fraction only. Although practically not at all achievable, this would lead to as many fractions as proteins, far too much to manage for a complex biological sample with tens to hundreds of thousands of proteins.

However, it is feasible to aim for the presence of any given protein in not more than two fractions with a negligible overlap to the previous and subsequent fractions. An ideal chromatographic peak, as shown in Figure 2.29, has a certain width at the base and at half height that can be expressed in time or volume and can be calculated and printed by state-of-the-art LC evaluation software packages.

For IEX this calculation results in some ten fractions: fraction 1 to collect the flow-through of the unbound proteins, fraction 2 to 9 during gradient elution and fraction 10 while washing down the remaining proteins with high salt concentration.

Decreasing the fraction size further, results in too many fractions, and an enormous successive workload without any tangible benefit.

In RPC, with its sharper peaks, some fifteen to twenty fractions will be sufficient.

By applying the recommendations from the introduction in Section 2.2 – ideally 30 to 300 proteins per sample (=fraction) – a sample with some 100,000 proteins would require approximately 300 to 400 fractions, which is well in-line with the performance of the LC techniques and the equipment used in protein pre-fractionation.

Naturally, less complex samples like yeast or *E. coli* with 4,000 to 6,000 proteins require a lower degree of protein pre-fractionation.

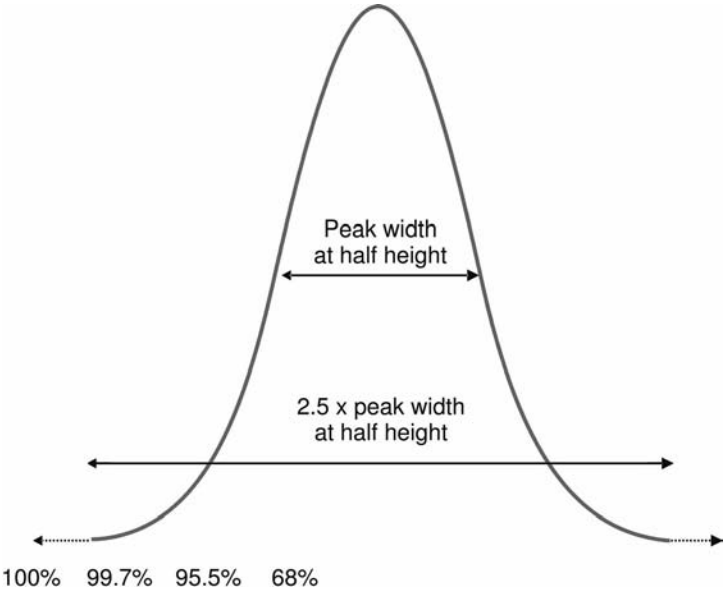


Fig. 2.29: In order to estimate the optimal fraction size for a protein pre-fractionation experiment a fraction size of twice to three times the peak width at half height can be recommended. If the apex of a peak is in the middle of a fraction, this fraction contains almost the entire peak volume. If a fraction change occurs at the peak maximum the peak is split in equal parts in not more than two fractions.

2.5
Critical Review and Outlook

An internal comparison run with an *E. coli* sample (unpublished data, work still in progress) has disclosed that identification rates and sequence coverage raises significantly when using protein pre-fractionation rather than applying shotgun approaches at peptide level only. A preliminary interpolation claims that a complete analysis of all fractions would give in the order of 2,000 identified proteins, a figure matching the entire *E. coli* proteome.

Furthermore, protein pre-fractionation has additional benefits:

- Subdivides complex protein mixtures into manageable fractions;
- Offers a wide dynamic range ;
- Low µg to high mg initial sample load;
- Very small to very large sample volumes;
- Maintains the correlation between intact parent proteins and their tryptic peptides;

- Facilitates convenient up-scaling of individual fractions if more material is required, e.g. for biomarker discovery;
- Generates more and more confident protein identification.

A sample that has undergone protein pre-fractionation, generally does not require – after tryptic digestion – another multi-dimensional separation at peptide level. Typically, a high-resolution one-dimensional nanoRPC separation, followed by MS^n analysis, will be the method of choice. Figure 2.30 illustrates such a workflow.

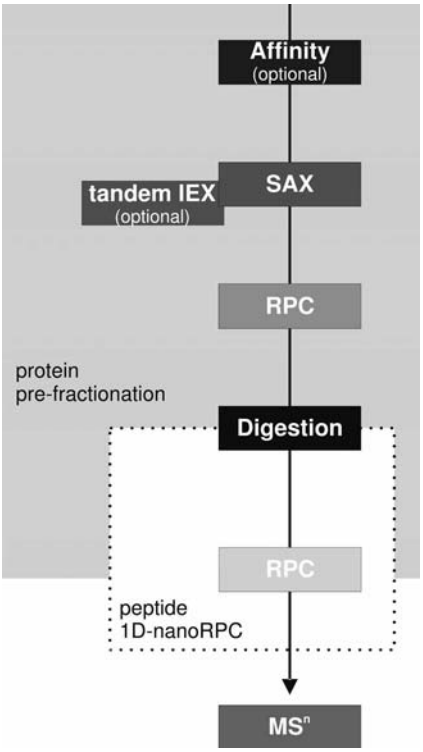


Fig. 2.30: Complete LC-based workflow after the sample has undergone a substantial de-complexification at protein level.

In the middle of the nineties of the last century there was hype for high-throughput approaches in proteome analysis. Now, some ten years later the success stories are still quite rare. Obviously, the challenges and requirements for meaningful proteomics have been underestimated. It seems as if there are no quick achievements in sight. However, time was not wasted as scientists have characterized

and cataloged thousands of proteins in data bases, strong and undisputable foundations for the future work in proteomics.

The extra workload associated with running multiple replicates of protein and peptide separations – electrophoresis or chromatography – has not always been appreciated. However, recently several papers can be found, which are requesting and proposing pre-fractionation strategies and techniques in order to cope with the enormous complexity of human samples for the deciphering of significant biomarkers; for instance the publications by Righetti *et al.*, Guerrier *et al.*, Wang and Hanash (all from 2005).

Righetti PG, Castagna A, Antonioli P, Boschetti E. Electrophoresis 26 (2005) 297–319.

Guerrier L, Lomas L, Boschetti E. J Chromatogr. 1073 (2005) 25–33.

Wang H, Hanash S. Mass Spec Reviews 24 (2005) 413– 426.

The strategies and application examples as outlined in the chapter above, if performed properly, are somewhat more time consuming compared to traditional concepts. It has proven that "quick and dirty" approaches can only give access to the "low hanging fruit". From a rational and logical standpoint it appears more likely that more intelligent sample preparation methods as e.g. protein pre-fractionation will fractionate and de-complexify samples to such an extent that consecutive separation- and detection-devices will be enabled to analyze the sample to the desired level. (This will – of course – require a certain amount of time and work effort.)

3
Mass Spectrometry

This section discusses the use of mass spectrometry (MS) for proteomics applications; protein identification, characterization and quantification. The section addresses key parameters for protein identification and the systems available to perform the listed applications.

As explained in previous chapters, large format two-dimensional gel electrophoresis enables the resolution of several thousand proteins in a reproducible fashion in a relatively short period of time. The instrumental developments in two-dimensional gel electrophoresis offered the momentum for proteomics. Similarly, technology advancement for protein identification was essential if proteomics was going to expand into the dominating field we know today. Edman sequencing was the principal method of protein identification and though it was used with considerable success for routine protein identification, the method was relatively slow (one or two peptides per day) and relatively insensitive (upper fmol to low pmol amounts; Pappin *et al*. 1995). Developments in the field of mass spectrometry addressed speed and sensitivity issues, which enabled biomolecular analysis, particularly the analysis of peptides and proteins to be performed routinely with confidence. Together with the development of database search engines and the population of sequence databases, researchers have employed MS with great success in proteomics. The evolution of MALDI and ESI ionization sources has impacted sensitivity significantly and mass analyzer development continues to extend the boundaries of mass accuracy, resolution and functionality.

Pappin DJC, Rahman D, Hansen HF, Bartlet-Jones M, Jeffery W, Bleasby AJ. Mass Spectrometry in the biological sciences. (1996) 135–150

Mass spectrometry is an analytical technique that measures the mass-to-charge ratio (m/z) of ions based upon their motion in an electric or magnetic field. Sample molecules are converted into ions in the gas phase and separated according to their m/z ratio; positively and negatively charged ions can be formed. This technique is performed by a mass spectrometer, which typically consists of three components (see Figure 3.1) and these components dictate the level of performance and the type of analysis a mass spectrometer can perform.

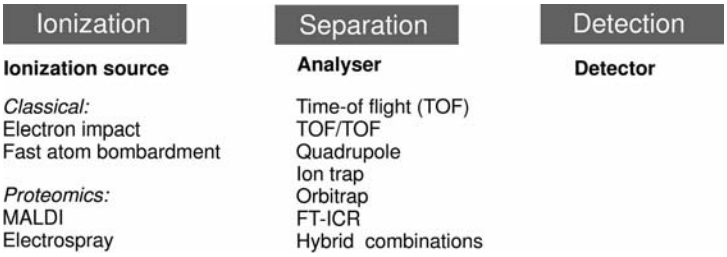


Fig. 3.1: A schematic indicating the three regions of a mass spectrometer: Ion source: the component which ionizes the sample in question, i.e. ESI and MALDI. Mass analyzer: the component that separate the ions according to their m/z ratio. Detector: the final component which records the signal produced by an ion.

Ions produced in the source are separated in the analyzer according to their mass-to-charge ratio (m/z) and the analyzer, to a large degree, determines the MS performance and functionality. Resolution, and mass accuracy are standard MS instrument specifications and tandem mass spectrometry is an essential functionality. All three are key for successful protein identification (ID) and characterization.

For instance, the higher the mass accuracy a mass spectrometer can afford, then the greater the tolerance limits can be set for database searches and ultimately the greater the confidence in the subsequent results. Resultantly, for protein identification and characterization it is essential to assign the mass of a given peptide as accurately as possible and as such a differentiation between the average and monoisotopic mass needs to be made.

Average and Monoisotopic Mass Assignment Amino acids consist of the elements carbon, hydrogen, nitrogen and oxygen, and to a lesser extent sulfur. Each of these elements exists naturally as a mixture of isotopes. For instance, carbon exists as a mixture of the ^{12}C isotope (98.9%) and the ^{13}C isotope (1.1%). As such the abundance of the two isotopes in a given compound will be reflected in a mass spectrum by the isotope envelope of the compound. There are two types of mass measurement for a given compound:

- **Average mass**: the measurement which reflects the contribution of the isotopes in an isotopic envelope. The average mass is taken at the centroid of the isotopic envelope.
- **Monoisotopic mass**: is the sum of the masses of the atoms in a molecule using the principle isotope mass of each atom instead of the isotope averaged atomic mass. In practice, this is the

mass of the first peak in a peptide isotopic envelope. With respect to carbon, this is termed the ^{12}C peak.

Figure 3.2 illustrates the predicted isotope distribution of the peptide HLKTEAEMK at resolution 5000 (FWHM).

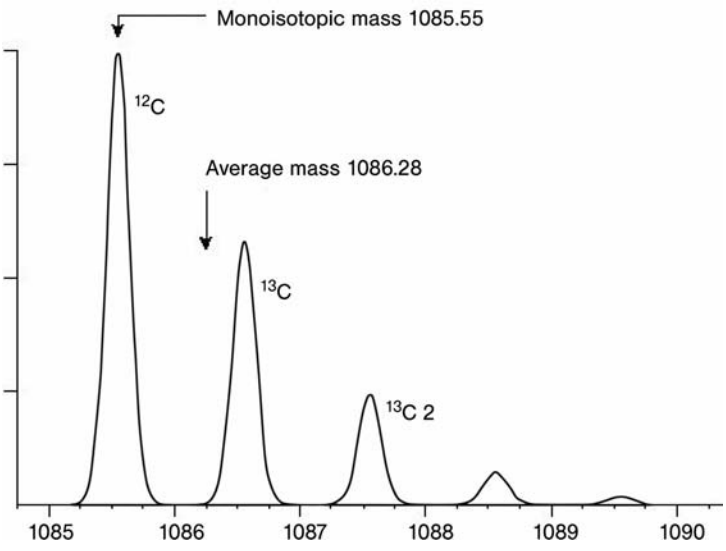


Fig. 3.2: Predicted isotope distribution of the peptide HLKTEAEMK at resolution 5000 (FWHM).

The second peak in the envelope represents the peptide structure where one of the carbon atoms in the peptide is now a ^{13}C atom.

This is a significant difference which can be capitalized on for protein identification by reducing the error tolerances for database searches.

Yates JR, Ciociorva D, Liao J, Zabrouskov V. Anal Chem 78 (2006) 493–500.
Macek B, Waanders, L, Olsen JV, Mann M. Mol Cell Proteomics 5 (2006) 949–958.
Olsen JV, de Godoy LMF, Li G, Macek B., et al.; Mol Cell Proteomics 4 (2005) 2010–2021.

The spectrum demonstrates the resolution of the ^{12}C and ^{13}C isotopes. As a result the monoisotopic mass, ^{12}C peak, can be clearly assigned.

The mass difference between the average and monoisotopic masses is 0.73 Da.

Resolution and mass accuracy specifications are determined in a large part by the analyzer technology. Improvements in analyzer performance have enabled the mono-istopic mass of a peptide, rather than the average mass of a peptide, to be almost exclusively used for protein identification, characterization and quantification. Mass measurement accuracies of less than 2 ppm have been reported in the routine analyzes of complex peptide mixtures and intact proteins. It is possible to increase this further with internal mass calibration, as described by Matthias Mann and co-workers, with mass accuracies of less than 1 ppm (r.m.s.) reported.

3.1 Ionization

The ion source is the region of the mass spectrometer where the gas phase ions are produced from sample molecules. The method of producing the ions is termed the ionization technique. Several ionization techniques have been developed; the earliest incarnations include electron impact and chemical ionization, which are useful for ionizing small molecular weight molecules, but less applicable for larger (bio)molecules. The first example of ionizing larger biomolecules was reported by MacFarlane and Torgerson in 1976 using the technique plasma desorption. The introduction of fast atom bombardment in 1981 (Barber *et al.* 1981) enabled the ionization and detection of a range of intact biomolecules with relatively good sensitivity. The FAB source was coupled with a magnetic sector analyzer, enabling biomolecule tandem mass spectrometry analysis to be performed with relatively good sensitivity, high resolution for the first time.

McFarlane R, Torgerson DF. Science 191 (1976) 920–925.

Barber M, Bordoli RS, Sedgwick RD, Tyler AN. Nature 293 (1981) 270–271.

However, FAB was supplanted by two ionization techniques developed in the late 1980s. Karas and Hillenkamp introduced matrix assisted laser desorption ionization (MALDI) in 1988 as a technique that could readily ionize (large) biomolecules in a very sensitive manner. MALDI is a pulsed ionization technique which utilizes the energy from a laser to desorb and ionize the analyte molecules in the presence of a light absorbing matrix. In another breakthrough, Fenn and co-workers demonstrated that electrospray ionization could also ionize large biomolecules with high sensitivity.

These two ionization techniques have become the standard for ionization of protein and peptide samples.

Fenn JB, Mann M, Meng CK, Wong SK, Whitehouse C. Science 246 (1989) 64–71.

Tanaka K, Ido Y, Akita S, Yoshida Y, Yoshida T. 35-kai Shitsuryo Bunseki Rengo Toronkai, Yoshishu (1987) 22–23.

Karas M Hillenkamp F. Anal Chem 60 (1988) 2299–2301.

3.1.1 Matrix Assisted Laser Desorption Ionization

Matrix Assisted Laser Desorption Ionization (MALDI) ions are created by mixing the analyte with a small, organic molecule which absorbs light at the wavelength of the laser, the matrix. The analyte becomes incorporated into the crystal lattice of the matrix and is then irradiated with a laser. The laser causes desorption and ionization of the matrix and analyte, either by protonation or cationation (positively charged ions) or by deprotonation (negatively charged ions; see Figure 3.3). The ions are then accelerated into the MS analyzer (see Section 3.2)

MALDI produces predominantly singly charged ions.

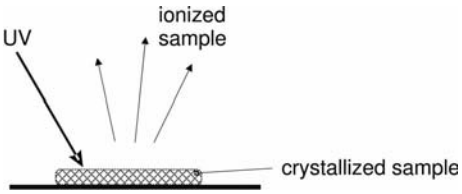


Fig. 3.3: Schematic of the MALDI process.

For a tryptic digest, α-cyano-4-hydroxy cinnamic acid is typically the standard matrix of choice (Beavis and Chait 1989). The matrix affords high sensitivity for the detection of peptides (and proteins) and exhibits negligible matrix adduction. A second matrix, 2,5-dihydoxybenzoic acid (DHB) initially described by Strupat *et al.* 1991; enables peptide analysis, detection of high molecular weight proteins and the analysis of oligosaccharides released from glycoproteins (Mock *et al.* 1991; Stahl *et al.* 1991; Harvey 1993).

Beavis, RC, Chait BT. Rapid Commun Mass Spectrom 3(1989) 432–435.

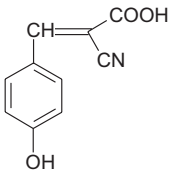
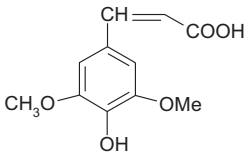
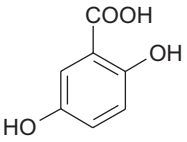
Strupat K, Karas M, Hillenkamp F. Int J Mass Spectrom Ion Proc 111 (1991) 89–102.

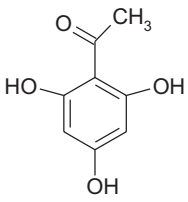
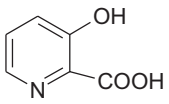
Mock KK, Davy M, Cottrell JS. Biochem Biophys Res Commun 177 (1991) 644–651.

Stahl B, Steup M, Karas M, Hillenkamp F. Anal Chem 63 (1991) 1463–1466.

Harvey DJ. Rapid Commun Mass Spectrom 7 (1993) 614–619.

Tab. 3.1: Common MALDI matrices used in biological applications.

Matrix	Matrix Structure	Application	
α-Cyano-4-hydroxy-cinnamic acid		UV laser: peptide analysis and protein digests. Analytes <10 kDa	*Beavis RC, Chaudhary T, Chait BT. Org Mass Spectrom 27 (1992) 156–158*
Sinapinic acid (4-hydroxy-3,5-dimethoxycinnamic acid)		Analysis of large polypeptides and proteins >10 kDa	*Beavis RC, Chait BT. Rapid Commun Mass Spectrom 3 (1989) 432–435.*
2,5-Dihydroxybenzoic acid (2,5 DHB)		UV laser: protein digests and proteins, oligosaccharides released from glycoproteins	*Strupat K, Karas M, Hillenkamp F. Int J Mass Spectrom Ion Proc 111 (1991) 89–102.*

Matrix	Matrix Structure	Application
2,4,6-Trihydroxyacetophenone (THAP)		UV laser: oligonucleotides <3 kDa
3-Hydroxy picolinic acid		UV laser: Oligonucleotides >3 kDa

Pieles U, Zurchner W, Schar M, Moser HE. Nucleic Acids Res 21 (1993) 3191–3196.

Due to the pulsed nature of MALDI, it has been predominantly coupled with the pulsed, time-of-flight (TOF) analyzer or configurations thereof. This combination, with its simple operation, ease of automation and robust performance has meant that MALDI-TOF instruments are common place for routine protein identification such as in the 2D PAGE-MS workflow where protein spots are picked from a 2DE gel (see Section 1.7.1). The use of MALDI-TOF for peptide mass fingerprinting (PMF) is more than sufficient for protein identification from these simple samples.

However, MALDI PMF is not a suitable method for protein identification when a complex mixture is under investigation. For the analyis of more complex samples, tandem mass spectrometry (MS/MS) is almost exclusively required and there is an absolute need to couple a chromatographic step with MS. MALDI has long been incompatible with liquid chromatography and as such a chromatographic step has typically been coupled with electrospray ionization equipped mass spectrometers for MS/MS applications. However, the innate features of MALDI still make it a very attractive and accessible technique. Thus, the introduction of the MALDI TOF/TOF configuration with its high performance MS/MS specifications and recent developments in liquid handling systems enabling nano-scale chromatography peptide separations to be coupled with MALDI system capable of MS/MS, have been a welcome addition to the proteomics toolbox. Importantly, this configuration supports both high performance PMF and MS/MS in a single system.

In this offline method, LC-MALDI, peptides are eluted from a nano-LC column in the standard fashion, but are then mixed with a matrix prior to spotting onto a MALDI plate. In a standard configura-

tion, such an LC-MALDI spotter will consist of a T-piece, syringe pump, and micro fan. A low dead-volume PEEK-T and mixer (dead volume <50 nL) connect the capillaries from the syringe pump and UV cell to the sample needle, providing reproducible mixing before spotting. The syringe pump delivers the matrix solution with continuous flow rates down to a few nanoliters per minute. Different syringe barrel sizes can be employed, eliminating the need for frequent refilling of small syringes. The length of the capillary connections from the UV cell and the syringe pump are short, resulting in a delay time as short as 3 minutes. Commercial LC-MALDI spotters are provided by a number of vendors.

An LC-MALDI spotter is significantly different to the conventional MALDI spotter available as part of the 2D PAGE-MS workflow for MALDI PMF.

LC-MALDI affords some attractive benefits to the researcher:

- Fixation of samples to the MALDI target, allows longer data collection and sample re-interrogation at any time, removing the temporal constraints of the LC dimension.
- An RPC separation will reduces the complexity of the sample as discussed previously, which will aid minimization of ion suppression.
- Conservation of samples.
- Probable separation of isobaric peptides (i.e. peptides with the same apparent molecular weight), which might have different hydrophobicities and would therefore be resolved by LC.
- Increased sequence coverage for higher confidence in protein identification.

The LC-MALDI technique is a powerful adjunct to ESI based mass spectrometers, with MALDI and ESI often providing complementary protein identification information; Bodnar WM *et al.* 2004. This publication reported a "significant degree of overlap" (63%) was observed between the proteins identified in the LC/ESI/MS/MS and LC/MALDI/MS/MS data sets in the analysis of mammalian mitochrondrial ribosomes. However, unique peptides and proteins were identified by each method; indicating that improved proteome coverage can be obtained using a combination of these ionization techniques.

Bodnar WM, Blackburn RK, Krise JM, Moseley MA. J Am Soc Mass Spectrom 14 (2003) 971–979.

The technique has been applied to a number of challenging and topical proteomics applications including the identification of membrane proteins (Zhang *et al.* 2004); identification and quantification

Zhang N, Li N, Li L. J Proteome Res 3 (2004) 719–727.

Ji C, Li L. J Proteome Res 4 (2005) 734–742.
Chen HS, Rejtar T, Andreev V, Moskovets E, Karger BL. Anal Chem 77 (2005) 2323–2331.

using differential stable isotope labeling (Ji and Li 2005) and the incorporation of high speed, high-resolution monolithic capillary columns into the technique. In this publication, 384 unique peptides were identified in a single 10 minute LC run from 2000 resolved components (Chen *et al*. 2005).

3.1.2 Electrospray Ionization

Fenn JB, Mann M, Meng CK, Wong SK, Whitehouse CM. Science 246 (1989) 64–71.

Fenn *et al*. (1989); described the use of electrospray ionization (ESI) for ionizing large biomolecules in 1989.

Electrospray (ESI) ions are produced at atmospheric pressure by applying sample dissolved in solvent to a narrow capillary tube, which is under the influence of an electric field. A potential difference is created between the capillary and the inlet of the mass spectrometer generating a force extending the liquid to form a cone from the capillary tip, from which a fine mist of droplets will emerge (Taylor cone; Taylor 1964). The cone is formed as a result of repulsive coulombic forces between the like charges. Subsequently, evaporation of the droplets reduces droplet size whilst the charge on the droplet remains constant. Eventually, the surface coulombic forces exceed the surface tension and the droplets fission into smaller droplets (the Rayleigh limit). This process continues until nanometer-sized droplets are produced (Figure 3.4).

Taylor G. Proc R. Soc Lond A280 (1964) 383–397.

The charges are statistically distributed over the analyte's potential charge sites, enabling the formation of multiply charged ions. Each multiply charged ion can be termed a charge state, and a distribution of charge states is characteristic of large macromolecules during ESI analysis.

ESI produces predominantly multiply charged ions.

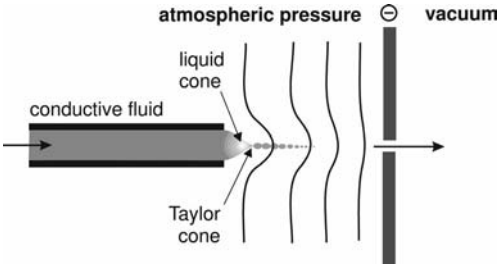


Fig. 3.4: Schematic of ESI.

This unique feature of ESI, multiple charging of analyte molecules, yields an effectively reduced mass for the analyte of interest. Simply, the observable mass range is lowered offering significant advantages for the analysis of large molecular weight analytes, particularly proteins. Figure 3.5 demonstrates the multiply charging of 150 KDa protein. With respect to proteins the resolution of each multiply charged ion is insufficient to determine the number of charges the ion possesses, from which can be determined its true mass. Therefore, the masses of at least two of the multiply charged ions in the envelope are used in a deconvolution algorithm to give the mass of the whole protein (lower panel).

Note the mass range and the number of charges (z value in m/z) on the protein in the spectrum).

Its mass is known from the spectrum, but without knowledge of the charge state, the real mass of the analyte remains unknown.

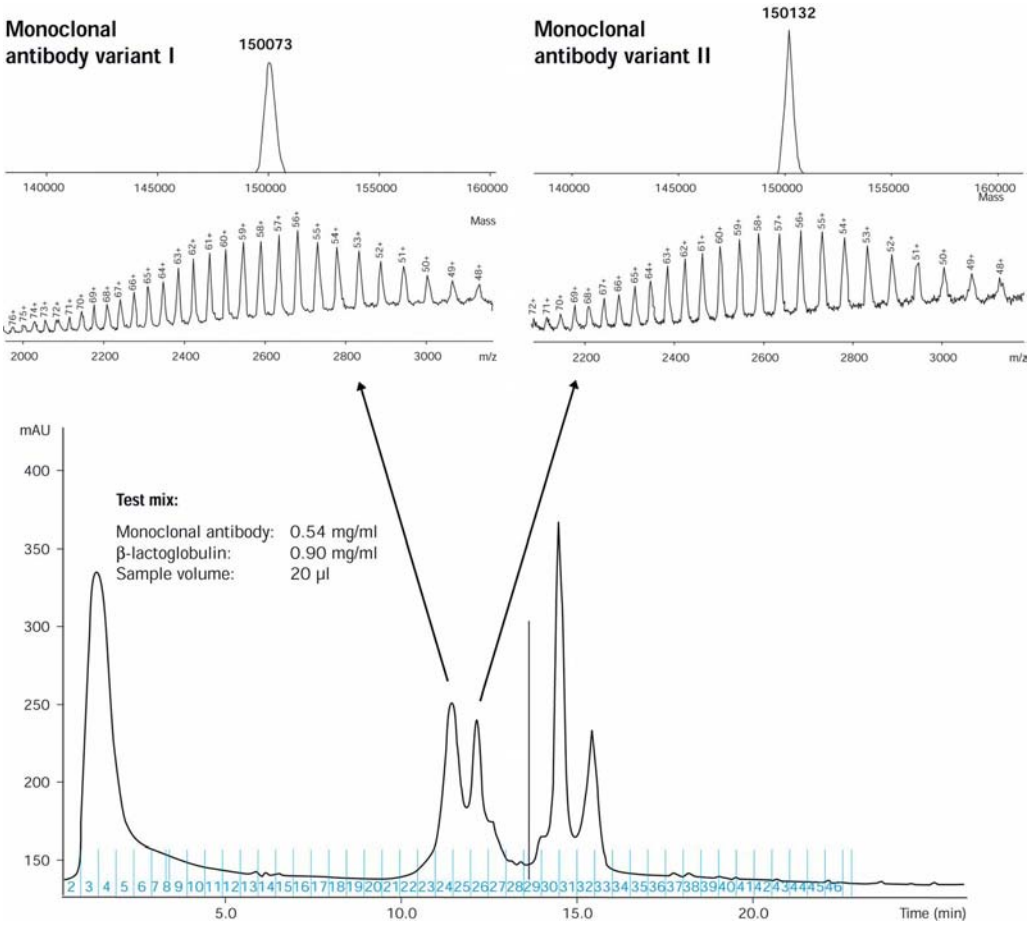


Fig. 3.5: Characteristic multiple charging of a full-length protein acquired using ESI. Upper panel: true mass of protein after deconvolution. Lower panel: multiple-charged spectrum. Note the number of charge states observed for this 150 kDa protein.

With respect to peptides, the charge state of the multiple-charged ion can be readily determined due to the resolution capability of current mass spectrometers, see Figure 3.6. The mass difference between the adjacent ^{12}C and ^{13}C isotopes is indicative of the charge state. For a single-charged ion, the mass difference between the ^{12}C and ^{13}C isotopes of a peptide isotopic envelope will be 1 Da. In the case of a double-charged peptide ion (m/z = m/2) the mass difference between the ^{12}C and ^{13}C isotopes will be 0.5 Da, for a triple-charged peptide (m/z = m/3) the mass difference between the ^{12}C and ^{13}C will be 0.33 Da and so on. With knowledge of the charge state, an accurate mass of the peptide can be determined.

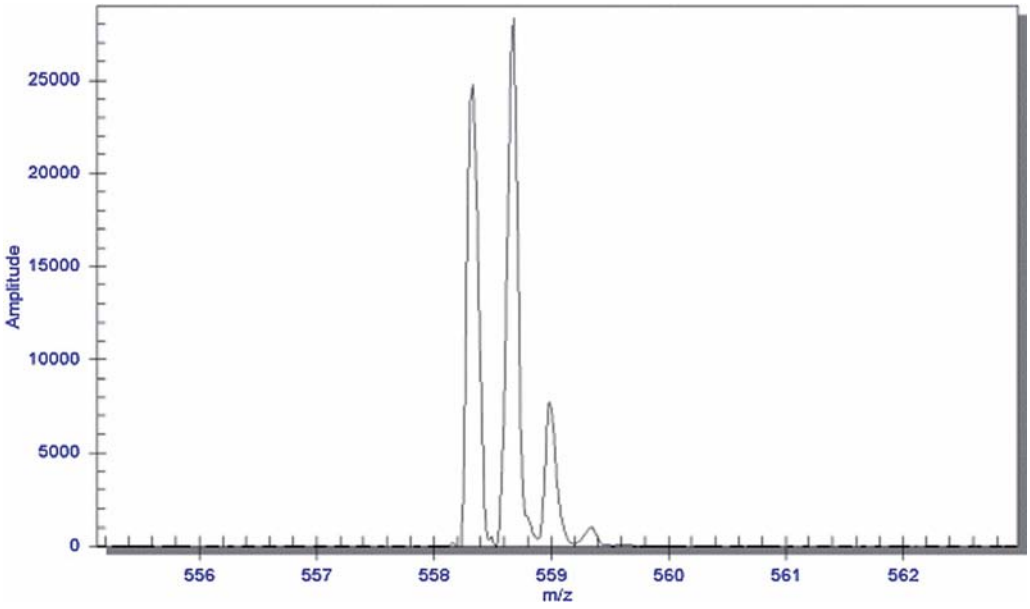


Fig. 3.6: Characteristic multiple charging of tryptic peptides: triple-charged neurotensin protein acquired using ESI.

Electrospray generates flow rates ranging from tens of microliters to hundreds of microliters per minute. The resultant high consumption rate was problematic for proteomic applications with respect to sensitivity and analysis time (high amount of sample is consumed in a short amount of time). In the early nineties, reductions in ESI low rates were reported; sub-microliter flow rates using continuous infusion affording significant increases in sensitivity (Emmett and Caprioli 1994). Wilm and Mann reported the use of a nanospray ionization source. (Wilm and Mann 1996) This miniaturization of electrospray generated flow rates in the order of tens of nanoliters per minute (typically 25–40 nL/min), by spraying the solvent from a narrow

bore coated needle (sub 10 μm tip diameter). This was a crucial development; delivering higher sensitivity and longer analysis times, enabling multiple MS/MS experiments to be performed per run.

Emmett MR, Caprioli RM. J Am Soc Mass Spectrom 5 (1994) 605–613.

Though nanospray generates flows of 25–40 nL/min, nanoLC (at least 75 mm i.d columns) only generates flow rates of 200 nL/min.

Wilm M, Mann M. Anal Chem 68 (1996) 1–8.

3.2 Ion Separation

A wide range of analyzers, compatible with both MALDI and ESI, are used for proteomics applications

3.2.1 Time-of-Flight Analyzer

The time-of-flight (TOF) analyzer is a pulsed analyzer and as such is routinely coupled with a MALDI ion source. (see Figure 3.7).

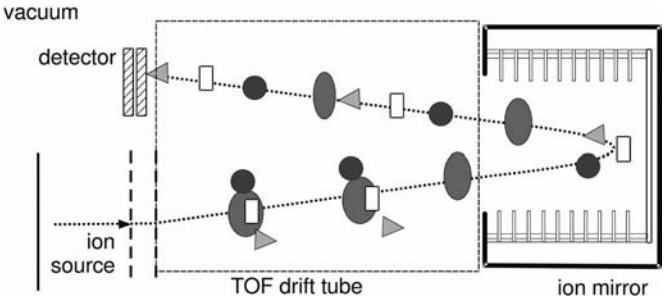


Fig. 3.7: Schematic of a reflectron TOF analyzer. This analyzer is typically coupled with MALDI to perform peptide mass fingerprinting.

Mass measurement is determined by measuring the time-of-flight of an ion in the analyzer region; the time-of-flight of an ion is proportional to the square root of its mass/charge ratio, given a constant accelerating voltage: Time of flight $k\sqrt{m/z}$.

Thus, the smaller the molecule the faster it will travel the distance of the flight tube to the detector.

The linear TOF analyzer is the simplest form and exhibits the lower performance with respect to resolution and mass accuracy. A linear TOF instrument is incapable of resolving the ^{12}C and ^{13}C isotopes of the peptide isotopic envelope across the range of the protein digest, 700–3500 Da, subsequently mass accuracy is poorer as the average mass, not the mono-isotopic mass, tends to be used for database searching.

The performance of TOF analyzers with respect to resolution and mass accuracy was vastly improved following two developments in the 1990s: delayed extraction and incorporation of a reflectron.

Wiley WC , McLaren IH. Rev Sci Instrum. 26 (1955) 1150–1157.

Brown R, Lennon JJ. Anal Chem. 67 (1995) 1998–2003.

Whittal RM, Li L. Anal Chem 67 (1995) 1950–1954.

Vestal ML, Juhasz P, Martin SA. Rapid Commun Mass Spectrom 9 (1995) 1044–1050.

Delayed extraction Resolution is improved by delaying the extraction of ions from the source for a short period of time. Wiley and McLaren, 1953; reported the method time lag focusing and noted the increased resolution. This was revisited by Brown and Lennon, Vestal *et al.* Whittal and Li in 1995 by applying it to MALDI. They observed that by delaying the extraction of ions from the source, a much tighter packet of ions arrive at the detector giving significantly higher resolution and mass accuracy.

Mamyrin BA. Int J Mass Spectrom 206 (2001) 251–266.

Incorporation of a reflectron A reflectron (Mamyrin 2001) improves resolution in two ways: (1) by acting as an ion mirror reversing the trajectory of the ions in the flight tube, effectively increasing the length of the flight tube; (2) by reducing an ion's kinetic energy spread. Ions of the same mass formed in the source can have different kinetic energies when they leave the source depending upon their position in the source when the accelerating voltage was applied, consequently, ions of the same mass arrive at the detector at different times, thus reducing resolution and mass accuracy. A reflectron can accommodate these small differences in kinetic energy with the result that ions of the same mass are better time-focused at the detector, greatly improving resolution and subsequently mass accuracy.

Protein database search confidence using MALDI TOF PMF data is improved with increased mass accuracy

Cornish TJ, Cotter RJ. Rapid Commun Mass Spectrom 8 (1994) 781–785.

Anderson UN, Colburn AW, Makarov AA, Raptakis EN, Reynolds DJ, Derrick PJ, Davis SC, Hoffman AD , Thomson S. Rev Sci Instrum 69 (1998) 1650–1660.

Delayed extraction coupled with a reflectron can routinely achieve resolutions greater than 10,000 (FWHM; Figure 3.8). This level of performance enabled MALDI-TOF to become established as the principle method of protein ID in the 2D PAGE-MS workflow (Section 1.7).

Reflectrons are incorporated as standard in most commercial TOF mass spectrometers and the different configurations include a conventional linear field reflectron, the curved field reflectron (Cornish and Cotter, 1994) and the harmonic or quadratic field described by Anderson *et al.* 1998.

Boyle JG, Whitehouse CM. Anal Chem 64 (1992) 2084–2089.

Although a TOF analyzer is commonly coupled with a pulsed MALDI source it has also been combined with electrospray, in the ESI-TOF instrument (Boyle and Whitehouse 1992; Mirgorodskaya *et al.* 1994; Verentchikov *et al.* 1994).

Mirgorodskaya OA, Shevchenko AA, Chernushevich IV, Dodonov AF, Miroshnikov AI. Anal Chem. 66 (1994) 99–107.

Verentchikov AN, Ens W, Standing KG. Anal Chem 66 (1994) 126–133.

A reflectron TOF instrument is typically used in MS mode, though a technique called post source decay (PSD) has been considered a pseudo MS/MS technique (see Section 3.3.4.4).

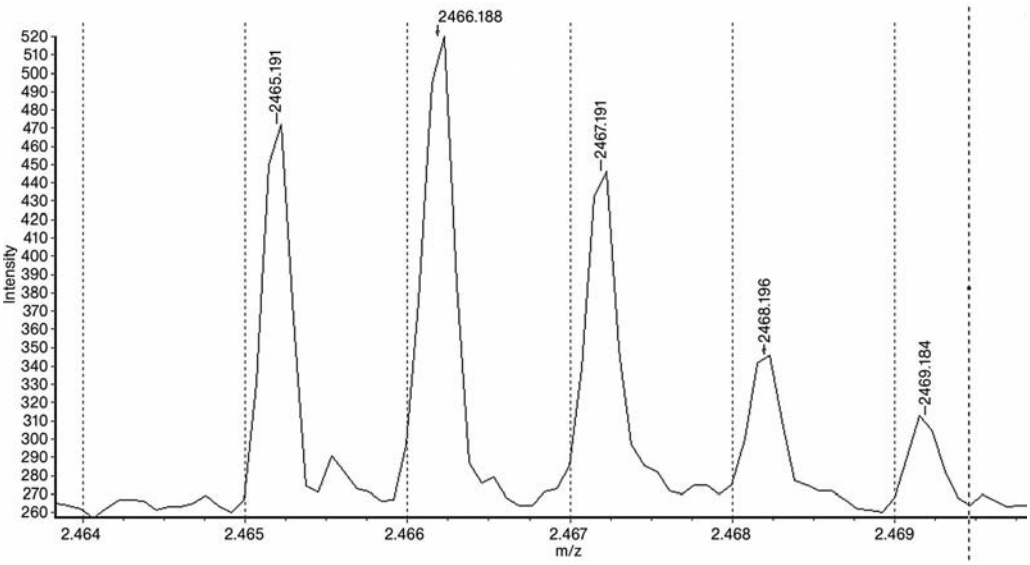


Fig. 3.8: Delayed extraction coupled with a reflectron can routinely achieve resolutions greater than 10,000 (FWHM).

3.2.2 Triple Quadrupole Analyzer

The quadrupole analyzer consists of four parallel hyperbolic rods, through which the gas phase ions have to achieve a stable trajectory. The analyzer is operated by the application of a voltage (DC) and an oscillating voltage (radio frequency, rf) to one pair of rods and DC voltage of opposite polarity and rf voltage of different phase to the opposite pair of rods. The alternating electric field helps to stabilize and destabilize the passing ions. The ions traverse through the space between the rods, and only at specific voltages applied to the rods will certain m/z values be allowed to pass through the rods and reach the detector. The voltages are scanned to allow a wide mass range to be observed.

A single quadrupole analyzer has negligible benefits for proteomic analysis, but if three quadrupoles are arranged in sequence, the subsequent triple quadrupole analyzer coupled with ESI has been demonstrated to be very useful for proteomics (von Haller *et al.* 2001) This configuration is almost exclusively used to provide structural information by tandem mass spectrometry (see Section 3.3.4). Here the first and third quadrupoles operate as the mass filters described above, with the second quadrupole operating as a collision cell (where the structural information is produced). The analyzer can be operated

von Haller PD, Donohoe S, Goodlett DR, Aebersold R, Watts JD. Proteomics 1 (2001) 1010–1021.

in three different ways to perform MS/MS experiments (see Section 3.3.4.1) and is typically used in MS/MS mode (see Figure 3.9).

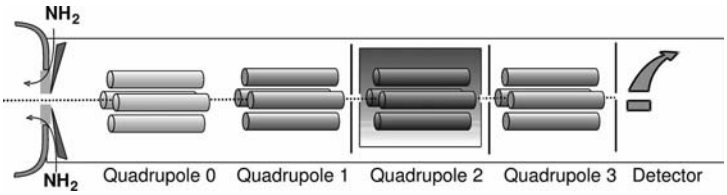


Fig. 3.9: Schematic of a triple quadrupole analyzer. Coupled with ESI, it can perform a range of MS/MS techniques including product ion, precursor ion and neutral loss scans.

3.2.3 Quadrupole Ion Trap

Stafford GC, Kelley PE, Syka JEP, Reynolds WE, Todd, JFJ. Int J Mass Spectrom Ion Proc 60 (1984) 85–98.

The quadrupole ion trap is based on the same theory as the quadrupole analyzer, with the quadrupole field generated within a three dimensional trap. The ion trap itself is filled with helium and comprises a ring electrode and two end cap electrodes, creating the three dimensional electric field by applying a large RF voltage to the ring electrode. The orbiting motion of the ions in the trap is governed by the large rf voltage and the cooling effects of collisions with the helium gas (the helium gas reduces the kinetic energy of the ions and helps focus the ions in the center of the trap). A mass spectrum is acquired by sequentially ejecting fragment ions from low m/z to high m/z. This is performed by scanning the rf voltage to make ion trajectories sequentially become unstable, the mass selective instability mode developed by Stafford *et al.* (1984). Ions are ejected and detected at the detector (see Figure 3.10).

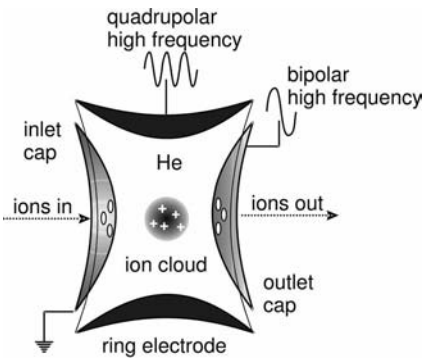


Fig. 3.10: Schematic of the fundamental operation of a 3D ion trap analyzer. Commonly coupled with ESI for proteomics applications.

A product ion MS/MS spectrum is obtained via resonance excitation. The precursor ion is isolated by the application of a waveform signal to the endcap electrodes. Fragmentation of the precursor ion is caused by the application of a small voltage across the endcap electrodes and collisions with helium gas. The resultant fragmentation is similar to that observed from a triple quadrupole mass spectrometer; a low-energy collisional regime with fragmentation predominantly at the peptide amide bonds (see Section 3.3.4.1). A drawback of the instrument for the acquisition of product ion MS/MS spectra is that the fragment ions in the lower third of the mass range (approximately $1/3^{rd}$ of the mass of the precursor ion) will not be detected.

An additional advantage of quadrupole ion traps and FT-ICR MS (see Section 3.2.7) is the unique ability to perform multiple stages of mass spectrometry (MS^n) (see Section 3.3.4.5). For a detailed review see Jonscher and Yates (1997).

Jonscher KR, Yates JR III. Anal Biochem 211 (1997) 1–15.

The performance characteristics of a quadrupole ion trap make it a highly attractive instrument for proteomic applications, particularly for tandem mass spectrometry applications, either interfaced with nanospray (Hoess *et al.* 1999) LC-MS/MS (Ho *et al.* 2002) 2DLC-MS/MS (Washburn *et al.* 2001) or MALDI (Qin *et al.* 1996). The ion trap is predominantly used in MS/MS mode.

Developments in ion trap technology have delivered the linear quadrupole ion trap, where ions are trapped in a two-dimensional quadrupole field, instead of a three-dimensional quadrupole field. Such a configuration affords significant benefits for protein ID and characterization including rapid cycle time which enables more information to be obtained from a sample in less time with high sensitivity (Yates *et al.* 2006). The linear ion traps also offer additional tandem mass spectrometry functionality (Macek *et al.* 2006). In addition, to MS^n, commercial ion trap systems are available that can perform a novel fragmentation technique, electron transfer dissociation or ETD (Thermo LTQ and Bruker Esquire HCT Ultra), see section 3.3.4.6. ETD, a peptide and protein fragmentation technique that preserves modifications, has found applications in PTM analysis such as phosphorylation or glycosylation and in top down proteomics. The technique extends the capability of the user with respect to protein sequencing and identification of type and location of various PTMs. The common approach is to use both collision induced dissociation and ETD, generating non-redundant sequence information.

Hoess M et al. 1999; Washburn MP, Wolters D, Yates JR. Nature Biotech 19 (2001) 242–247.

Qin J, Steenvorden RJJM, Chait BT. Anal Chem. 68 (1996) 1784–1791.

Hoess M, Robins P, Naven TJP, Pappin DJC, Sgouros T, Lindahl T. EMBO J 18 (1999) 3868–3875.

Macek B, Waander L, Olse, JV, Mann M. Mol Cell Proteomics 5 (2006) 949–958.

Yates JR, Cociorva D, Liao J, Zabrouskov V. Anal. Chem. 78 (2006) 493–500.

3.2.4
Quadrupole Time-of-Flight

Morris HR, Paxton T, Dell A, Langhorne J, Berg M, Bordoli RS, Hoyes J, Bateman RH. Rapid Commun Mass Spectrom 10 (1996) 889–896.

Shevchenko A, Chemushevich IV, Ens W, Standing KG, Thomson B, Wilm M, Mann M. Rapid Commun Mass Spectrom 11 (1997) 1015–1024.

Bell AW, Ward MA, Blackstock WP, Freeman HNM, Choudhary JS, Lewis AP, Chotai D, Fazel A, Gushue JN, Paiement J. J Biol. Chem 276 (2001) 5152–5165.

Gavin et al. Nature 415 (2002) 141–147.

Initially described in 1996 (Morris *et al*. 1996) for oligosaccharide analysis and more recently by Lobada *et al*. (2000), these quadrupole time-of-flight (QTOF) instruments rapidly became the instrument standard for MS/MS applications within proteomics. By combining a mass filtering quadrupole analyzer and a collision cell (rf-only quadrupole) with a non-scanning reflectron TOF analyzer, the user is able to acquire MS and, most notably MS/MS data with high mass accuracy, resolution and sensitivity. The instrument is typically coupled with HPLC (Bell *et al*. 2001; Gavin *et al*. 2002) and is predominantly used in MS/MS mode (see Figure 3.11).

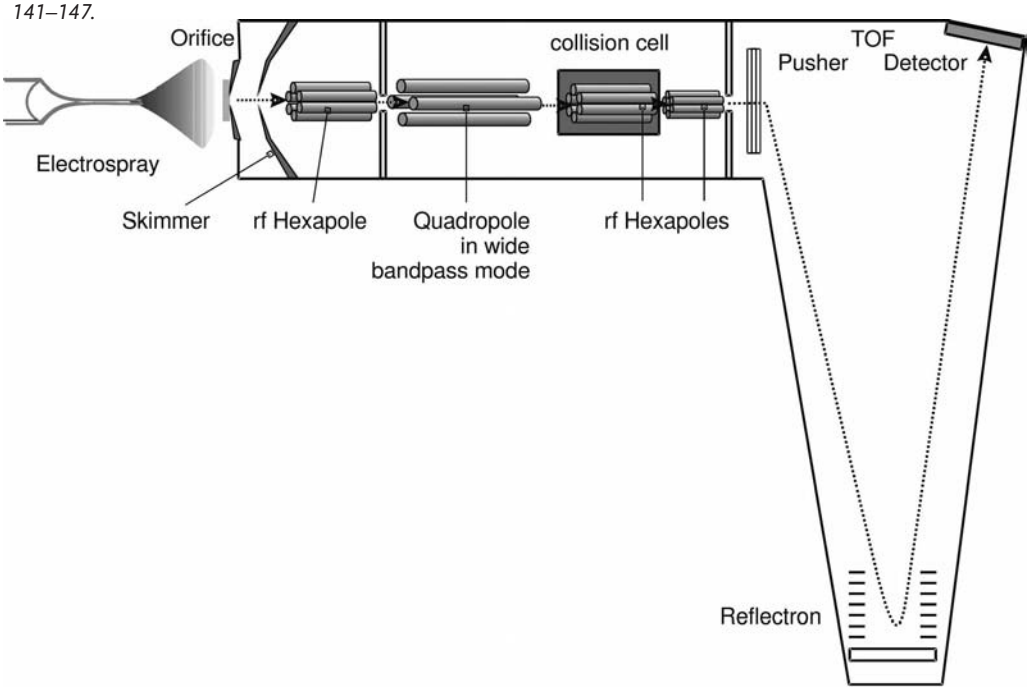


Fig. 3.11: Schematic of a quadrupole TOF hybrid analyzer. It is commonly coupled with ESI for proteomics applications.

Later developments have seen a MALDI ion source coupled with a hybrid instrument enabling similar MS/MS performance to be attained from singly charged ions produced by MALDI. (Lobada *et al*. 2000; Shevchenko *et al*. 2000; Baldwin *et al*. 2001).

Lobada A, Krutchinsky A, Bromirski M, Ens W, Standing KG. Rapid Commun Mass Spectrom 14 (2000) 1047–1057.

This configuration enables a peptide mass fingerprint and MS/MS data with high resolution and mass accuracy to be acquired using the same instrument. A similar approach has been developed with the TOF/TOF analyzer.

Shevchenko A, Loboda A, Shevchenko A, Ens W, Standing KG. Ana. Chem 72 (2000) 2132–2141.

3.2.5
Hybrid Triple Quadrupole Linear Ion Trap

Baldwin MA, Medzihradszky KF, Lock CM, Fisher B, Settineri TA, Burlingame AL. Anal Chem 73(2001) 1707–1720.

As the name suggests, the hybrid triple quadrupole linear ion trap (QTRAP) instrument combines the features of a triple quadrupole and a linear ion trap analyzer. For proteomics applications, this equates to fast scans with high sensitivity, accurate quantification and complete product ion, precursor ion and neutral loss tandem mass spectrometry functionality. The additional scan functionality has been shown to be useful for post translational modification analysis (Sandra *et al*. 2004).

Sandra K, Devreese B, Van Beeumen J, Stals I, Claeyssens M. J Am Soc Mass Spectrom 15 (2004) 413–423.

The QTRAP is predominantly used in MS/MS mode.

3.2.6
TOF/TOF Analyzer

A TOF/TOF analyzer coupled with a MALDI ion source (Medzihradszky *et al*. 2000) enables the generation of peptide mass fingerprint data and peptide sequence data in a single instrument. Basically, two TOF analyzers are separated by a collision cell, with the first TOF analyzer, used for precursor ion selection. (see Figure 3.12). High energy collisions occur within the collision cell, and the second TOF analyzer resolves the ions. The configuration allows for high sensitivity and high resolution in both MS and MS/MS modes, and is capable of reducing protein identification to a one tier process. When coupled with nano-LC using an LC-MALDI spotter, this configuration is a powerful tool for the proteomics researcher.

Medzihradszky KF, Campbell JM, Baldwin MA, Falick AM, Juhasz P, Vestal ML, Burlingame AL. Anal Chem 72 (2000) 552–558.

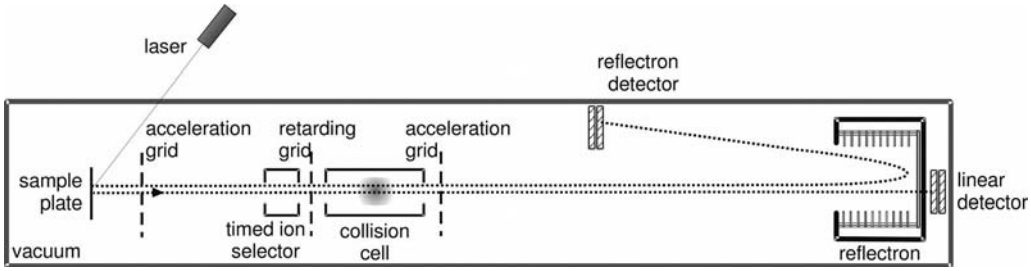


Fig. 3.12: Schematic of a TOF/TOF analyzer. Coupled with MALDI to perform peptide mass fingerprinting and peptide sequence analysis by MS/MS. Often coupled with a nanoLC spotter in LC-MALDI.

3.2.7
Fourier Transform Ion Cyclotron

Similarly to an ion trap, a Fourier transform ion cyclotron (FT-ICR) instrument is capable of trapping and storing ions (see Figure 3.13). The ICR cell resides in a strong magnetic field and consists of three parallel plates arranged in a cube. In the cell, ions of a given m/z ratio have a given cyclotron frequency of a given orbit radius. On applying an rf voltage at the same frequency as the cyclotron frequency, the respective ions absorb energy and are accelerated to a larger orbit radius. When the rf voltage is removed the energized, accelerated ions still rotate at a constant radius. Ions which have a different cyclotron frequency remain unexcited and hence ions of differing mass can be separated. As the cyclotron frequency of an ion is determined by its mass to charge ratio, a Fourier transform can be performed on the signal to determine the mass of the ion. FT-MS affords high sensitivity and exceptional resolution and thus mass accuracy.

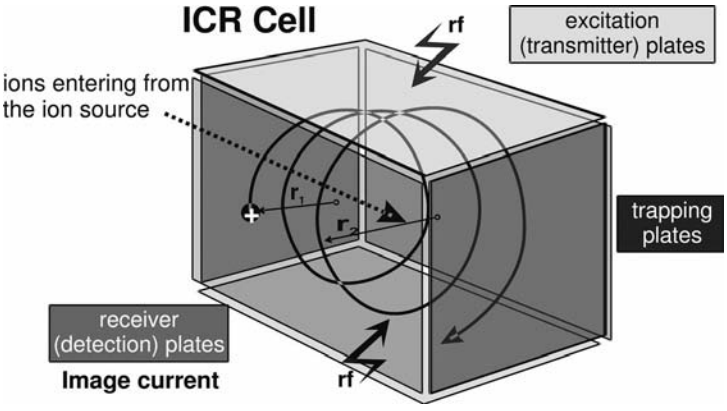


Fig. 3.13: Schematic of the fundamental operation of an FT ICR mass spectrometer. It is commonly coupled with ESI for proteomics applications.

3.2.8
Orbitrap™

The Orbitrap™ analyzer is the most recently of developed analyzers (commercially available from Thermo Scientific since 2005). It is not a conventional ion trap, in that there is no RF or magnet to hold the ions in the cell. The analyzer traps ions in an electrostatic field, with the attraction towards a central electrode. The electrode confines the ions so that they move or orbit in complex spiral patterns (Makarov 2002, and Hardman and Makarov, 2003). A Fourier Transform is used to extract the masses accurately from the oscillation frequencies This type of analyzer affords high resolution, high mass accuracy and an increased dynamic range, similar in performance to an FT-ICR instrument and high sensitivity (Olsen *et al*. 2005). This type of mass spectrometer can be used in both MS and MS/MS modes.

Hardman M, Makarov A. Anal Chem 75 (2003) 1699–1705.

Makarov A. Anal Chem 72 (2000) 1156–1162.

Olsen JV, de Godoy LMF, Li G, Macek B, Mortensen P, Pesch R, Makarov A, Lange O, Horning S, Mann M. Mol Cell Proteomics 4 (2005) 2010–2021.

The Orbitrap™ has recently been coupled with a linear ion trap (Finnigan LTQ-Orbitrap™, Thermo) (see Figure 3.14). This configuration is a hybrid consisting of the two mass analyzers, both capable of detecting ions and recording spectra: the two analyzers can be used independently or in conjunction. The instrument can be used for both bottom up (peptide based approach to protein ID) and top down (protein based approach to protein ID) approaches (see Section 3.6.2).

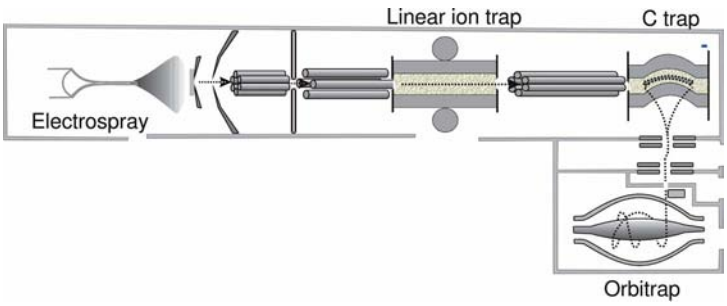


Fig. 3.14: Schematic of the hybrid LTQ-Orbitrap™ analyzer.

3.3
Generating MS Data for Protein Identification

Generally, two different approaches, or combinations thereof, can be used for protein identification (ID): peptide mass fingerprinting and tandem mass spectrometry (MS/MS). In the first instance, the mass/charge ratio (m/z) of the analytes are measured and the calculated masses are indicative of the composition of the individual analytes.

In the second instance, tandem mass spectrometry (MS/MS) is used to acquire peptide sequence information, which is used to identify and characterize proteins with high specificity (see Figure 3.15).

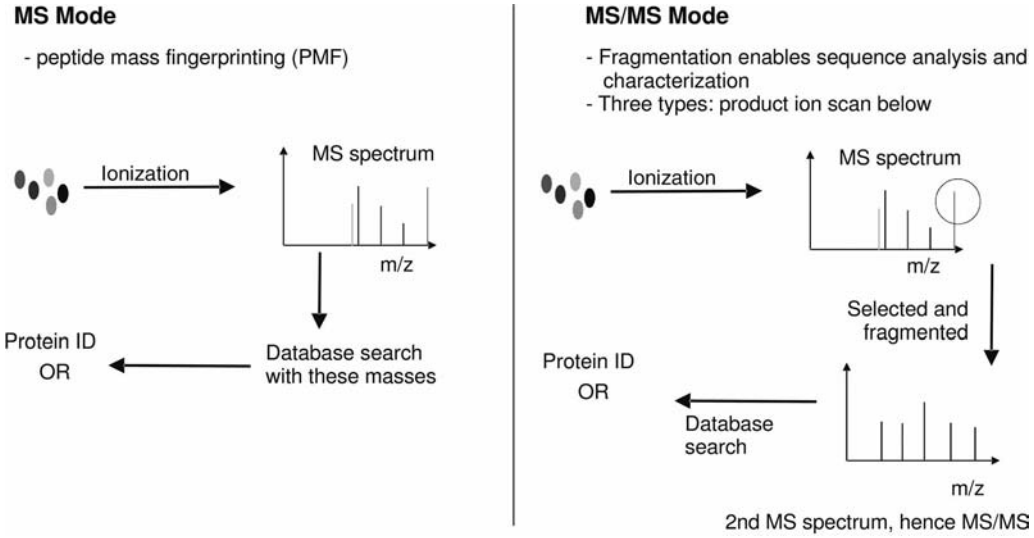


Fig. 3.15: Schematic of the methods of protein identification using a mass spectrometer: Peptide mass fingerprinting is performed in MS mode and peptide sequence analysis in MS/MS mode.

3.3.1 Peptide Mass Fingerprint

The technique originally described in 1993 comprises protein digestion, MALDI TOF analysis and sequence database search algorithms. (Pappin *et al*. 1993; Henzel WJ *et al*. 1993; Mann *et al*. 1993; Yates *et al*. 1993; James *et al*. 1993).

Simply, every protein in the protein database (or in the genome under investigation) is theoretically digested with the cleavage reagent used in the digestion reaction, generating many hundreds of thousands of theoretical peptides. The experimental peptide masses derived from the MS spectrum, the peptide mass fingerprint (PMF) is then compared to the theoretical peptide masses and a score is calculated and assigned. The score reflects the match between the theoretically and experimentally determined masses; the protein identified as the most probable is the one that gives the best match between the experimental and the theoretical peptides. The number of peptides observed in the PMF and the accuracy to which they are measured determines the confidence of the protein identification.

Pappin DJC, H jrup P, Bleasby A. Curr Biol 3 (1993) 327–332.

Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C. Proc Natl Acad Sci USA 90 (1993) 5011–5015.

Mann M, Hojrup P, Roepstorff P. Biol Mass Spectrom 22 (1993) 338–345.

Yates JR III, Speicher S, Griffin PR, Hunkapiller T. Anal Biochem 214 (1993) 397–408.

James P, Quadroni M, Carafoli E, Gonnet G. Biochem Biophys Res Commun 195 (1993) 58–64.

The incorporation of reflectron technology and delayed extraction (timed delay ion extraction) into MALDI-TOF instrumentation has enhanced the performance of peptide mass fingerprinting considerably. The ^{12}C isotope of the peptide isotopic envelope, the mass used in a PMF search, can be unambiguously assigned across the peptide mass range of interest, significantly enhancing mass measurement of this peak (sub 50 ppm mass accuracy is routine). The improved mass accuracy further constrains the database search, reducing the potential for ambiguous protein identifications (Jensen *et al*. 1996; Clauser *et al*. 1999).

Jensen ON, Podtelejnikov A, Mann M. Rapid Commun Mass Spectrom 10 (1996) 1371–1378.

Clauser KR, Baker P, Burlingame AL. Anal Chem 71 (1999) 2871–2882.

Several programs are available to perform this type of search, varying in the execution of the task (including MASCOT at www.matrixscience.com; and Phenyx at http://phenyx.vital-it.ch/pwi/login/login.jsp). Accuracy, reliability and speed determine the program of choice. Regardless which program is used, four user variables are important for a PMF search:

- Peptide mass list.
- Specification of the cleavage agent.
- Error tolerance. The accuracy of mass measurement is determined by the calibration, the higher the mass accuracy the greater the specificity.
- Knowledge of peptide modifications i.e. methionine oxidation.

For this approach to give an unambiguous result, a significant number of experimentally determined peptide masses have to match the experimental masses, the peptides have to be a result of the correct cleavage specificity of the cleavage agent used and the protein in question has to exist in the protein database. As a result, the PMF approach is highly suited to highly characterized genomes and has been a core proteomics technique. Shevchenko *et al*. (1996) demonstrated that up to 90% of proteins selected from a 2D gel of an E coli lysate were identified by peptide mass fingerprinting.

Shevchenko A, Jensen ON, Podtelejnikov A, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H, Mann M. Proc Natl Acad Sci USA 93 (1996) 1440–1445.

Although an unambiguous match may be returned from a database search, not all the peptides within the peptide mass fingerprint may be identified. It is important to identify the remaining unidentified peptide peaks, which may arise from:

- The presence of other digested proteins within the sample (in the case of 2D gel, comigration to the same spot);
- Peptides arising from the simultaneous digestion of contaminant proteins such as keratin;

Though many keratin peaks are commonly known and these peptides, if present, can be subsequently omitted from the search.

- Incorrect cleavage, protease acting in a non-predicted fashion;
- Post-translationally modified peptides, altering the mass of the native peptide rendering it unrecognizable in the database search;
- The identified protein may be (highly) homologous to the protein under study, but not exactly the same and thus unmatched peptides will exist.

MALDI-TOF PMF is fast and simple method of protein identification, but the success of the method can be compromised in a number of ways:

Common for small molecular weight proteins, basic proteins if digested with trypsin low abundant proteins.

Axelsson J, Boren M, Naven TJP, Fenyö D. Proceedings of the 49th ASMS conference on mass spectrometry and allied topics, Chicago (2001).

- Insufficient peptides are observed in the peptide mass fingerprint to submit to a database search, i.e. insufficient information to identify the protein.
- The sample maybe a mixture of proteins. Although it is possible to analyze simple mixtures (Axelsson *et al*. 2001) there may not be a significant number of peptides from each protein component in the mixture to yield a successful identification. As several peptides are required to give a statistical match, this approach is not readily suited to complex mixtures
- Excessive post-translational modifications of the protein can result in masses, which are not predicted in the theoretical digestion of the proteins in the database as well as precluding peptides from the fingerprint.
- Very little homology can be found with another protein in the database or the protein in question may not actually exist in the protein database.
- If the above is true, peptide mass fingerprinting cannot be used with confidence for searching against the EST databases (which is the next stage of protein identification if a search of the protein database is unsuccessful). The probability that a significant number of peptides from the peptide mass fingerprint will find matches with a single EST yielding an unique protein identification is small.

If any of these situations arise, then more specific information is required to identify the protein.

3.3.2
Peptide Mass Fingerprint Combined With Composition Information

Historically, it has been possible to combine PMF information with compositional information. This was a common technique prior to the introduction of delayed extraction and reflectron technology. The composition information provided orthogonal information which provided additional specificity for a database search by enabling further constraints on that search (Fenyö *et al*. 1998). For instance, this publication demonstrated that with the knowledge of the presence of a cysteine residue in a tryptic peptide of mass 2000 Da (measured to 0.5 Da mass accuracy) the number of matching proteins in S cerevisiae was reduced by a factor of five. A number of approaches have been employed to gain compositional information. In one example reported by Pappin, a PMF is acquired and the peptide masses noted. A subsequent methyl esterification reaction is performed on a small aliquot of the sample esterifying the carboxyl side-chain of the acidic residues, glutamic and aspartic acid, and the carboxylic group of the C-terminal residue present in each peptide of the digest. A PMF spectrum is re-acquired. The subsequent mass changes between corresponding peptides are indicative of the number of acidic groups each peptide contains. This information was combined with the original PMF using a MOWSE composition search potentially increasing search discrimination by orders of magnitude (Pappin *et al*. 1995).

Fenyö D, Qin J, Chait BT. Electrophoresis 19 (1998) 998–1005.

Pappin D, Rahman D, Hansen HF, Bartlet-Jones M, Jeffrey W, Bleasby AJ. Methods in Biological Sciences (1996) 135–150.

The number of exchangeable hydrogens within a peptide offers composition information. Again, this procedure requires the acquisition of a PMF, subsequent labeling of the mixture with a deuterium solution (D_2O) and finally re-acquisition of a second spectrum. The mass increase of each peptide by the number of exchangeable hydrogens is indicative of amino acid composition (Sepetov *et al*. 1993; James *et al*. 1994).

Sepetov NF, Issakova OL, Lebi M, Swiderek K, Stahl DC, Lee TD. Rapid Commun Mass Spectrom. 7 (1993) 58–62.

James P, Quadroni M, Carafoli E, Gonnet G. Protein Sci 3 (1994) 1347–1350.

Moreover Goodlett *et al*. (2000) demonstrated composition information and high mass accuracy can be very specific information for unambiguously identifying proteins. By labeling cysteine residues with a specific isotopic label, IDEnT (the tag contained chlorine, which has a specific isotopic profile owing to the relative abundance of ^{35}Cl and ^{37}Cl) the distinctive isotopic pattern of the labeled peptide could be simply recognized. With this specific composition information and mass accuracy measured to within 1 ppm using an FT-ICR MS, the mass of a single peptide was sufficient to unambiguously identify a protein from the whole yeast database (1 peptide from a possible in ~345,000 peptides).

Goodlett DR, Bruce JE, Anderson GA, Rist B, Pasa-Tolic L, Fiehn O, Smith RD, Aebersold R. Anal Chem 72 (2000) 1112–1118.

Cysteine was chosen as the labeled residue because it is one of the rarest amino acid residues, allowing constrained database searching.

Sechi S, Chait BT. Anal Chem 70 (1998) 5150–5158.

The isotopical labeling of cysteine residues has also been exploited for extra composition information. Sechi and Chait (1998) reported a method for the modification of the cysteinyl thiol group with isotopically labeled acrylamide.

Clauser KR, Baker P, Burlingame AL. Anal Chem 71 (1999) 2871–2882.

Additionally, composition information can be inferred from immonium ions contained within a MALDI PSD spectrum, this is particularly discriminating information. Immonium ions present in a MALDI PSD spectrum indicate the presence of certain amino acid residues; again this can be included in a composition search as described above. (Clauser *et al*. 1999). This information is also available in a product ion MS/MS spectrum acquired with a MALDI QTOF or TOF/TOF instrument.

However, with the ubiquitous MS/MS functionality available in many laboratories, these approaches are seldom used today.

3.3.3 Peptide Mass Fingerprint Combined With Partial Sequence Information

Again, historically, supplementing PMF data with some partial sequence has been an attractive approach; PMF and sequence information can be used in the same search using the sequence query function with the MASCOT search engine. An unsuccessful search of the protein database with the peptide mass fingerprint data may be overturned with some partial sequence if the protein exists in the protein database.

The data required for this type of database search can be obtained with a MALDI-TOF instrument capable of post-source decay. Post-source decay (PSD) is a technique performed on a MALDI TOF instrument equipped with a reflectron, though not genuine MS/MS, it can generate stretches of peptides sequence which can be used in a search engine querry of a protein database. A worked example is demonstrated below whereby a PMF is acquired prior to the chemical derivatization of the peptide digest with chemically assisted fragmentation (CAF; see Section 3.3.4.4) reagent and subsequent CAF-PSD is performed.

An unsuccessful result was obtained from the PMF search (Figure 3.16). Following chemical derivatization, PSD was performed on two of the derivatized peptides. The spectra of each peptide include several sequence specific C-terminal fragment ions (Figures 3.17a, 3.18a), which is indicative of partial amino acid sequence can be inferred for both peptides from. Each sequence is combined with the peptide mass fingerprint in a sequence query search using MASCOT.

Combining the interpreted sequence derived from the PSD spectrum of peptide m/z 1569.79 with the original PMF in a database sequence query search, yields an unambiguous match (Figure 3.18b). Similarly, an unambiguous match is returned when the determined sequence from the second peptide (peptide m/z 1157.57) is combined with the PMF in a sequence query search (Figure 3.18c). When both sets of sequence data are combined with the peptide mass fingerprint the confidence in the match returned from the database search increases dramatically (Figure 3.18c). Close inspection of the PSD spectra demonstrated that the sequence can readily determined using the CAF chemistry. The CAF reagent generates only C-terminal fragment ions (see Section 3.3.4.4), which makes the sequence relatively simple to interpret. In the case of the second peptide, there is some ambiguity whether the residue is a glutamine or a glutamic acid (experimentally measured difference 128.7 Da) in part of the determined sequence. However, the MASCOT search query allows both amino acid residues to be inputted into the search query (see Step 15 on page 407). As the figures demonstrate a short stretch of determined sequence combined with the PMF is a powerful approach for reducing ambiguous protein identifications; particularly if genuine MS/MS analysis is not an option.

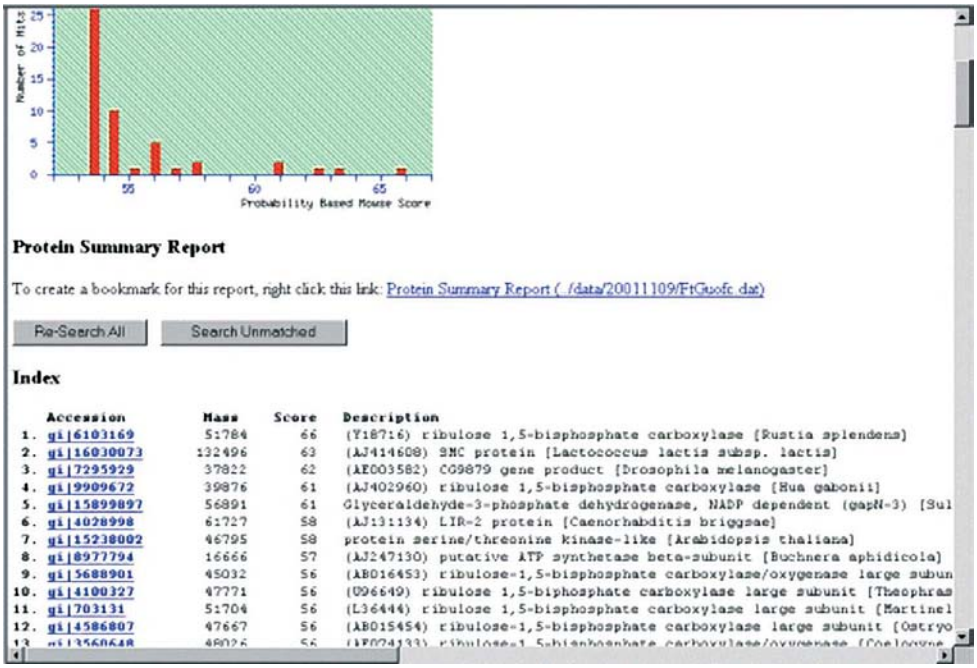
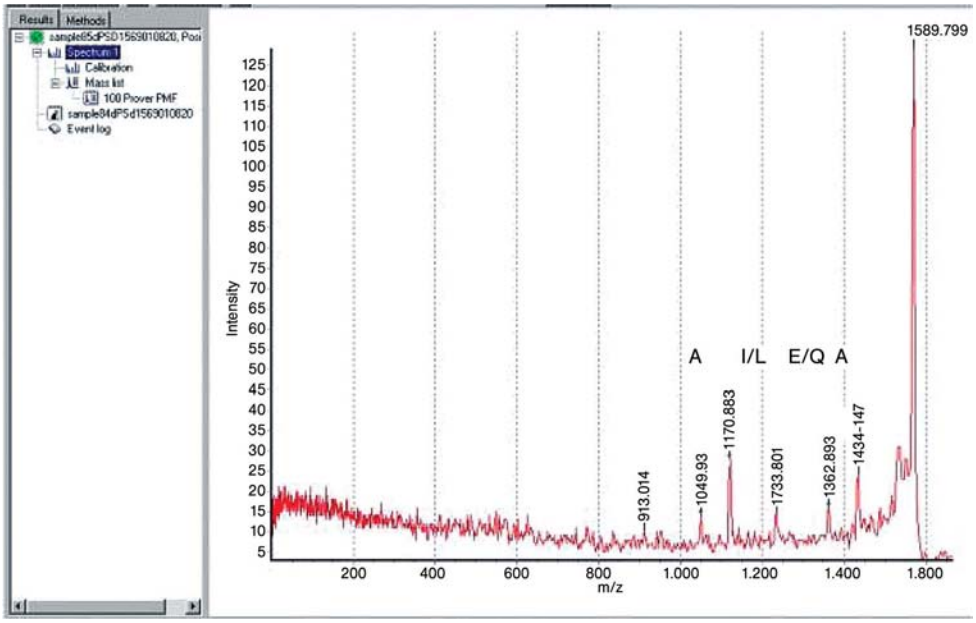
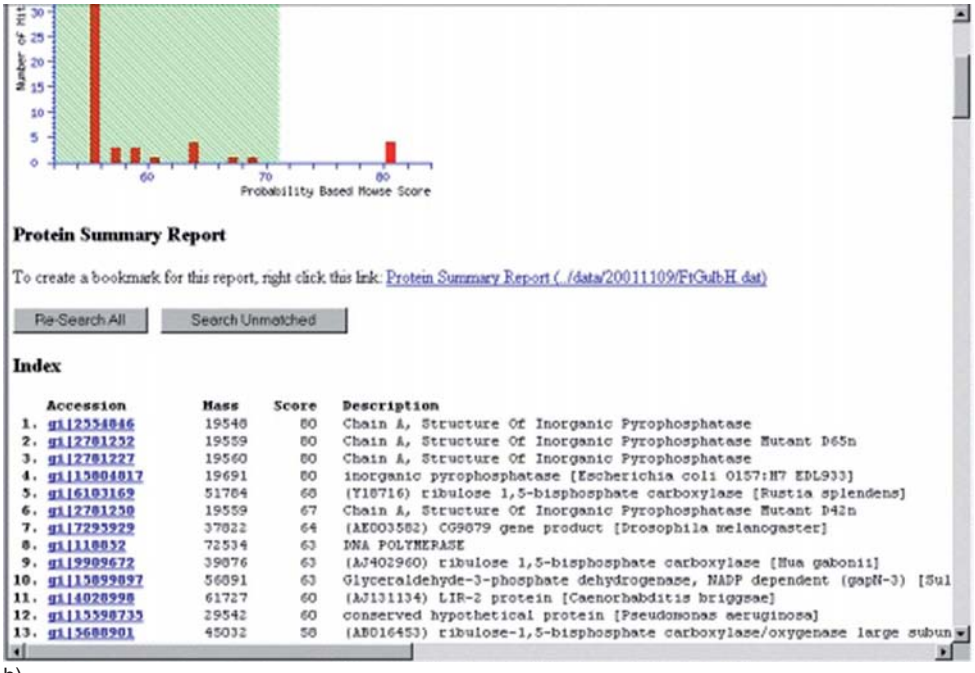


Fig. 3.16: An unsuccessful PMF search of the protein database.

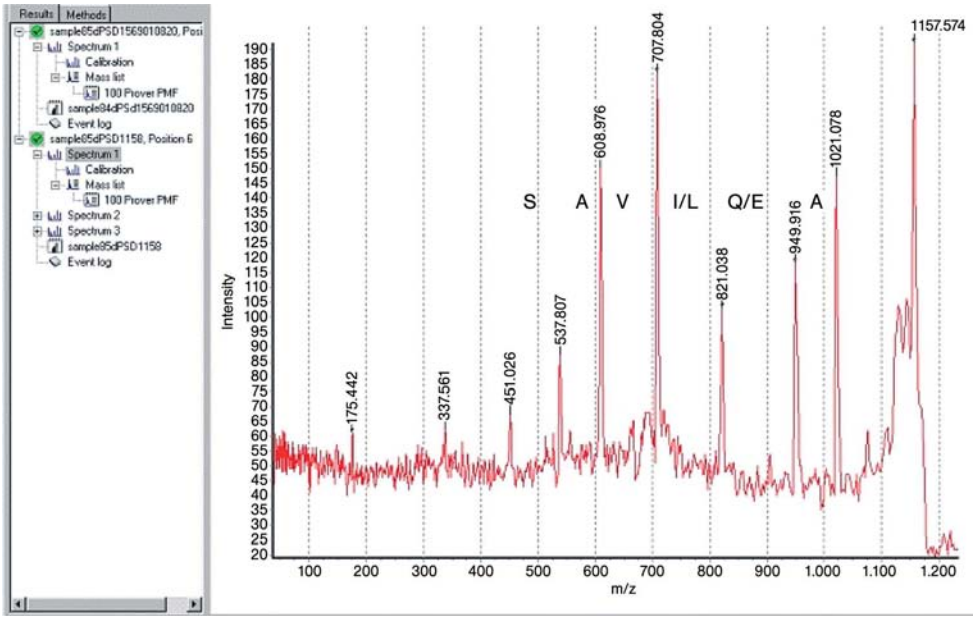


a)

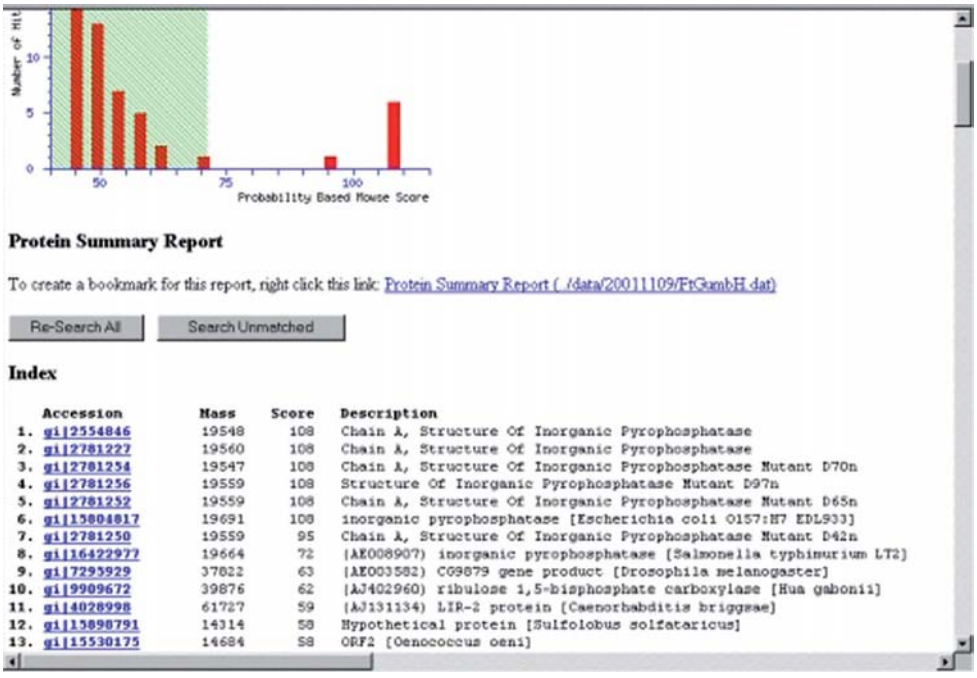


b)

Fig. 3.17: (a) PSD spectrum of the derivatized peptide, *m/z* 1569.79. The sequence – A[IL]EA was determined from the spectrum and combined with the PMF as before. The result of the combined search is demonstrated in Figure 3.17b. An unambiguous result is returned from the search. (b) MASCOT search result from the combined PMF and partial sequence determined from the PSD spectrum in Figure 3.17a.



a)



Protein Summary Report

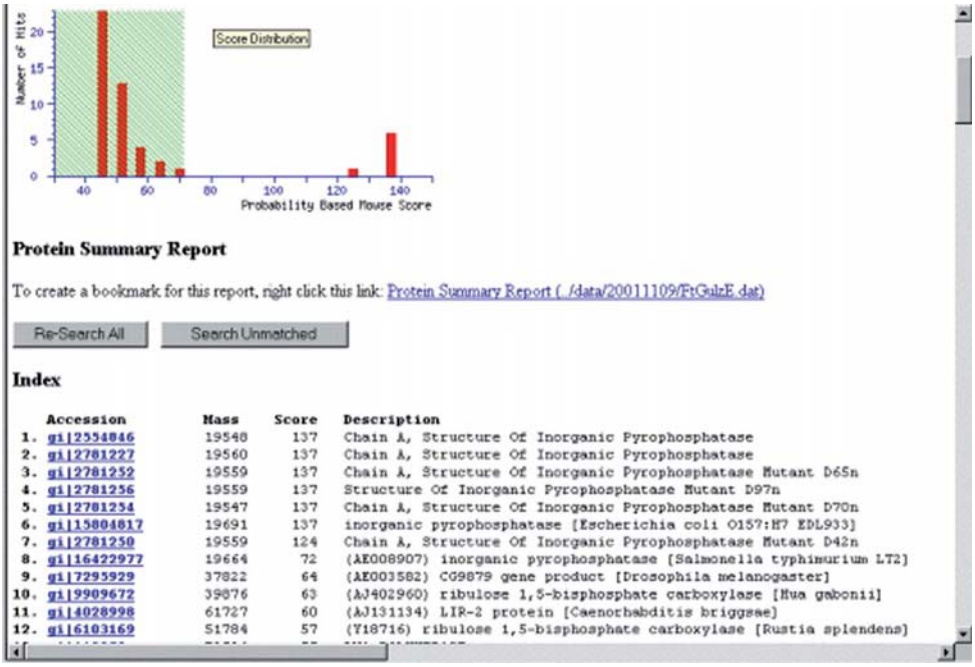
To create a bookmark for this report, right click this link: Protein Summary Report (../data/20011109/FrGambH.dat)

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	gi|2554846	19548	108	Chain A, Structure Of Inorganic Pyrophosphatase
2.	gi|2781227	19560	108	Chain A, Structure Of Inorganic Pyrophosphatase
3.	gi|2781254	19547	108	Chain A, Structure Of Inorganic Pyrophosphatase Mutant D70n
4.	gi|2781256	19559	108	Structure Of Inorganic Pyrophosphatase Mutant D97n
5.	gi|2781252	19559	108	Chain A, Structure Of Inorganic Pyrophosphatase Mutant D65n
6.	gi|15804817	19691	108	inorganic pyrophosphatase [Escherichia coli O157:H7 EDL933]
7.	gi|2781250	19559	95	Chain A, Structure Of Inorganic Pyrophosphatase Mutant D42n
8.	gi|16422977	19664	72	(AE008907) inorganic pyrophosphatase [Salmonella typhimurium LT2]
9.	gi|7295929	37822	63	(AE003582) CG9879 gene product [Drosophila melanogaster]
10.	gi|9909672	39876	62	(AJ402960) ribulose 1,5-bisphosphate carboxylase [Hua gabonii]
11.	gi|14028998	61727	59	(AJ131134) LIR-2 protein [Caenorhabditis briggsae]
12.	gi|15898791	14314	58	Hypothetical protein [Sulfolobus solfataricus]
13.	gi|15530175	14684	58	ORF2 [Oenococcus oeni]

b)



c)

Fig. 3.18: (a) PSD spectrum of the derivatized peptide, m/z 1157.57. The sequence – SAV[IL]EA was determined from the spectrum and combined with the PMF as before. The result of the combined search is demonstrated in Figure 3.18b. An unambiguous result is returned from the search. (b) MASCOT search result from the combined PMF and partial sequence determined from the PSD spectrum in Figure 3.17a. (c) MASCOT search result of the combined PMF and partial sequence from two peptides.

3.3.4 Tandem Mass Spectrometry

Tandem mass spectrometry (or MS/MS) is performed with a certain type of mass spectrometer. Such an instrument needs to be capable of selecting ions of a particular m/z value and subjecting the selected ions to fragmentation within the mass spectrometer. Generally, these experiments are performed successfully on two types of instrument; those where analyzers are in series (tandem in space) such as the triple quadrupole and hybrid quadrupole-TOF configurations described earlier in this section; and secondly those instruments which employ ion trapping mechanisms such as the quadrupole ion trap and FT-ICR analyzer (tandem in time).

Typically, fragmentation is performed by collision induced dissociation (CID), a mechanism of fragmenting molecular ions in the gas phase. Peptide molecular ions are allowed to collide with neutral gas molecules (helium, nitrogen or argon) within a cell in the mass spectrometer. As a result of the collision, some of the kinetic energy possessed by the molecular ion is converted into internal energy which results in bond breakage and the fragmentation of the molecular ion into smaller fragments.

Traditionally, three types of MS/MS experiments are performed routinely within proteomics to determine peptide sequence and sites of post-translational modifications (i.e. protein identification and characterization). The configuration used in triple quadrupole- and hybrid quadrupole-TOF instruments will be used to explain the three types of experiment (see Figure 3.19). Effectively the analyzer region of these instruments can be regarded in three parts: MS1, the collision cell and MS2.

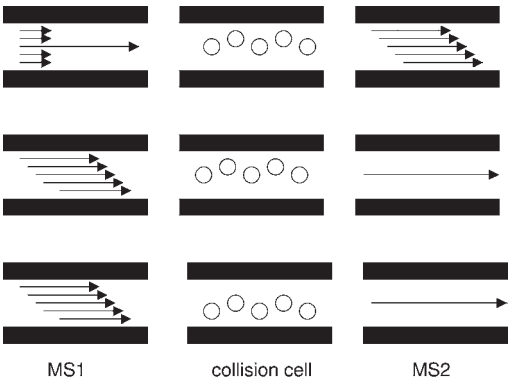


Fig. 3.19: Schematic of the three types of MS/MS experiment. Top panel: product ion scan; middle panel: parent ion scan; bottom panel: neutral loss scan.

3.3.4.1 **Product Ion Scan**

In a product ion scan, the first part of the analyzer, MS^1, is used to specifically select the ion of interest, the precursor ion (i.e. a peptide). The precursor ion is allowed into the collision cell where it undergoes collision induced dissociation (CID) (Figure 3.19, upper panel). Here, the peptide precursor ions collide with molecules of the collision gas yielding a distribution of fragment ions, or product ions. These product ions are resolved by the third part of the analyzer, MS^2, before detection at the detector producing a product ion spectrum. With respect to a triple quadrupole analyzer, MS^2 is a mass filtering quadrupole and scans the selected mass range to enable detection of the

product ions. In the case of a hybrid quadrupole TOF analyzer, MS^2 is a reflectron TOF analyzer, in which the entire mass range is not scanned, but collected simultaneously with significant improvements in sensitivity and resolution. With respect to the TOF/TOF configuration, the first TOF acts as MS1 and the second TOF acts as MS^2. Figure 3.20 demonstrates the experimental workflow, where an ion of interest is selected and isolated by MS1 and fragmented, yielding the product ion MS/MS spectrum.

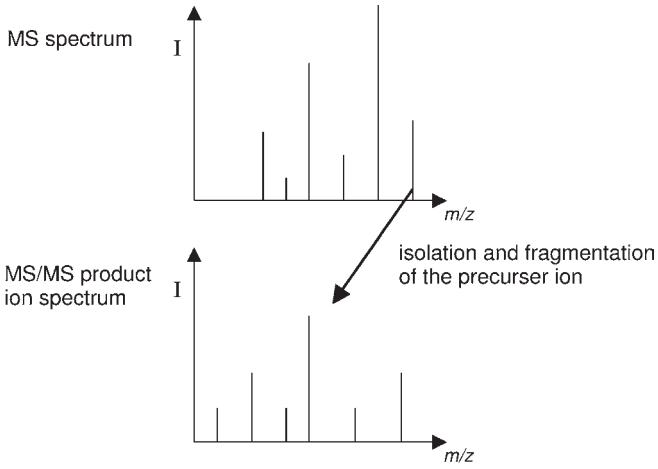


Fig. 3.20: Product ion MS/MS experimental workflow.

Collision induced dissociation (CID) can be further defined by the energy of the collisions observed in the collision cell. Fragmentation within triple quadrupole, quadrupole ion trap and hybrid quadrupole TOF analyzers occurs at collisional energies in the order of 10–100 eV range, whilst fragmentation within a magnetic sector or TOF/TOF analyzer occurs at collisional energies at least an order of magnitude higher in the keV range. The former is described as low-energy CID whilst the latter is described as high-energy CID.

As a result of the low-energy collisional fragmentation, the peptide precursor ion fragments predictably at each peptide amide bond along the peptide backbone yielding a distribution of product ions in two complementary ion series forming a ladder which is indicative of the peptide sequence. This fragmentation nomenclature was described by Roepstorff and Fohlman (1984; see Figure 3.21). The two complementary ion series are:

- N-terminal ion series, or b-ion series. The ions of the n-terminal ion series will contain the N-terminal amino acid and extensions from this residue (Figure 3.22 and Table 3.2).

- C-terminal ion series, or y-ion series. The ions of the C-terminal ion series will contain the C-terminus of the peptide and extensions from this residue (Figure 3.21 and Table 3.2).

Roepstorff P, Fohlman J. Biomed Mass Spectrom 11 (1984) 601.

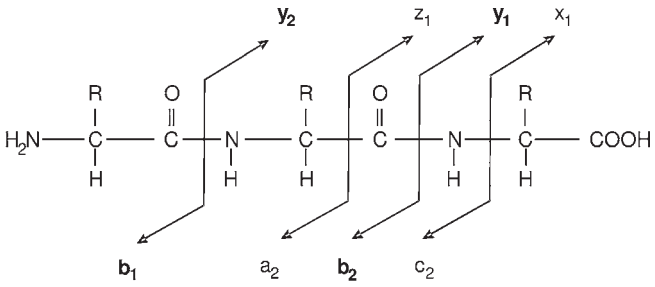


Fig. 3.21: Fragmentation nomenclature acc. to Roepstorff and Fohlmann.

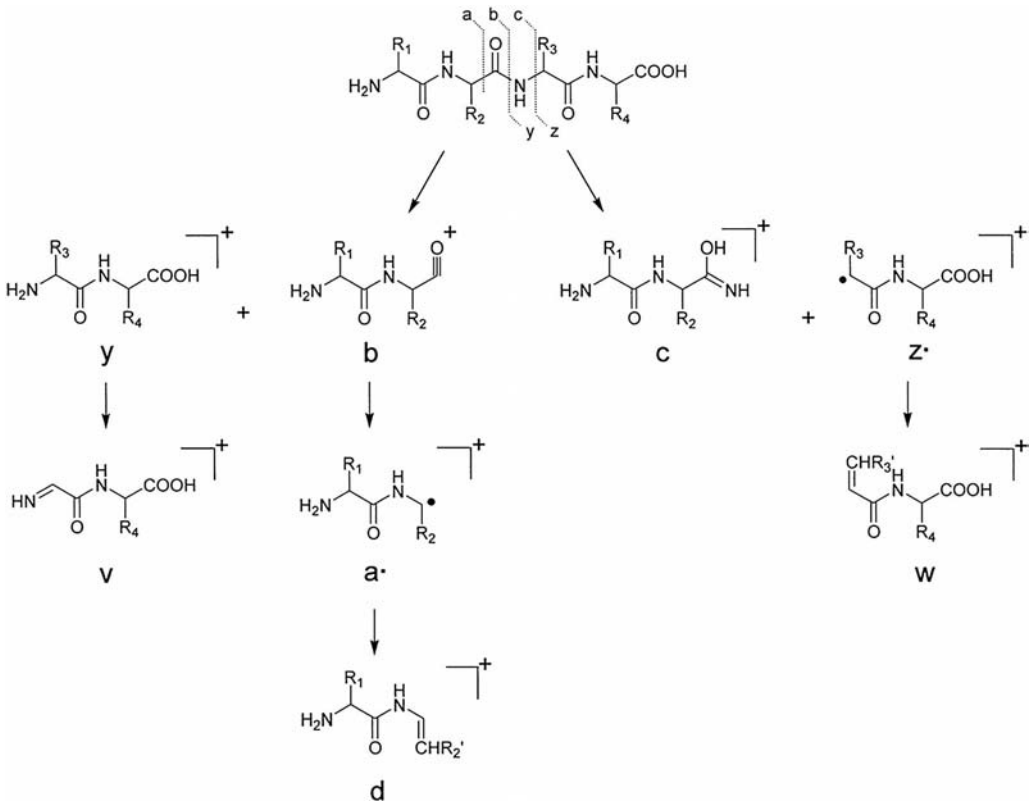


Fig. 3.22: Structure of the observed b, y, a●, c, and z● ions and d, w, and v side-chain fragment ions. R2′ and R3′ represent partial side-chain loss (from. Cournoyer *et al.* 2005).

Cournoyer JJPittman JL, Ivleva VB, Fallows E, Waskell L, Costello CE, O'Connor PB. Protein Sci. 14 (2005) 452–463.

Tab. 3.2: Common fragment ions formed during low-energy CID and the respective formulae to calculate the mass of each ion.

Fragment ion	Formula
a-ion	Total residue mass of the amino acids present –28 (for the loss of CO)
b-ion	Total residue mass of the amino acids present
y-ion	Total residue mass +18 +1

Johnson RS, Martin SA, Biemann K. Int J Mass Spectrom Ion Proc 86 (1988) 137–154.

At higher collisional energies, the peptide is additionally fragmented at the amino acid side-chains (Johnson *et al.* 1988), the subsequent fragmentation pattern can be used to differentiate the isobaric reisudes, leucine and isoleucine. Similarly, electron capture dissociation, an alternative fragmentation technique, delivers similar side-chain specific fragmentation. (see Section 3.3.4.7).

With the knowledge of how peptides fragment within a mass spectrometer, the subsequent sequence information derived from product ion MS/MS (or PSD experiments) can be used in three ways:

Perkins DN, Pappin DJC, Creasy DM, Cottrell JS. Electrophoresis 20 (1999) 3551–3567.

- Manual interpretation of the sequence (generally from the b- and y-ion series of fragment ions) where the determined sequence can be inputted as a search query (Perkins *et al.* 1999).

Mann M, Wilm M. Anal Chem 66 (1994) 4390–4399.

- Manual interpretation of a short stretch of sequence used together with the accurate mass of the precursor ion and the masses of the first and last fragment ions in the stretch of the sequence (knowledge of these two masses, gives the masses of the uninterpreted sequence in the peptide), a method termed the peptide sequence tag (Mann and Wilm 1994).

Eng JK, McCormack AL, Yates JR III. J Am Soc Mass Spectrom. 5 (1994) 976–989; Yates JR III, Eng JK, McCormack AL, Schieltz D. Anal Chem 67 (1995) 1426–1436.

Spahr CS, Susin SA, Bures EJ, Robinson JH, Davis MT, McGinley MD, Kroemer G, Patterson SD. Electrophoresis 21 (2000) 1635–1650.

- The automatic, uninterpreted MS/MS search (Eng *et al.* 1994; Yates *et al.* 1995; Perkins *et al.* 1999). The process of selecting, isolating and fragmenting ions of interest is now routinely performed in an automated fashion, following earlier publications demonstrating the ability to perform data dependent fragmentation (Spahr *et al.* 2000).

As sequence information is significantly more discriminating than a molecular mass alone, a few peptides of sufficient length are sufficient to identify a protein from a well characterized genome (Susin *et al*. 1999; Shevchenko *et al*. 2000).

Once a CID spectrum has been matched, a theoretical tryptic digest of the identified protein can be generated and the presence of other tryptic peptides can be checked and confirmed.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Brothers G, Snow B, Jacotot E, Constantini P, Larochette N, Goodlett DR, Aebersold R., Pietu G, Prevost MC, Siderovski D, Penninger J, Kroemer G. Nature 397 (1999) 441–446.

Shevchenko A, Loboda A, Shevchenko A, Ens W, Standing KG. Anal Chem 72 (2000) 2132–2141.

Using product ion MS/MS as a protein ID tool, less peptides are generally required to unambiguously identify a protein from a known genome than with MALDI PMF. Resultingly, today's proteomics research uses this particular MS/MS technique in almost all protein identification strategies which generate peptide sequence information.

However, even a product ion MS/MS analysis may not generate unambiguous protein identification from a protein database search query. In these cases, a further tool for protein identification is the expressed-sequence tag (EST) databases (Mann, 1996). ESTs are short stretches of nucleotide sequence, which peptide sequence data can be used to search against. Peptide sequence data can be used to search the EST databases if a search against the protein database is unsuccessful. A PMF search against the EST database is inappropriate, as it would not be sufficiently discriminating for unambiguous identification.

Mann M. Trends Biol Sci. 21 (1996) 494–495.

In the rarer instances, whereby a protein is not identified from any database search query then genuine *de novo* sequencing needs to be performed.

This is often the case if the genome of the organism in question is not fully sequenced or poorly characterized.

De novo sequencing requires the analysis of a peptide fragmentation spectrum without recourse to a protein database. Other applications for *de novo* sequencing include the characterization of splice variants. Though the peptide sequences probably exist in the database, they may not exist in a consecutive order. Thus the sequencing of peptides, which span two exons, could be very useful for determining splice variants (B. Küster, personal communication).

However, this is not a straightforward task. The fragmentation pattern of a peptide in a product ion MS/MS spectrum is indicative of its sequence. If a significant number of continuous residues can be determined from the spectrum (greater than 12 residues) oligonucleotide probes can be developed. However, under low-energy CID conditions the peptide precursor ion fragments to yield y- and b-ion series of varying lengths. Neither series may be long enough to generate the sufficient stretch of continuous residues required for subsequent oligonucleotide probe construction. In such circumstances it is important to detect both ion series in the spectrum, as they are complementary. For instance, the residue indicated by the first b-ion, the N-terminal residue, b_1, is the same residue indicated by the final y-ion and vice versa. In this manner, the complementary ion series can be used to fill in the gaps that may exist in the sequence of one of the ion series.

However, the presence of two ion series can complicate the interpretation of the spectrum, even though they are complementary. Thus, for efficient *de novo* sequencing it is very important, if not essential, to be able to differentiate these two series. Once this has been achieved the presence of the two ion series is not a burden, the interpretation of the spectrum is simplified significantly.

Hunt DF, Yates JR III, Shabanowitz J, Winston S, Hauer CR. Proc Natl Acad. Sci USA 83 (1986) 6233–6237.

A range of different approaches have been taken to perform *de novo* sequencing by mass spectrometry. The first report of peptide *de novo* sequencing by ESI mass spectrometry was described by Hunt and colleagues as early as 1986, where tryptic peptides of apolipoprotein B were successfully sequenced using FAB-triple quadrupole mass spectrometry (Hunt *et al*. 1986). In this method, the group used differential modification of the peptides to differentiate each ion series, simplifying interpretation and confirming the peptide sequence. Specifically, product ion MS/MS spectra were acquired of the native peptides. Subsequently the peptide fractions underwent methyl esterification, and the product ion MS/MS spectra of the corresponding derivatized peptides were acquired. This reaction esterified the C-terminus of each peptide (and all subsequent acidic resides in each peptide, aspartic and glutamic acids). Resultantly, the starting point of the y-ion series (C-terminal containing ions), y_1, was shifted by 14 Da (and hence the remainder of the y-ion series) whilst the starting point of the b-ions series remained unchanged (unless the n-terminal residue, b_1-ion was a glutamic or aspartic acid). Hence, the y-ion series became recognizable and the two ion series could be differentiated.

However, with this method the sample has too be split and two product ion MS/MS experiments are required for each peptide. The presence of aspartic or glutamic residues internal to the sequence would also change by the esterifying mass, and subsequent y- and b-ions internal to the sequence

Schnolzer M, Jedrzejewski P, Lehman WD. Electrophoresis 17 (1996) 945–953.

Qin J, Herring CJ, Zhang X. Rapid Commun. Mass Spectrom 12 (1998) 209–216.

Shevchenko A, Chemushevich IV, Ens W, Standing KG, Thomson B, Wilm M, Mann M. Rapid Commun Mass Spectrom 11 (1997) 1015–1024.

Uttenweiler-Joseph S, Neubauer G, Christoforidis S, Zerial M, Wilm W. Proteomics 1 (2001) 668–682.

A second approach described the incorporation of an isotopic label during the peptide digestion. Specifically, the protein of interest is digested as normal but in a 1:1 mixture of ^{16}O:^{18}O digestion buffer. Subsequently all peptides will appear as ^{16}O:^{18}O isotope doublets; with the label being incorporated into the carboxyl group of the C-terminal residue. When this isotopic doublet is fragmented during a product ion MS/MS experiment, all C-terminal product ions, the y-ion series, will exhibit this isotopic doublet and hence can be immediately differentiated from the b-ion series and other non C-terminal containing fragment ions.

Wilm and co-workers developed this approach further, combining the isotopic labeling with a technique calling differential scanning enabling improved assignment of the y-ion series (Uttenweiller *et al*. 2001). The method requires two product ion MS/MS spectra to be acquired; one where the whole ^{16}O:^{18}O peptide envelope is selected for fragmentation and the second where only the ^{18}O labeled ions are selected for fragmentation. Using a software algorithm the y-ion series can be filtered automatically.

However, both approaches do not affect the fragmentation pattern and hence the b-and y-ion series still fragment in the same manner.

Furthermore, both approaches are only applicable to enzymatic methods and to proteins which can be digested; it is not applicable to those samples that are presented, such as MHC class peptides.

A wide range of peptide derivatization protocols have been reported for altering the peptide fragmentation pattern and fragmentation efficiency in order to aid interpretation of product ion MS/MS spectra. Derivatization of the peptide digest at the N-terminus with phosphonium quaternary tags improved sensitivity and yielded a single ion series, the b-ion series (Roth *et al.* 1998); Munchbach *et al.* 2000; described a derivatization protocol to aid the *de novo* sequencing of peptides.

Roth KDW, Huang ZH, Sadagopan N, Throck Watson J. Mass Spectrom Reviews 17 (1998) 255–274.

Munchbach M, Quadroni M, James P. Anal Chem 72 (2000) 4047–405.

Keough and co-workers developed a strategy to obtain a single y-ion series, particularly for PSD analysis, with impressive results (see Section 3.3.4.4). Long stretches of consecutive y-ions from the y-ion series can be readily observed with this technique and has the potential to be useful for *de novo* sequencing. However, as only one of the ion series is observed a long uninterrupted sequence is necessary.

Another derivatization approach to facilitate *de novo* sequence analysis is the derivatization of peptide digests with basic NHS esters. Pyridyl quaternary NHS ester reagents were reported for the derivatization of peptide digests for PSD analysis facilitating charge localization at the N-terminus, subsequently yielding only the b-ion series. (Spengler *et al.* 1997; Cardenas *et al.* 1997). These NHS ester reagents were further developed to produce a series of gas phase basic reagents to quantitatively label the peptide digest (N-succinimidyl morpholino acetate and N-succinimidyl pyridyl acetate; SMA and SPA respectively). Each of these tags defined the starting points for both the b- and y-ion series, enabled the observation of often complete y-ion series for tryptic peptides (the final y-ion is observed in all cases) and long stretches of the b-ion series. Hence, both the starting and end points of the peptide are known and the ion series are subtley differentiated. Armed with these pieces of information *de novo* sequencing is significantly simplified. Furthermore, because the two ion series are complementary the determined sequence can be readily checked in each ion series. This proved highly beneficial for uninterpreted, automated MS/MS database searching and essential for *de novo* sequencing (Tugal *et al.* 1998; Kondo *et al.* 1997; Hoess *et al.* 1999).

Spengler B, Luetzenkirchen F, Metzger S, Chaurand P, Kaufmann R, Jeffrey W, Bartlet-Jones M, Pappin DJC. Int J Mass Spectrom Ion Proc 169 (1997) 127–140.

Cardenas MS, Van der Heeft E, De Jong APJM. Rapid commun Mass Spectrom. 11 (1997) 1271–1278.

Tugal T, Zou-Yang XH, Gavin K, Pappin D, Canas B, Kobayashi R, Hunt T, Stillman B. J Biol Chem 273 (1998) 32421–32429.

Kondo H, Rabouille C, Newman R, Levine TP, Pappin D, Freemont P, Warren G. Nature 388 (1997) 75–78.

Hoess M, Robins P, Naven TJP, Pappin DJC, Sgouros T, Lindahl T. EMBO J 18 (1999) 3868–3875.

In today's research, MS suppliers provide automated *de novo* sequencing software to support this application. Automated de novo sequencing is supported considerably by high mass accuracy and high resolution improves considerably the results, providing higher confidence in assignment of the correct amino acid sequence.

3.3.4.2 Precursor Ion Scan

For proteomics applications, a precursor ion scan is employed to locate sites of protein modifications, such as phosphorylation. In this experiment the first part of the MS analyzer, MS^1, is set to transmit all the components of the mixture in to the collision cell to undergo CID. The third part of the analyzer, MS^2, is fixed at a specific mass value, so that only analytes, which fragment to give a fragment ion of this specific mass will be detected at the detector (see Figure 3.19, middle panel). In this manner, the precursor ion scan can be used to selectively identify certain species in a complex mixture, such as peptides containing a phosphorylated residue.

Wilm M, Neubauer G, Mann M. Anal Chem. 68 (1996) 527–533.

Carr SA, Huddleston MJ, Annan RS. Anal Biochem 239 (1996) 180–192.

Annan RS, Huddleston MJ, Verna R, Deshaies RJ, Carr SA. Anal Chem 73 (2001) 393–404.

Steen H, Küster B, M Fernandez, Pandy A, Mann M. Proceedings of the 49th ASMS conference on mass spectrometry and allied topics, Chicago (2001).

The presence of serine, threonine or tyrosine phosphorylated peptide in a complex peptide digest can be determined using a precursor ion scan. A phosphorylated peptide fragments during low-energy collisional fragmentation to give a fragment ion of 79 Da (the PO_3^- group). Therefore to specifically identify phosphorylated peptides in a mixture the third part (MS^2) of the analyzer is set to 79. Thus, only species which fragment to give a fragment ion of 79 are detected and hence indicate the presence of a phosphorylated residue (Wilm *et al.* 1996; Carr *et al.* 1996; Annan *et al.* 2001).

This experiment has been historically restricted to a triple quadrupole instrument, but recent applications have been demonstrated using a hybrid quadrupole TOF instruments (Steen *et al.* 2001). The method has been exploited for the specific detection of tyrosine phosphorylated peptides in complex mixtures.

3.3.4.3 Neutral Loss Scan

Similarly, a neutral loss scan is employed in proteomics applications to locate peptides containing a protein modification. In this technique, the 1st and 3rd parts of the analyzer are scanned synchronously, but with a specific *m/z* offset. Once more the second part of the analyzer is used as the collision cell and the entire mixture is allowed to enter the collision cell, only those species which fragment to yield a fragment ion with the same mass as the offset will be detected (see Figure 3.20). For instance, serine and threonine phosphorylated peptides readily lose phosphoric acid during low-energy CID. Phosphoric acid has a mass of 98 Da, thus the offset for a doubly charged peptide would be set at 49. Hence any species which loses 49 Da from a doubly charged ion would be observed at the detector and be indicative of a phosphorylated peptide (Covey *et al.* 1991; Schlosser *et al.* 2001). This type of experiment is typically carried out with a triple quad or Q-TOF type instrument. Phosphopeptide analysis in an ion trap is

Covey TR, Huang EC, Henion JD. Anal Chem 63 (1991) 1193–1200.

Schlosser A, Pipkorn R, Bossemeyer D, Lehman WD. Anal Chem 73 (2001) 170–176.

typically performed with a MS^3 approach. Both approaches have different specific benefits, which are not pointed out here.

3.3.4.4 MALDI-TOF Post Source Decay

Though not a genuine MS/MS technique, MALDI-TOF post source decay (PSD) is capable of generating sequence specific information, which can be used for protein identification. Described by Spengler and co-workers in the 1990s (Spengler 1997; Chaurand *et al*. 1999), this technique demonstrated some reasonable success as a protein identification tool. The quality of data is less than that acquired with conventional MS/MS instruments, as described earlier (Silles *et al*. 2000; Gevaert *et al*. 2001), though still sufficient for protein ID from relatively simple samples such as those derived from a 2D PAGE gel.

Spengler B. J Mass Spectrom. 32 (1997) 1019–1036.

Chaurand P, Luetzenkirchen F, Spengler B. J Am Soc Mass Spectrom 10 (1999) 91–103.

Silles E, Mazon MJ, Gevaert K, Goethals M, Vandekerckhove, Lebr R, Sandoval IV. J Biol Chem. 275 (2000) 34054–34059.

Gevaert K, Demol H, Martens L, Hoorelbeke B, Puype M, Goethals M, Van Damme J, De Boeck S, Vandekerckhove J. Electrophoresis 22 (2001) 1645–1651.

In the technique, the peptide of interest is specifically selected from a complex mixture using an ion gate. In a conventional linear reflectron, the configuration can only accommodate a small range of KE differences i.e the reflectron will only be able to focus those ions that are similar in mass to the parent ion (Figure 3.23). In this instance, only PSD ions that are close in mass to the precursor ion will be mass measured correctly. Thus, to perform PSD with a conventional reflectron the voltage on the reflectron has to drop sequentially to allow the fragment ions of lower mass (and lower KE) to become focussed at the detector. This is termed stepping the reflectron. In a conventional reflectron, several different voltages or segments, (as many as 12 segments may be required) are required to focus the entire mass range of product ions (Figure 3.24a). The individual segments are then "stitched" together using the instrument software, generating the complete product ion spectrum. (Figure 3.24b).

The use of a quadratic field reflectron for PSD analysis attempts to address the issue of stepping the reflectron. It is capable of accommodating ions of widely different kinetic energies in a single spectrum (Figures 3.22–3.23), because in a quadratic field reflectron an increased voltage is applied, non-linearly, to create a perfect quadratic field across the reflectron. Resultantly, all the product ions created during the post source decay of a particular precursor ion are focused at the detector, irrespective of their energy, over the entire range of m/z. Hence, a complete PSD spectrum is obtained under the same experimental conditions with each pulse of the laser, without the need for data stitching.

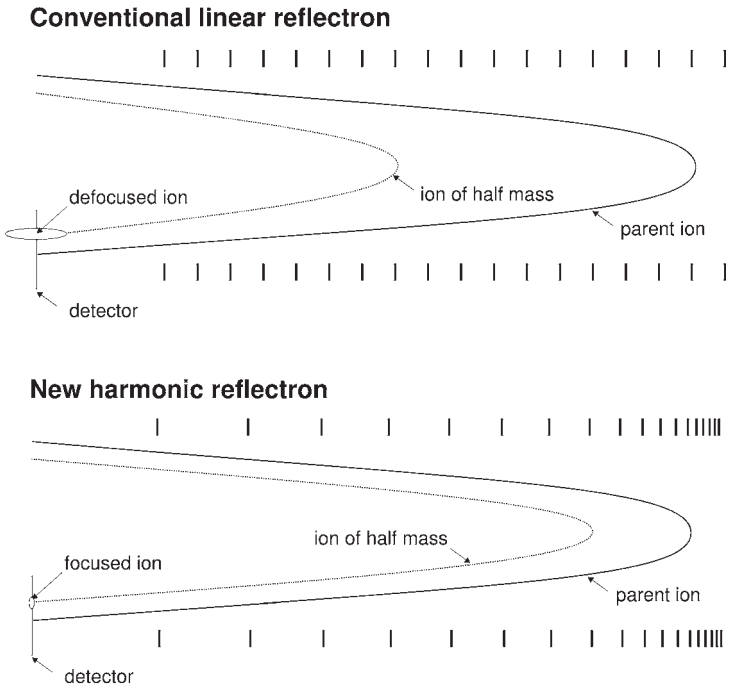


Fig. 3.23: Schematic demonstrating the passage of the precursor ion and product ion through two types of reflectron. Upper panel: conventional linear reflectron. Lower panel: quadratic field reflectron.

Under typical timed ion delay MALDI conditions, peptide fragmentation observed during PSD is reduced (Kauffman *et al.* 1996). Keough and co workers described a derivatization method for peptide sequence analysis by MALDI PSD that facilitated fragmentation during PSD, increasing sensitivity significantly. The tag was designed to promote efficient charge-site initiated fragmentation of the peptide bonds. Consequently, only a single ion series, the y-ion series, is observed in the PSD spectrum, simplifying interpretation.

The theory proposed that under MALDI ionization conditions, the strong basic residue at the C-terminus of tryptic peptides (arginine) would be protonated and the strong acidic group at the C-terminus would be deprotonated (Figure 3.25). The additional proton (almost exclusively MALDI ions in the peptide mass range are singly charged) would then be free to randomly ionize and subsequently fragment the peptide amide bonds of the peptide backbone. Hence, when the fragment ions are formed only the y-ions are observed because, the formal positive charge typical of a b-ion is neutralized by the negative charge on the sulfonic acid group, hence b-ions are not observed.

Kauffman R, Chaurand P, Kirsch D, Spengler B. Rapid Commun Mass Spectrom 10 (1996) 1199–1208.

Keough T, Youngquist RS, Lacey MP. Proc Natl Acad Sci USA 96 (1999) 7131–7136.

Keough T, Lacey MP, Fieno AM, Grant RA, Sun Y, Bauer MD, Begley KB. Electrophoresis 21 (2000) 2252–2265.

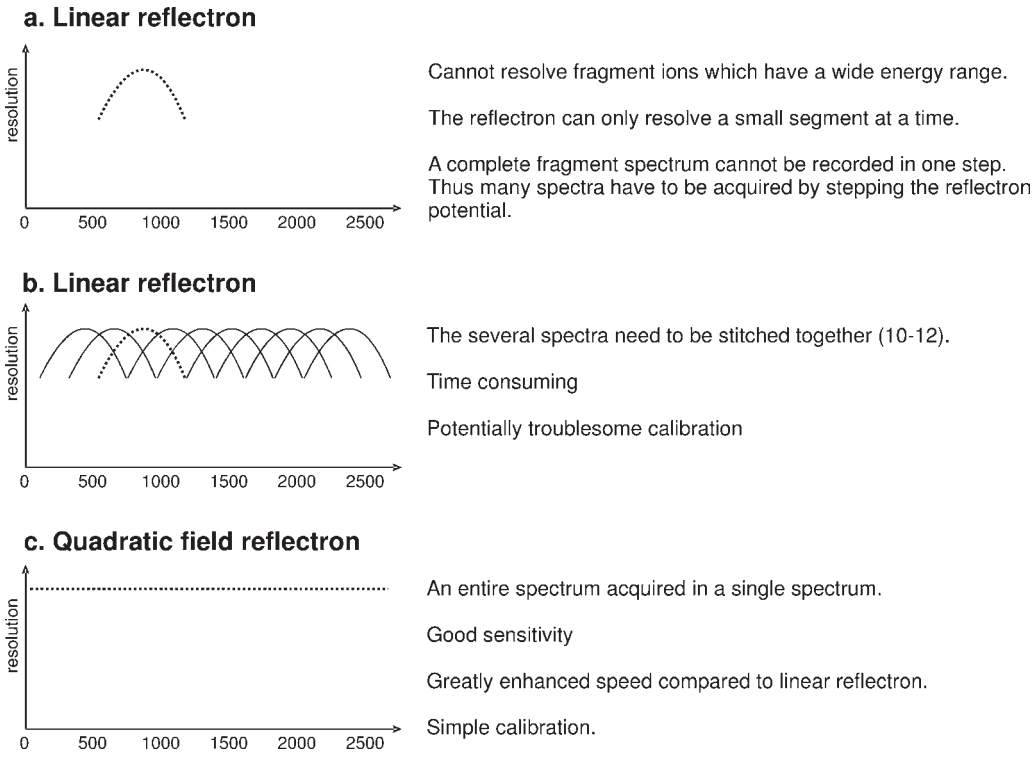


Fig. 3.24: Schematic of the time focusing of a conventional and quadratic field reflectron.
Upper panel: only a small segment of the mass range can be focussed at one time with a conventional linear reflectron.
Middle panel: multiple segments acquired to obtain the whole mass range of fragment ions using a conventional linear reflectron.
Lower panel: quadratic field reflectron allows the focussing of the mass range in a single TOF spectrum.

$^{-}O_3S–A–A–A–A–A–R^{+}$ **(a)**

$^{-}O_3S–CHR–CO^{+}$ $[H_2NCHR–CO_2H]^{+}$ **(b)**

b_1 ion y_1 ion

(R = guanidino side-chain of arginine)

Fig. 3.25: The effect of a strongly acidic group at the N-terminus of an arginine C-terminating peptide.

In the original method, the peptide digest was derivatized with 2-sulfonyl acetyl chloride, labeling the α-amino group at the N-terminus of each peptide (and the ε-amino group of the each lysine side-chain) with the very strong acid, sulfonic acid (pKa<2).

Labeling of arginine-terminating tryptic peptides greatly facilitates fragmentation, particularly fragmentation by MALDI PSD. Figure 3.26 demonstrates the extent of fragmentation during a PSD experiment. In the case of this synthetic peptide, 16 consecutive residues can be readily determined. The derivatization technique is compatible with protein digests derived from 2D gels (see Sections 3.4.3–3.4.4).

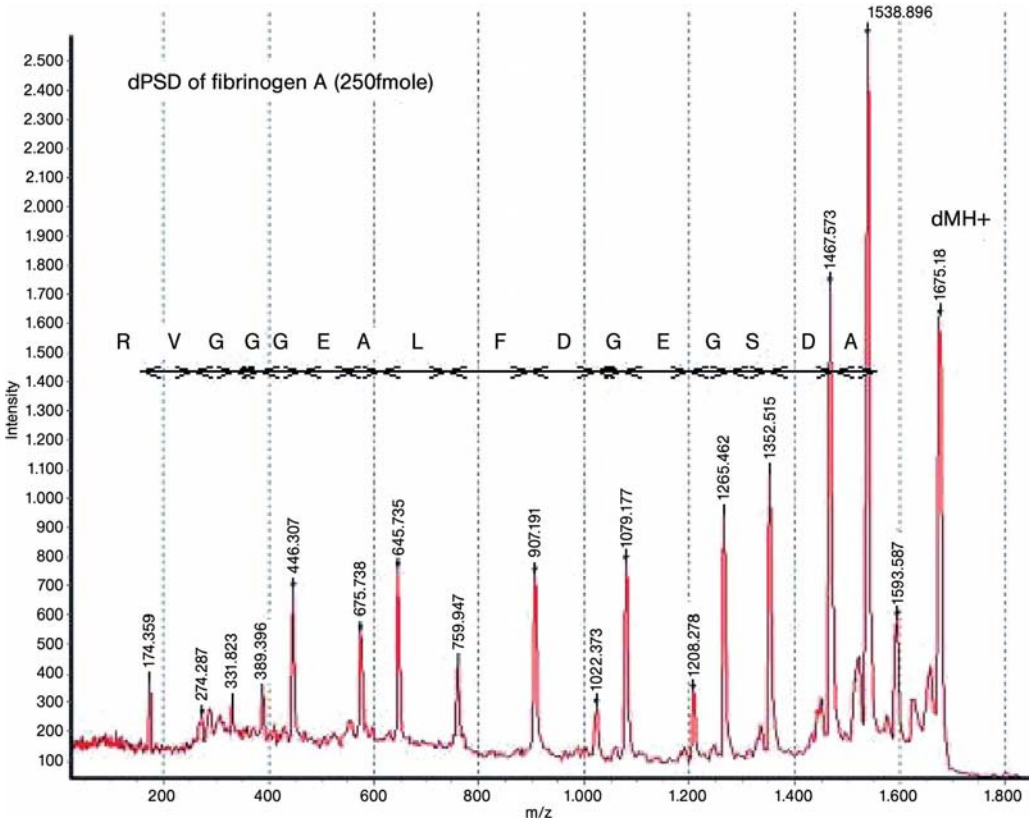


Fig. 3.26: PSD spectrum of a CAF derivatized synthetic peptide acquired with the Ettan MALDI-TOF Pro (250 fmol applied to the target). The 16 consecutive residues are readily determined. Though Leucine (L) is specified in the sequence, this cannot be determined from the experiment (Isoleucine and leucine are isobaric). dMH^+ is the precursor ion. Note the intensity of the precursor ion. In a typical PSD experiment, fragment ion abundance is a very low percentage of the precursor ion abundance.

As the derivatization strategy also causes the derivatization of the ε-amino group of the lysine side-chain, therefore labeling the lysine with a strong acidic group, PSD of lysine C-terminating peptides becomes inefficient. To extend the applicability of this method to lysine terminating peptides, the lysine must first be modified to the homo-arginine derivative. This has recently been reported by a number of groups to increase lysine tryptic peptide detection in peptide mass fingerprinting (Brancia *et al*. 2000; Keough *et al*. 2000). If this modification of the lysine peptides is performed prior to the sulfonation reaction then the sulfonation methodology is equally applicable to lysine C-terminating peptides.

Brancia FL, Oliver SG, Gaskell SJ. Rapid Commun Mass Spectrom 14 (2000) 2070–2073.

Keough T, Lacey MP, Youngquist RS. Rapid Commun Mass Spectrom 14 (2000) 2348–2356.

The sulfonation method has further been improved with the development of a water stable sulfonic NHS ester reagent (Figure 3.27; Liminga *et al*. 2001). Subsequent PSD spectra acquired from peptides derivatized in this manner is termed chemically assisted fragmentation (CAF).

Liminga M, Borén M, Åström J, Carlsson U, Keough T, Maloisel JL, Palmgren R, Youngquist S. Proceedings ASMS conference on mass spectrometry and allied topics, Chicago, USA (2001).

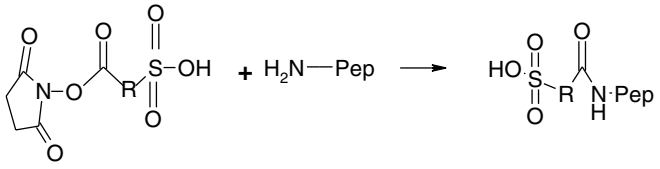


Fig. 3.27: Reaction scheme for the water stable sulfonic NHS ester reagent.

One of the drawbacks of the original method was the hygroscopic nature of the acetyl chloride reagent, requiring the derivatization procedure to be performed under stringent conditions in a non-aqueous environment. The novel watersoluble reagent allows the quantitative derivatization of the peptide digest at sub pmol quantities of starting material and is compatible with the lysine modification step affording a one pot derivatization (see Step 10). The derivatization step is amenable to automation. This derivatization approach enables peptide mass fingerprinting data and subsequent sequence information to be acquired on the same instrument with good sensitivity (see Section 3.5.1).

This derivatization strategy is equally applicable to any MS/MS instrument, which employs MALDI as the ionization source, enabling the simple interpretation of the peptide sequence from the fragmentation of a singly charged ion.

Despite the efforts to improve the performance of MALDI PSD, the development of MALDI TOF/TOF systems and the performance and automation of LC-ESI-MS/MS systems, the use of MALDI PSD has become considerably reduced.

3.3.4.5 Multi-stage Tandem Mass Spectrometry

Multi-stage tandem mass spectrometry (MS^n) involves multiple stages of mass selection, separated by some form of fragmentation. This type of fragmentation experiment is restricted to quadrupole ion traps and FT-ICR MS. A fragment ion produced in the product ion MS/MS spectrum can be selected, isolated and fragmented a second time generating even further sequence information in a third spectrum. This spectrum is called an MS/MS/MS product ion spectrum, or MS^3 (see Figure 3.28; Louris *et al.* 1989). This type of experiment is useful for identification of phosphorylated or glycosylated residues if sufficient material exists. This process can be repeated a number of times, resulting in a series of MS^n spectra where 'n' represents the number of times the fragmentation cycle has been performed.

Louris JN, Amy JW, Ridley TY and Cooks RG. Int J Mass Spectrom Ion Proc 88 (1989) 97–111

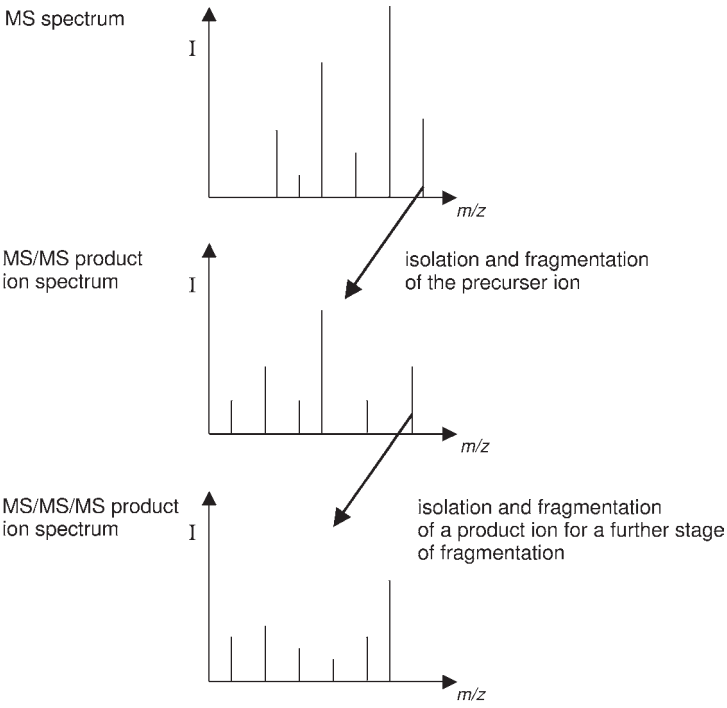


Fig. 3.28: Schematic demonstrating the workflow in an MS^3 experiment.

3.3.4.6 Electron Transfer Dissociation

Electron transfer dissociation (ETD) is a new fragmentation technique that fragments peptides by transferring electrons to positively charged peptides, yielding a strech of sequence ions derived from

cleavage at the amide groups along the peptide backbone. Due to its non-ergodic nature, amino acid side-chains and important modifications such as phosphorylation are not fragmented and therefore important discriminatory information is maintained. The technique also has the potential to analyze large peptides and proteins and therefore support the top dpwn approach to proteomics. ETD is now available in two commercially available ion traps.

3.3.4.7 **Electron Capture Dissociation**

Electron capture dissociation (ECD) is an alternative fragmentation method to CID, but it is restricted to FT-ICR instruments (Zubarev *et al*. 1998; McLafferty *et al*. 2000). In the ECD method, multiply charged ions are irradiated with low-energy electrons produced by an emitter cathode located behind the ICR cell. Upon capture of electrons, reduced radical cations $[M+nH]^{(n-1)+}$ are generated which dissociate by fast and facile fragmentation of the N–C_a bond of the peptide chain, producing mainly c and z fragment ions. The ECD mechanism exhibits extensive peptide cleavage, retention of sites of modification (Shia *et al*. 2001) and side-chain specific cleavage enabling the differentiation of isobaric residues such as leucine and isoleucine (Kjeldsen *et al*. 2003) by producing predominantly *c* and *z* type fragment ions (Cooper *et al*. 2005); again it is non-ergodic in nature and in that sense similar to ETD.

The technique supports the top down approach to proteomics (Section 3.6.2), where intact protein molecular ions are fragmented within the mass spectrometer enabling protein sequence and PTM information to be acquired without performing proteolysis.

The top-down proteomics approach is further supported by the infra red multiphoton dissociation (IRMPD) dissociation technique (Breuker *et al*. 2002). This technique is used for ion activation prior to ECD. Ion activation prior to ECD in this fashion, results in greater fragmentation of the protein backbone enabling more sequence information to be determined.

Generation of the product ion MS/MS spectra alone does not identify the protein, this requires a database search query using one of the common search engines. MS experiments for protein identification rely heavily on computational data analysis and database searching algorithms. These algorithms have been developed to assign peptide sequences based on automated interpretation of MS/MS spectra (Eng *et al*. 1994; Perkins *et al*. 1994; Fenyo *et al*. 2002; Creasy *et al*. 2002).

Zubarev RA, Kelleher NL, McLafferty FW. J Am Chem Soc 120 (1998) 3265–3266.

McLafferty FW, Horn DM, Breuker K, Ge Y, Lewis MA, Cerda B, Zubarev RA, Carpenter BK. J Am Soc Mass Spectrom. 12 (2001) 245–249.

Shi SDH, Hemling ME, Carr SA, Horn DM, Lindh I, McLafferty FW. Anal Chem. 73 (2001) 19–22.

Kjeldsen F, Haselmann K F, Sorensen ES, Zubarev RA.. Anal. Chem. 75 (2003) 1267–1274.

Cooper HJ, Håkansson K, Marshall AG. Mass Spectrum. Reviews 24 (2005) 201–222.

Breuker K, Oh H, Horn DM, Cerda B, McLafferty, FW. J Am Chem Soc 124 (2002) 6407–6420.

Eng JK, McCormack AL and Yates JR 3rd, J Am Soc Mass Spectrom 5 (1994) 976–989.

Perkins DN, Pappin DJC, Creasy DM, Cottrell JS. Electrophoresis 20 (1999) 3551–3567.

Fenyo D, Beavis RC.Trends Biotechnol. 20 (2002) 35–38.

Creasy DM, Cottrell JS. Proteomics 2 (2002) 1426–1434.

Lisacek, F. A guide of good practices for protein-centric bioinformatics. WILEY-VCH, Weinheim (2007).

Automated analysis of peptide MS/MS spectra is now routine with hundreds of MS/MS spectra being analyzed in a single LC-MS run. However, with hundreds of assignments being returned by the search query for every LC-MS run it is important to be confident that the assignment is correct and not a false identification. For a comprehensive review of database mining for proteomics applications, please refer to the monography of Lisacek.

3.4 Protein Characterization

Protein characterization extends further than simply protein identification and often refers to the identification and location of post-translational modifications (PTMs). PTM analysis is one of the pillars on which proteomics is built, as PTM information cannot be determined at the DNA level. Post-translational modification of proteins is an essential route to modify the function/activity of that particular protein, with the process being governed by a series of enzymes including kinases and phophatases. There are several hundred examples of post-translational modifications (Garavelli, 2004), but the most common modifications studied are reversible phosphorylation and glycosylation Specifically, reversible phosphorylation of proteins is a key mechanism for the regulation of major cellular processes such as proliferation, differentiation or apoptosis through highly dynamic and complex signalling pathways. It has been estimated that 100,000 potential phosphorylation sites exist in the human proteome, with the large majority poorly characterized (Zhang *et al*. 2002).

Garavelli JS. Proteomics 4 (2004) 1527–1533.
Zhang H, Zha X, Tan Y, Hornbeck PV, Mastrangelo AJ, Alessi DR, Polakiewicz RD, Comb MJ. J Biol Chem 277 (2002) 39379–39387.

The key challenges in PTM analysis are to isolate/enrich for the modified protein/peptide from a complex mixture, to quantify the phospoprotein expression, determine the stoichiometry or occupancy and to pinpoint the site of modification. To further complicate the analysis, post-translationally modified proteins are often expressed with low abundance. Traditionally, PTMs have been identified using radiolabeling and Edman sequence analysis, amino acid analysis and immunochemistry. However, due to the low protein expression of target proteins and the complexity of starting samples methods with greater sensitivity of detection for PTMs are required.

As we have described previously in this chapter, the evolution of mass spectrometry instrumentation over the last 20 years has had a sigificant impact on protein ID and proteomics. Moreover, mass spectrometry is well placed to support the identification of PTMs.

MS provides the sensitivity needed to study PTMs, though the sample preparation prior to MS is absolutely essential to benefit from this sensitivity capability.

As a post-translational modification will cause a change in the mass of protein, specifically to the amino acid it is attached to, mass spectrometry can detect this. Further, tandem mass spectrometry techniques such as a precursor ion scan and a neutral loss scan can be used to identify peptides that contain a certain PTM within a complex mixture. Once the PTM containing peptide has been detected, a product ion tandem mass spectrometry scan can be performed to determine the location of the PTM within that particular peptide (see Fig. 3.19).

3.4.1 Phosphorylation Analysis

Protein phosphorylation is arguably the most studied PTM. Unlike N-linked glycosylation, there is no specific sequon which indicates phosphorylation, though phosphorylation commonly occurs on serine, threonine and tyrosine and less commonly on histidine residues. Many methods have been reported and examples exist where the state of phosphorylation of a single protein has been determined. However, the mapping of an entire phosphoproteome and relative quantitative analysis of phosphoproteins could be considered something of a holy grail in current proteomics method development and here has been considerable development in pursuit of this goal (Sachon *et al*. 2006; Goshe *et al*. 2001; Blagoev *et al*. 2004; Gruhler *et al*. 2004; Tao *et al*. 2005; Zhou *et al*. 2001; Reinders *et al*. 2005).

Strategies that are specifically designed to identify sites of phosphorylation, attempt to isolate or at least enrich for phosphopeptides or phosphoproteins upstream of tandem mass spectrometry.

Tandem mass spectrometry is the key tool for identifying the site of phosphorylation.

The isolation/enrichment method is either based on an affinity chromatography step or a chemical derivatization approach and most approaches to date are applied at peptide level (Figure 3.29).

Sachon E, Mohammed S, Bache N, Jensen ON. Rapid-Commun Mass Spectrom 20 (2006) 1127–1134.

Goshe MB, Conrads TP, Panisko EA, Angell NH, Veenstra TD, Smith RD. Anal Chem 73 (2001) 2578–2586.

Blagoev B, Ong SE, Kratchmarova I, Mann M. Nat Biotechnol 22 (2004) 1139–1145.

Gruhler A, Olsen JV, Mohammed S, Mortensen P, Færgeman NJ, Mann M, Jensen ON. Mol Cell Proteomics 4 (2005) 310–327.

Tao WA, Wollscheid B, O'Brien R, Eng JK, Xiao-jun L, Bodenmiller B, Watts JD, Hood L, Aebersold R Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. Nat Methods 2 (2005) 591–598.

Zhou H, Watts JD, Aebersold R. Nat Biotechnol. 19 (2001) 375–337.

Reinders J, Sickmann A. Proteomics 5 (2005) 4052–4061.

Though for some simpler samples, it maybe possible to employ a shotgun method, fractionating the sample prior to tandem mass spectrometry.

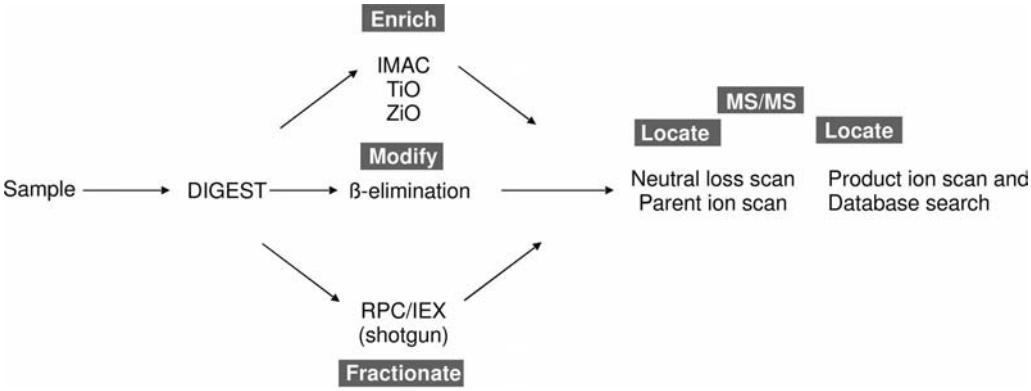


Fig. 3.29: Schematic of the methods used for phosphorylation analysis. Unless the sample is very simple and abundant, then enrichment of the phosphopeptide(s) is necessary prior to MS analysis.

Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A.. Trends Biotechnol. 20 (2002) 261–268.

The enrichment step is potentially very important, as unfractionated phosphorylated peptides often suffer from signal suppression in positive ion ESI MS analysis (Mann *et al*. 2002).

3.4.2 Affinity Chromatography

Again there are many studies which highlight the use of affinity chromatography for phospopeptide isolation/enrichment, with varying specificities. These techniques, of which common examples described below, have been specifically applied to phosphopeptides.

3.4.2.1 Immobilized Metal Ion Affinity Chromatography

Nuwaysir LM, Stults JT. J Am Soc Mass Spectrom 4 (1993) 662–669.

Posewitz MC, Tempst P. Anal Chem 71 (1999) 2883–2892.

Zhou W, Merrick BA, Khaledi MG, Tomer KB. J Am Soc Mass Spectrom. 11 (2000) 273–282.

Stensballe A, Jensen ON. Proteomics 1 (2001) 955–966.

Immobilized metal ion affinity chromatography (IMAC) is a technique that can be used to selectively enrich phosphopeptides in protein digests, which resultantly increases the sensitivity of phosphopeptide detection by MS. Traditional IMAC typically involves two components: an immobilized chelating group and a metal species. The chelating group binds to and presents one face of the metal, allowing interaction and separation of phosphorylated species. A variety of metals have been immobolized, with Ga^{3+} and Fe^{3+} proving to be the most effective. The technique has been reported on many occasions for the enrichment of phosphorylated peptide (Nuwaysir *et al*. 1993; Posewitz *et al*. 1999; Zhou *et al*. 2000; Stensballe *et al*. 2001).

In the technique, protein samples are reduced, alkylated and digested, typically with trypsin. The peptide mixture is then applied to an immobilized metal affinity chromatography column under

acidic conditions (pH 2.5–3.5), unbound nonphosphopeptides are removed from the column with an acidic wash solution and phosphopeptides are eluted under alkaline conditions. Posewitz *et al.* evaluated a variety of metals, including Fe^{3+},Ga^{3+},Al^{3+} and Zr^{3+}, for immobilized metal affinity chromatography enrichment of phosphopeptides. Optimal performance, with respect to selectivity and recovery was observed with iminodiacetate (IDA) resins complexed with Ga^{3+}.

Increased binding of nonphosphopeptides maybe observed when loading is performed outside the pH 2.0–3.5 range or if overloading is allowed.

Heydon CE, Eyers PA, Lauren D. Aveline-Wolf, Resing KA, Maller JL and Ahn NG. Mol Cell Proteomics 2 (2003) 1055.

Selectively, enrichment and recovery of phosphopeptides using IMAC depends heavily on the the metal ion and the chelating resin and is biased towards multiply phosphorylated peptides (Heydon *et al.* 2003). The technique is often affected by the non-selective enrichment of acidic peptides, though this can be addressed with methyl esterification of the peptide samples, improving the selectivity of enrichment (Ficarro *et al.* 2002).

Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. Nat Biotechno. 20 (2002) 301–305.

3.4.2.2 **Titanium Dioxide**

Several papers have been published demonstrating the ability of titanium dioxide (TiO_2) to selectively enrich for phosphopeptides from complex peptide digests, via bidentate binding at the TiO_2 surface (Pinkse *et al.* 2004; Larsen *et al.* 2005). When combined with multiple protein digests and LC-MS/MS, titanium dioxide enrichment has successfully been used for site identification at a low fmol scale.

Phosphopeptides with several basic amino acids have been shown to have a lower affinity for titanium dioxide:

Pinkse MWH, Uitto PM, Hilhorst MJ, Ooms B, Heck AJR. Anal Chem 76 (2004) 3935–3943.

Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. Mol Cell Proteomics 4 (2005) 873–886.

3.4.2.3 **Zirconium Dioxide**

Porous zirconium dioxide (ZiO) provides high selectivity for phosphopeptides under an acidic binding condition (pH 2; Kweon *et al.* 2006).

Schlosser A, Vanselow JT, Kramer A. Anal Chem 77 (2005) 5243–5250.

Kweon HK, Hakansson K. Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. Anal Chem 78 (2006) 1743–1749.

3.4.3 **Chemical Derivatization**

Several methods have been reported for the enrichment of phopsphopeptides. One common method exploits the characteristic that phosphoserine and phosphothreonine undergo undergo β-elimination, losing the phosphate group which renders them susceptible to attack by a range of nucleophilic reagents. These reagents are designed with a tag to either enable enrichment prior to MS or to aid detection of

A similar β-elimination approach could also be used to isolate O-glycoylated Serine and Threonine, though it become difficult to distinguish between the two PTMs

Oda Y, Nagasu T, Chait BT. Nat Biotechnol 19 (2001) 379–382.

Knight ZA, Schilling B, Row RH, Kenski DM, Gibson BW, Shokat KM. Nat Biotechnol 21 (2003) 1047–1054.

Goshe MB, Conrads TP, Panisko EA, Angell NH, Veenstra TD, Smith RD. Anal Chem 73 (2001) 2578–2586.

Zhou H, Watts JD, Aebersold R. Nat Biotechnol. 19 (2001) 375–37.

No detectable interference from other modifications such as glycosylation was reported.

Tao WA, Wollscheid B, O'Brien R, Eng JK, Xiao-jun L, Bodenmiller B, Watts JD, Hood L, Aebersold R. Nat Methods 2 (2005) 591–598.

the phosphopeptide in the mass spectrometer (Oda *et al*. 2001; Knight *et al*. 2003 and Goshe MB *et al*,. 2001). The advantage of using a chemical derivatization approach is that stable isotopes can be incorporated into the mass tag, enabling relative quantification. However, several steps are typically involved in these derivatization strategies.

Another approach, reported by Tao and colleagues, enables the selection of methylated phosphotyrosine peptides as well as methylated phosphoserine and phosphothreonine residues using an amended, solid phase derivatization approach which reduces the number of steps involved. With this method, the phosphate group remains on the peptide, which supports identifying the point of modification within the peptide. Methy esterification is a key step in the method as it protects carboxyl groups from becoming modified during the reaction and enables stable isotopes to be incorporated for relative quantification studies. As a result, non-phosphorylated residues are not retained on the support and selectivity for phosporylated species is high.

Following any of these enrichment steps, the phosphopeptide sample(s) are typically analyzed by LC-MS/MS. It is advantageous if the particular system can perform the full range of tandem mass spectrometry techniques: product ion scan for peptide identification and PTM site location and neutral loss and precursor ion scanes to detect the peptides containing the modification. As explained previously a mass spectrometer can be configured to detect certain diagnostic fragment ions during the fragmentation or CID process.

3.4.3.1 **Precursor Ion Scan**

With respect to phosphorylation analysis, a precursor ion scan can be performed on the fragment ion 79. In this experiment, effectively the whole sample is allowed to pass into the collsion cell to undergo fragmentation. Only peptides which fragment to generate a fragment ion of 79 Da will be detected at the detector. Such a fragment is indicative of a phosphate group and as such is diagnostic of a phosphorylated peptide. This is a sensitive method for identifying phosphorylated peptides and its application has been reported on a number of occasions (Neubauer and Mann, 1999).

Neubauer G, Mann M. Anal Chem 71 (1999) 235–242.

3.4.3.2 **Neutral Loss Scan**

With respect to phosphorylation, a neutral loss scan can be performed to detect the loss of phosphoric acid (H_3PO_4) during the fragmentation process. The mass spectrometer is configured to detect this, using knowledge of the probable charge states of the peptide (i.e. for doubly charged phosphorylated peptides the figure should be set to

49; 98/2). This same fragmentation process can be utilized in a multi-stage, MS^3, tandem mass spectrometry experiment (Beausoleil *et al*. 2004, Chang *et al*. 2004).

A recent publication comprehensively compared several affinity methods for phosphoproteome wide analysis (Bodemiller *et al*. 2007). The paper described the use of IMAC, titanium oxide, zirconium doxide, and a solid phase chemical derivatization method for the analysis of a cytosolic fraction of *Drosophila melanogaster* Kc167 cells.

They discovered that each of the methods enriched for phosphopeptides with an acceptable level of reproducibility. However, each method essentially enriched for different phosphopeptides, though there was some partial overlap, which were not detectable without the enrichment step. Using all the methods, a total of 887 unique phosphorylation sites were identified; ~62% of these were identified in the IMAC samples, ~60% in the chemical derivatisated samples, ~41% in the $pTiO_2$ samples and ~17% in $dhbTiO_2$ samples. Roughly $1/3^{rd}$ of the identified phosphorylation sites were identical between chemically derivatized and IMAC, $1/3^{rd}$ beween chemically derivatized and $pTiO_2$ and $1/3^{rd}$ between IMAC and $pTiO_2$. They concluded that none one method could exhaustively represent or analyze a whole phosphoproteome.

Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villén J, Li J, Cohn MA, Cantley LC, Gygi SP. Proc Natl Acad Sci USA 101 (2004) 12130–12135.

Chang EJ, Archambault V, McLachlin D., Krutchinsky AN, Chait, BT. Anal Chem 76 (2004) 4472–4483.

Bodenmiller B, Mueller LN, Mueller M, Domon B, Aebersold R. Nature Methods 4 (2007) 231–237.

3.4.4 Glycosylation

Glycosylation is an important PTM, with the composition of the glycans crucial for the function of many proteins in cell signalling and host–pathogen interactions. A number of factors make it a particularly challenging PTM to analyze, including glycoprotein enrichment, sensitivity of MS analysis, heterogeneity, occupancy, isobaric masses for many of the sugar residues and, arguably limited chemical derivatization potential (compared to phosphorylation). Glycosylation is either N-linked or O-linked. N-linked glycosylation occurs at a very specific sequon: Asn-Xxx-Ser/Thr, where Xxx is any residue except a proline. In addition, N-linked glycans all have a common basic structure. On the other hand, O-linked glycosylation does not have a specific sequon, otherthan it occurs at serine or threonine residues.

Once more, identifying glycoproteins in a complex mixture can be challenging. Glycoproteins have long been enriched using lectin affinity chromatography (Gabius *et al*. 2002). Alternatively, digestion of the sample in question, followed by precursor ion scanning, as described above, can be used to locate glycosylated peptides using the diagnostic fragment ions of hexoses and hexosamines, 163 and 204 respectively (Huddleston *et al*. 1993).

Gabius HJ, Andre S, Kaltner H, Siebert HC. Biochim Biophys Acta 1572 (2002) 165–177.

Huddleston MJ, Bean MF, Carr SA. Anal. Chem. 65 (1993) 877–884.

Kaji H, Saito H, Yamauchi Y, Shinkawa T, Taoka M, Hirabayashi J, Kasai K, Takahashi N, Isobe T. Nat Biotechnol 21 (2003) 667–672.

Hägglund P, Bunkenborg J, Elortza F, Jensen ON, Roepstorff P. J Prot Research 3 (2004) 556–566.

Methods combining glycoprotein enrichment with glycoprotein digestion and tandem mass spectroimetry analysis have successfully identified sites of glycosylation. Kaji *et al.* (2003) utilized lectin affinity chromatography and glycoprotein digestion in ^{18}O-labeled water for site-specific analysis; whilst Hagglund *et al.* (2004) employed lectin affinity for glycoprotein enrichment and hydrophilic interaction chromatography (HILAC) for glycopeptide enrichment prior to digestion and MS/MS analysis.

Unfortunately, if there is nore than one N-linked site, the specific heterogeneity at each site cannot be assigned.

The use of the enzyme PNGase F can be used to pinpoint the site of N-linked glycosylation. This enzyme will cleave off the N-linked glycans from every site in the protein and in the process convert the aspartamine residue to aspartic acid, a 1 Da mass difference. A simple peptide map will then identify the peptide(s) that contained the site of N-linked glycoyslation. Additionally, the cleaved N-linked glycans can then be analyzed by MALDI-TOF to highlight the heterogeneity of N-linked glycosylation for that specific protein.

Wells L, Vosseller K, Cole RN, Cronshaw JM, Matunis MJ, Hart GW. Mol Cell Proteomics 1 (2002) 791–804.

Methods for investigating O-linked glycosylation include β-elimination as described for phosphorylation. Wells *et al.* (2002) described O-linked analysis using affinity enrichment and tandem mass spectrometry.

3.5 Protein Quantification Using Mass Spectrometry

Protein quantification by mass spectrometry is an exciting application. To qualify, this is using mass spectrometry to detect the difference in protein expression between two samples. This can be performed using stable isotope labeling or by a non-labeling, software approach.

3.5.1 Stable Isotope Labeling Approaches

Yao X, Freas A, Ramirez J, Demirev PA & Fenselau C. Anal Chem 73 (2001) 2836–2842.

Conrads TP, Issaq HJ & Veenstra TD. Biochem Biophys Res Commun 290 (2002) 885–890

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Nature Biotechnol 17 (1999) 994–999.

Stable isotope labeling is a technique that has long been used in mass spectrometry. Mass spectrometry exploits the characteristic that chemically identical analytes with a different stable isotope composition can be separated on a mass spectrometer, due to the mass difference associated with the different isotopes. The ratio of the different analytes signal intensity is indicative of their relative abundance. Many methods have been reported for just such an analysis of protein expression. Stable isotopes have been incorporated into the protein/peptide analyte using digestion in ^{18}O labeled water (Yao *et al.* 2001); via metabolic labeling using heavy amino acid acids (Conrads *et al.* 2002); using isotope coded affinity tags (ICAT™) (Gygi *et al.* 1999)

and more recently with isobaric mass tags (iTRAQ™) which enables both relative and absolute quantification (Ross *et al.* 2004). The tagging of proteins as described in the latter examples have proven to be the most common methods used, though SILAC, stable isotope labeling with amin acids in cell culture is a prominant method (Ong *et al.* 2002). Two further methods, AQUA according to Stemman *et al.* (2001) and Gerber *et al.* (2003), and QconCAT according to Pratt *et al.* (2006) have been reported for absolute quantification.

Importantly, all the methods except iTRAQ™ measure the relative abundance in MS mode, whereas iTRAQ measures the relative abundance in MS/MS product ion mode.

Ross PL, Huang YLN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Mol Cell Proteomics 3 (2004) 1154–1169.

Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A. Mann M. Mol Cell Proteomics 1 (2002) 376–386.

Pratt JM, Simpson DM, Doherty MK, Rivers J, Gaskell SJ, Beynon RJ. Nat Protocols 1 (2006) 1029–1043.

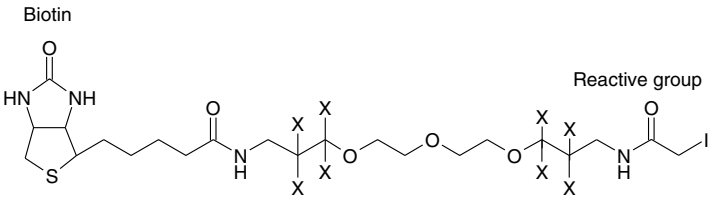
Stemmann O, Zou H, Gerber SA, Gygi SP, Kirschner MW. Cell 107 (2001) 715–726

Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Proc Natl Acad Sci USA 100 (2003) 6940–6945.

3.5.2 Isotope-coded Affinity Tags

This technique was reported by Gygi *et al.* (1999) describing the site-specific labeling of cysteine residues with isotope-coded affinity tags (ICAT™; Figure 3.30). The reagent has three constituent groups; *first* biotin which provides affinity for binding with an avidin column, *second* a linker which incorporates stable isotopes, and *third* a reactive group, iodoacetamide, which labels cysteine residues.

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Nat Biotechnol 17 (1999) 994–999.



X is either hydrogen (light chain) or deuterium (heavy chain)

Two tags exist, one with no deuteriums in the linker (light) and one with eight deuteriums in the linker (heavy). The strategy requires that a two protein mixture, control and test are labeled with the light

and heavy tag respectively. The two derivatized fractions are then combined and digested before affinity isolated using an avidin column. The cysteine labeled peptides are separated by RP-HLPLC and eluted into the mass spectrometer. The two forms of the same peptide will differ in mass by 8 Da, and the ratio between the two determines the relative quantification. Using data dependent analysis the product ion MS/MS spectrum is acquired to identify the protein from the derivatized peptide.

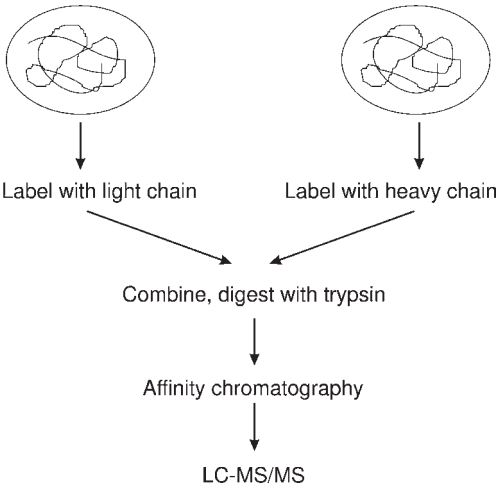


Fig. 3.30: ICAT reagent and workflow.

3.5.3 Stable Isotope Labeling with Amino Acids in Cell Culture

Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A. Mann M. Mol Cell Proteomics 1 (2002) 376–386.

Stable isotope labeling with amino acids in cell culture (SILAC) is a simple approach for in vivo incorporation of a label into proteins for MS-based quantitative proteomics. This technique reported by Ong *et al*. describes the metabolic labeling of two cell states, with the incorporation of amino acids with substituted stable isotopic nuclei (e.g. deuterium, ^{13}C, ^{15}N); for instance normal and deuterated amino acid leucine.

When this labeled analog is provided to cells in culture, it is incorporated into all newly synthesized proteins. After a number of cell divisions, each instance of this particular amino acid will be replaced by its isotope labeled analog. As there is negligable chemical difference between the labeled amino acid and the natural amino acid isotopes, cells behave normally.

In the experimental design, the samples are mixed, digested and analyzed by LC-MS/MS. Relative abundance is derived from leucine-

containing peptides, which will appear as heavy and light peaks, representing those leucine-containing peptides that contain deuiterium and those containing hydrogen. A key limitation is that it can only be used on cultured cells.

3.5.4 AQUA™

In contrast to the strategies described above, AQUA™ provides absolute quantification by employing synthetic peptides containing stable isotopes. The authors describe the steps: selection of the internal peptide standard selection. The peptide is selected on its amino acid sequence and then synthesized with one of the residues being replaced with the "heavy" version which contains the stable isotopes. Resultantly, this peptide is chemically identical to its counterpart in question, but distinguishable in the MS.

Stemmann O, Zou H, Gerber SA, Gygi SP, Kirschner MW. Cell 107 (2001) 715–726.

Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Proc Natl Acad Sci USA 100 (2003) 6940–6945

The AQUA peptide is analyzed by LC-MS/MS to study its fragmentation pattern, which is identical to its counterpart peptide. Subsequent LC–MS/MS analysis using an SRM experiment produces highly accurate measurement of both the internal standard and analyte.

3.5.5 iTRAQ

The iTRAQ reagents are designed to allow labeling of up to four samples with four reagents of the same mass (isobaric).

Ross PL, Huang YLN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Mol Cell Proteomics 3 (2004) 1154–1169.

The reagent consists of three components: an N-hydroxysuccinimide (NHS) ester group which reacts with primary amines at the amino-termini and lysine side-chains (alpha and epsilon amino groups):

a balance group;
a reporter group.

Fragmentation of these four tags in product ion tandem mass spectrometry, produces four reporter ions (m/z 114.1, 115.1, 116.1, 117.1 Da) which are used for quantification of the four samples. As a result, up to four different biological samples may be labeled simultaneously. iTRAQ reagents label the alpha amino group of digested peptides and epsilon amino group of the lysine side-chain. Resultantly, a greater coverage of peptides is observed (also non-cysteine containing proteins can be detected). The design of the tag and workflow are shown in Figure 3.31.

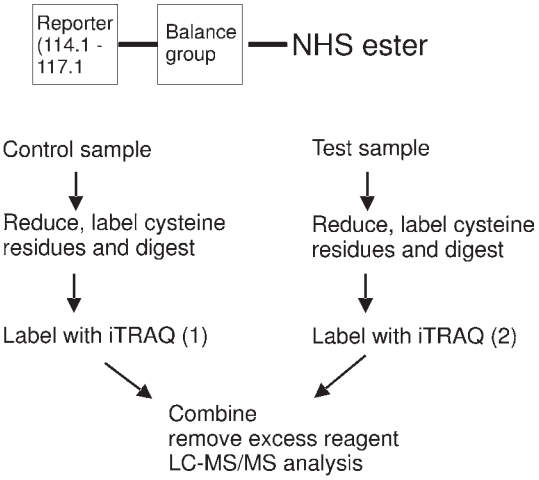


Fig. 3.31: iTRAQ™ workflow for 2 samples.

3.5.6 Non-labeling Software Approaches

This software package is based on similar algorithms like the Decyder 2D software for the evaluation of DIGE gels, which are described in Section 1.6.2.2.

All of the above methods require a degree of sample manipulation to deliver relative and absolute quantification. It can be argued that such sample preparation can introduce error and as a result the methods could be measuring experimental variation rather than real biological variation. A number of software approaches have been developed which attempt to perform relative quantification without any labeling by comparing consecutive LC-MS profiles: Non-commercial (like MSight) and commercial. Such a software package, DeCyder™ MS, is provided by GE Healthcare. This novel software integrates visualization, detection, comparison, and statistical tools. It simplifies the evaluation of large LC-MS and LC-MS/MS data sets for the relative quantification of peptides, based on profile MS spectra. DeCyde MS converts a conventional LC-MS chromatogram into a two- and three-dimensional intensity map, as displayed in Figures 3.32 and 3.34 respectively. Identification results from the MS/MS spectra can be imported into the software and used for simple sorting of peptides belonging to the same protein.

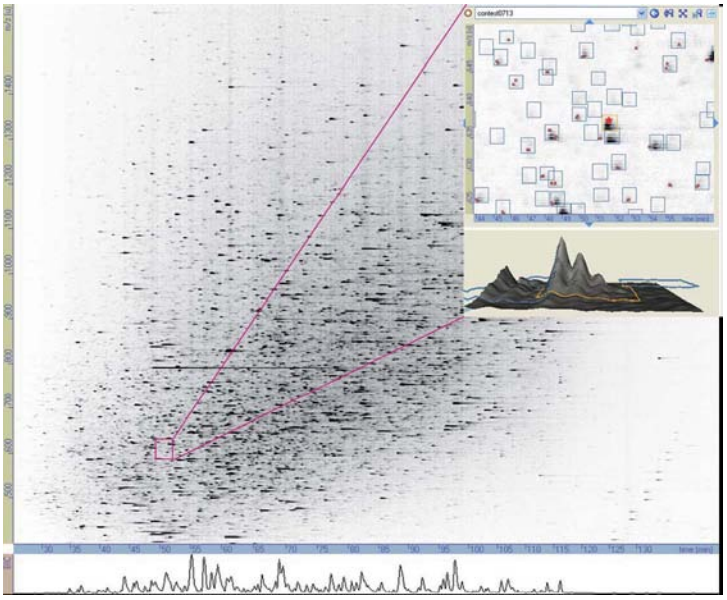


Fig. 3.32: Two-dimensional intensity map of an LC-MS run generated by DeCyder MS. The inset illustrates a zoomed section where the charge states of detected peptides can be seen as well as the MS/MS events (red stars). Courtesy of A. Parbel GE Healthcare.

DeCyder™ MS performs two main analysis procedures:

- **Peptide detection**. The PepDetect module provides accurate *m/z* signal detection, background subtraction, isotope and charge-state deconvolution, and reporting of the eluted peptides from the column. Relative quantification is obtained by summing up the relevant peak volumes for each peptide in all charge states.
- **Run-to-run matching.** The PepMatch module aligns the individual charge states from the same peptide in different runs. According to the experimental setup ratio values and statistical significance for each of the peptides is calculated. Various normalization and filtering techniques can be applied to report easily the requested results.

A workflow is highlighted in Figure 3.33, and an example of difference in protein expression is shown in Figure 3.34. This intensity map approach also acts as a valuable visualization tool enabling qualitative analysis of an LC-MS run as shown in Figure 3.35.

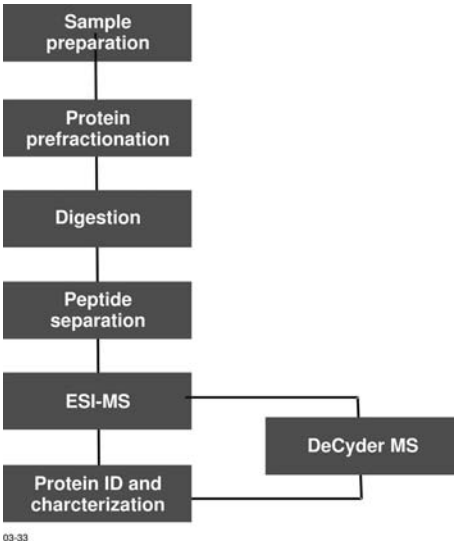


Fig. 3.33: The workflow for DeCyder MS. DeCyder MS uses data derived from an electrospray MS experiment.

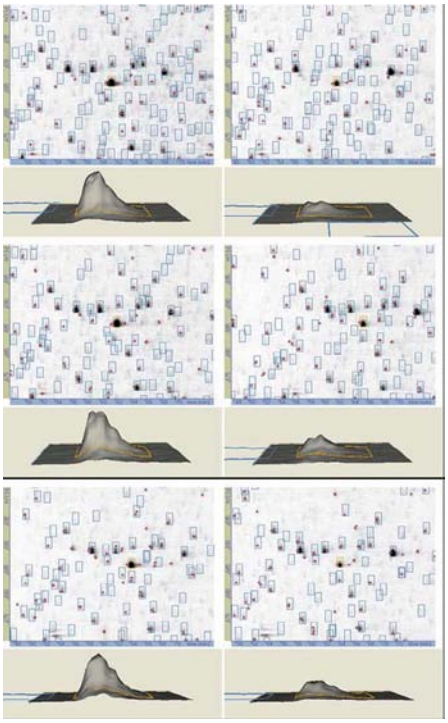


Fig. 3.34: DeCyder MS PepMatch module (matching module) showing the different expression of one particular peptide in three repeats (courtesy of A. Parbel GE Healthcare).

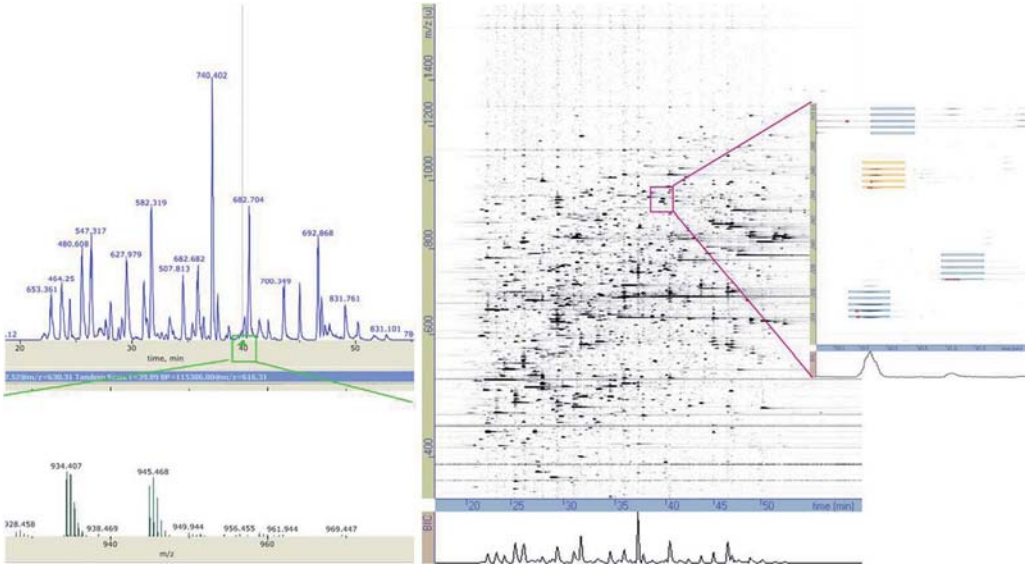


Fig. 3.35: Comparison of traditional LC-MS profile to that of DeCyder MS, whereby peptide components can be clearly detected with DeCyder™ MS. This visualization of an LC-MS profile in this fashion is useful for a range of troubleshooting and monitoring needs. (courtesy of A.Parbel GE Healthcare).

3.6 MS Strategies

Effectively there are two strategies, bottom up and top down. The difference, essentially, is what is the state of the sample when it enters the mass spectrometer i.e. is it a peptide or a protein?

3.6.1 Bottom up Approach

In this approach, separated proteins or complex protein mixtures are digested and the resultant peptides analyzed by MS in order to identify the native protein. The MS analysis is carried out either by peptide mass fingerprinting or by sequence analysis using tandem mass spectrometry. This is probably historical: 2D PAGE separated proteins can only be extracted from the gel by digestion; high resolution, nanoscale RPC of peptides supports high sensitivity MS analysis and the performance and specification of mass spectrometers with respect to peptides was higher than that for proteins. Fragmentation of peptides, rather than whole proteins, has been more practical with

mass spectrometers and mass accuracy greater for peptide analysis. However, it does have its limitations. Acquiring complete or extensive sequence coverage can be an issue, which can make protein characterization problematic. Furthermore, the digestion process is largely limited to trypsin, due to the fact that it locates basic residues to the C-terminus of the peptide which is beneficial for fragmentation and sensitivity, using CID tandem mass spectroscopy.

3.6.2 Top down Approach

This approach utilizes whole proteins and not digested peptides. This approach is performed with mass spectrometers equipped with ETD and ECD fragmentation. These techniques enable fragmentation of large polypeptides and proteins.

Using this approach, accurate measurement of the molecular weight and extensive sequence information can be acquired in one analysis. Additionally, PTM information can be generated.

4
Functional Proteomics: Studies of Protein–Protein Interactions

It is well known that the biological function cannot be linked to a single protein. In fact proteins act in complexes and interact with other proteins. Therefore the next step after expression proteomics is the analysis of protein interactions. A few selected approaches are described in brief in the following section. Many of these technologies, including SPR, are described in much more detail in the book "*Protein microarray technology*" edited by Kambhamti (2003).

Kambhampti, D (ed) Protein Microarray Technology. Wiley-VCH, Weinheim (2003)

4.1
Non-immunological Methods

4.1.1
Separation of Intact Multi-protein Complexes

Because it provides a higher resolution than sucrose gradient centrifugation, the electrophoretic separation of intact protein complexes with attached Coomassie brilliant blue dye in native polyacrylamide gels has become well established in many proteomics laboratories. Blue native PAGE had been developed by Schägger and von Jagow (1991) for the separation of mitochondrial membrane proteins and complexes in the mass range of 1,000 kDa to 10,000 kDa. Details on the technique can be found in Section 1.2.3. In an alternative and complementary approach multi-protein complexes are analysed by electrospray ionization mass spectrometry (Lamond and Mann 1997).

Schägger H, von Jagow G. Anal Biochem. 199 (1991) 223–231.

Lamond AI, Mann M. Trends Cell Biol 7 (1997) 139–142.

4.1.2
Probing with Interaction Partners

"Far Western blotting" according to Burgess *et al.* (2000) is a non-antibody-based detection method for specific complex partners with a purified bait protein after a native or a denaturating electrophoretic separation.

Burgess R, Arthur TM, Pietz BC. Methods Enzymol. 328 (2000) 141–157.

4.1.3
Surface Plasmon Resonance

Fägerstam LG, Frostell-Karlsson Å, Karlsson R, Persson, B, Rönnberg, I. J Anal Chromatography 597 (1992) 397–410.

Surface plasmon resonance (SPR) measures biomolecular binding events in real time without the use of labels by detecting mass concentration changes on a sensor surface. SPR has become an established technique for measuring biomolecular interaction, in particular protein interactions. SPR occurs when surface plasmon waves are excited at a metal/liquid interface (*the sensor surface*). Light is directed at, and reflected from, the side of the surface not in contact with sample. SPR causes a reduction in the reflected light intensity at a specific combination of angle and wavelength (*generating a refractive index-dependent SPR signal*). Molecules binding to the sensor surface cause changes in the refractive index close to the surface which are detected as changes in the SPR signal. Specifically, in an SPR experiment a reactant (ligand) is immobilized on a surface and its interaction with a second component (the analyte) is measured. Effectively the association and dissociation of the ligand–analyte interaction is measured (Fägerstam *et al.* 1992; see Figure 4.1). An SPR detector is capable of measuring real time label-free biomolecular interactions.

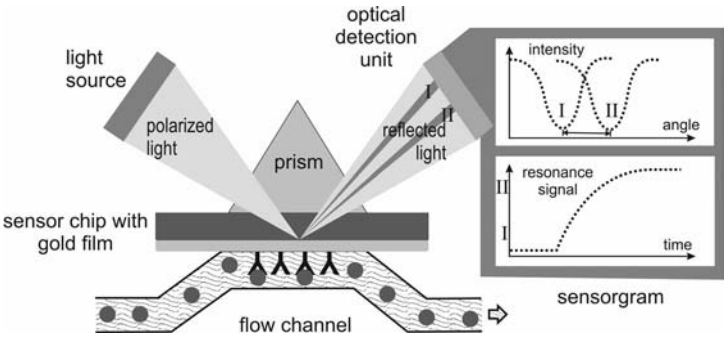


Fig. 4.1: SPR principal of detection. SPR is used to measure the change in refractive index as the analyte binds to the ligand (from Biacore seminar).

A number of applications have been developed for the SPR analysis of proteins with the objective of further characterizing protein interactions. In particular, real time binding data is often essential to understand the dynamic interactions between proteins and other biomolecules that drive and regulate biological processes. Specific questions may include what does the protein of interest bind with or to, where does it bind, when does it bind and how does it bind? Interestingly, studies with SPR can elucidate binding strength (affinity) speci-

ficity of interaction and the rates at which interactants bind and dissociate or kinetics (on and off rates).

Using SPR, complex association and dissociation are detected in real time and data are presented graphically in an interaction profile known as a sensorgram, which is rich in information on dynamics of molecular interactions (see Figure 4.2). A typical experiment takes about 30 minutes to immobilize or capture a ligand protein, 3 minutes for sample injection and kinetic binding measurement, and 1 minute for regeneration. The latter two steps are repeated for a series of analyses.

The major benefit of SPR technology is the information gained from the kinetics of the interaction.

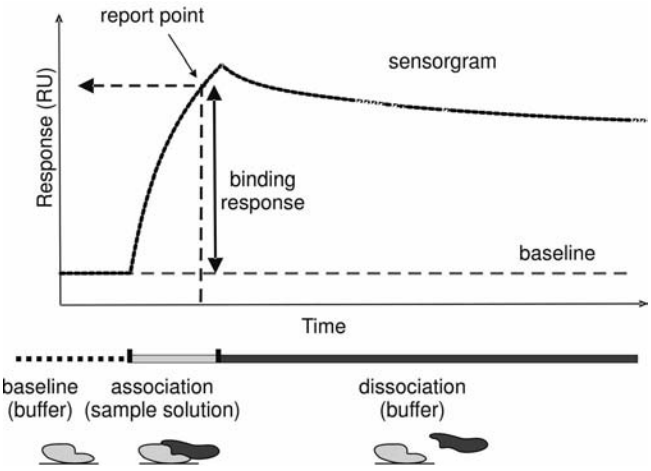


Fig. 4.2: Typical sensorgram of a label-free protein interaction analysis with SPR. The signal height (measured in RU) is also prortional to the mass of protein binding to the ligand.

Application areas in proteomics include the studies of protein complexes, cell adhesion, signal transduction pathways, transcription, regulation, and antibody recognition.

4.2 Antibody-based Techniques

4.2.1 Western Blotting and Dot Blots

However without pre-fractionation of the proteins cross-reactions during the detection procedure cannot completely be excluded.

The procedure of "Western blotting" is intensely described in Section 1.3. In many cases the efforts of electrophoretic separation of the samples and transfer of the separated proteins onto the blotting

membrane is not necessary: The sample mixture possibly containing the protein to be detected can also be applied directly on a blotting membrane as a dot using dot blot or slot blot manifolds. The probing for proteins is performed as described in Section 1.3.2. These dot blots allow parallel screening of many samples.

4.2.2 Protein Microarrays

A critical step is the immobilization of the proteins onto the support. Random orientation of the proteins can cause lossof biological activity and/ or recognition of its epitope by the antibody.

The concept described above, also called a "macroarray", has meanwhile evolved into microarray technologies, using microscope slide format (25×75 mm). The basic technology for microarrys had been developed for nucleic acid analysis in genomics and transcriptomics studies. Similar methodologies are applied on expression profiling of proteins in non-fractionated mixtures: Proteins are arrayed on membranes or on microscope glass slides (protein chips) in very low volumes in tiny spots using instruments similar to inkjet printers. Either antibodies are printed on the carrier to capture specific analytes from body fluids, cell and tissue lysates; or – vice versa – the sample lysates are immobilized on the carrier and antibodies are used to detect specific sample components. Detection is usually performed with a secondary antibody labeled with a fluorescent tag, like cyanine dyes as used in the DIGE approach. In this way multiplexed protein expression profiling can be carried out in a single assay, and relative quantification is enabled. The spotting is fully automated by employing pipetting robots, which apply the solutions from 384-well plates. The images require high resolution multifluorescence scanners.

Also their ease of use and their possibility of automation protein microarrays pledge to be more suitable for diagnostic applications than the classic proteomic separation technologies.

Protein microarrays allow studying many biological events simultaneously with very small sample volumes, resulting in molecular fingerprints on the protein level. Protein microarrays show still some methodological limitation, however there is a huge potential for a wide area of applications from high-throughput proteomics to clinical diagnostics. Figure 4.3 shows a typical microarray image after scanning with different wavelength fluorescence detection.

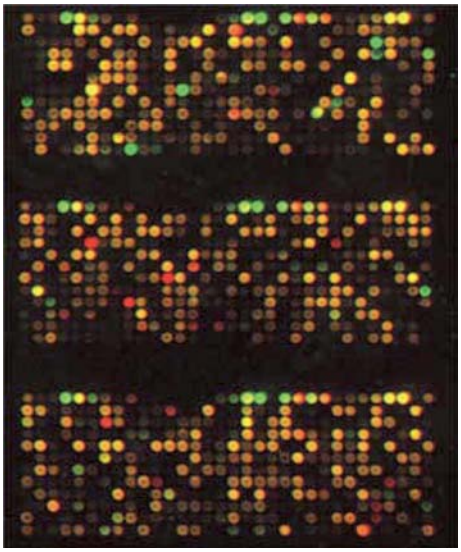


Fig. 4.3: Typical microarray image using Cy3 and Cy5 fluorescent labels after scanning with a high-resolution version Typhoon multifluorescence scanner.

Part II:
Practical Manual of Proteome Analysis

Equipment, Consumables, Reagents

Sample Preparation

Instrumentation

Freezer	–20 °C, –80 °C
Laboratory centrifuge	for 1.5 mL tubes, at least 13,000 rpm
Heating block	for 1.5 mL tubes, at least 95 °C
Micropipettes adjustable	from 2 µL to 1,000 µL

Optional (not always needed):
Sonicator probe

Consumables

Disposable powder free gloves

Reagents

Urea, thiourea, CHAPS, Dithiothreitol (DTT), Bromophenol blue, SDS, Tris-base, Glycerol (85%), Pharmalytes pH 3–10 (~40% w/v), IPG buffer,
Ettan™ 2D cleanup kit
Iodoacetamide

Optional (not always needed):
Molecular grinding kit,
PlusOne™ microdialysis kit
Ettan™ 2D quantification kit
Desoxyribonuclease I (DNase I)
Ribonuclease I (RNase A and RNase B)
Ribonuclease I "A" (RNase A)
Protease inhibitor mix
Phosphatase inhibitors: Na-vanadate, NaF
Sulfobetains

2-D DIGE

Instrumentation

Typhoon™ 9400, Trio	multifluorescence and phosphoimaging scanner
or:	
Ettan™ DIGE Imager (EDI)	

Reagents

Dimethylformamide
CyDyes for minimal labeling, lysine
CyDyes for saturation labeling, HCEP

2-D Electrophoresis

Instrumentation

IPGphor™	IEF system for 2-D electrophoresis
Regular strip holders 18 cm, 24 cm	for standard IEF in Immobiline DryStrips
Ceramic Manifold 24 cm	for basic gradients and very high sample loads
Reswelling tray	for rehydrating 12 IPG strips 7–24 cm
Ettan™ DALT twelve	SDS PAGE system for high-throughput 2-D electrophoresis
Ettan™ DALT six	SDS PAGE system for medium-throughput 2-D electrophoresis
with:	
Multitemp™ III	thermostatic circulator
EPS 3501 XL	programmable power supply 3,500 V
or:	
EPS 600	power supply 600 V
Gel caster for 14 gels	
Gel caster for 6 gels	
Precast gel cassette	1.0 mm
Gel casting cassette	1.0 mm
Gel casting cassettes low-fluorescence (for DIGE gels)	1.0 mm
Gel casting cassette	1.5 mm
Blank cassette insert	
Separator sheets 0.5 mm	

Filler sheets 1.0 mm
Cassette rack
Equilibration tube set 24 cm
Thin plastic ruler (300–500 µm)

Heating block — for 1.5 mL tubes, at least 95 °C
Magnetic stirrer
Laboratory shaker
Ring stand
Hand roller, scissors, spatulas, forceps with bent pointed tips
Assorted glassware: beakers, measuring cylinders, erlenmeyers, test tubes etc.
Magnetic stirrer bars in different sizes
Squeeze bottle, finger-pumped plant sprayer (ca. 500 mL)
Graduated pipettes of 5 mL and 10 mL + pipetting device (e.g. Peleus ball)
Micropipettes adjustable from 2 µL to 1,000 µL
Waterproof pen, black, like Edding 3000

Optional:
FilmRemover — apparatus for removing gels from support films
Gradient maker — for multiple gel caster
Laboratory platform, adjustable, Pinchcock clamp

Consumables

Paper electrode strips, paper bridge pads, loading cups,
Disposable gloves, tissue paper, filter paper, pipette tips, microcentrifuge tubes, test tubes with screw caps 15 mL and 50 mL,

Reagents

IPG DryStrips, 18 cm or 24 cm, different pH gradients
DeStreak™ (HED)
IPG cover fluid
IPGphor™ strip holder cleaning solution (neutral detergent)

Ettan™ DALT II gels and buffer kit:

DALT II Gel 12.5% homogeneous 6/pk
DALT II buffer kit
Urea, thiourea, CHAPS, Dithiothreitol (DTT), Bromophenol blue, SDS, Tris, Pharmalytes™ pH 3–10 (~40% w/v), IPG buffers

Acrylamide IEF solution (40%*T*, 3%*C*), or Acrylamide PAGE 40% solution, N,N′-methylenebisacrylamide (Bis), Glycerol (85%), Ammonium persulfate, TEMED, Glycine, GelSeal silicone grease, Agarose NA
Molecular weight markers range 14,400–94,000
IEF sample application pieces
Gel labels cut from a printed paper or overhead film
Bind-Silane, Decon™ 90
Thiourea, alternative detergents, sulfobetains, isopropanol
Acid violet 17, phosphoric acid, trichloroacetic acid, imidazole, zinc sulfate, EDTA-Na_2

Staining

Instrumentation

Processor Plus with large tray	apparatus for automated silver staining
Staining kit with set of trays	for multiple gel staining

Orbital shaker
Stainless steel staining trays (for Coomassie and silver staining)
Heating stirrer
Kitchen foil welding apparatus
Dark plastic tray (for Deep Purple™)

Gel dryer	gel drying frames and loading platform

Consumables

Disposable gloves	
Transparent smooth sheet protectors	A4 or letter format

Reagents

PhastGel blue tablets	Coomassie brilliant blue R-350

Acetic acid
PlusOne silver staining kit
Coomassie brilliant blue G-250, Tris, o-phosphoric acid, ammonium sulfate, methanol
Deep Purple

Evaluation

Instrumentation

ImageScanner	desk top scanner modified for electrophoresis gels
Typhoon 9000	multifluorescence and phosphoimaging scanner
Ettan DIGE Imager (EDI)	
Computer	(Windows NT , 2000 or XP)
Printer	

Software

DeCyder™	2D evaluation software for DIGE gels
DeCyder™ EDA	Extended data analysis with multivariate statistical tools
ImageMaster™ Platinum	2D evaluation software
Gray step tablet	

Spot Picking

Instrumentation

Ettan™ spot picker

Consumables

Powder-free gloves
500 µL Eppendorff tubes
Scalpel
Surgical needles

Reagents

Ammonium bicarbonate
Acetonitrile
MilliQ water

Digestion

Instrumentation

Ettan™ digester

Consumables

Reagents

DDT
Iodoacetamide
Sequence grade trypsin
Ammonium bicarbonate
Trifluoro-acetic acid
Acetonitrile
MilliQ water

Mass Spectrometry

Instrumentation

Ettan™ MALDI-ToF Pro

Consumables

Ettan™ MALDI-ToF Pro target slides
Gel loader pipette tips
ZipTips

Reagents

α-Cyano-4-hydroxycinnamic acid
2,5 Dihydroxybenzoic acid
Heptafluorobutryic acid
RP resin
Trifluoroacetic acid
Acetonitrile
Methanol
Formic acid
MilliQ water

Step 1: Sample Preparation

To explain here all ways of sample preparation in detail would be too much for this book. Therefore the following instructions should be seen as a starting point. For instance very hydrophobic proteins or plant proteins require more sophisticated extraction procedures.

Sample preparation is the most sensitive step in the entire procedure. Proteins, which got lost here, cannot be identified. Modifications of proteins lead to wrong conclusions in proteome analysis.

- To avoid protein losses, the treatment of the sample must be kept to a minimum.
- To avoid protein modifications, the sample should be kept as cold as possible.
- To avoid losses and modifications, the preparation time should be kept as short as possible.

It should also be noted that there are more than one possible procedure to treat a sample. A 100% representation of the proteins contained in a cell will never be obtained in practice. Usually the method, which will display the highest number of different proteins, is chosen. When there is special interest in a certain group of proteins, which are under-represented with the default method, an alternative procedure has to be applied.

Example: When – for a given sample – the highest number of proteins gets extracted under alkaline conditions, a number of basic proteins will not be included, because their solubility close to their isoelectric points is low. This means: For the analysis of the basic proteins acidic extraction conditions have to be employed.

The procedure described for basic proteins in this chapter is based on a procedure developed for optimal extraction and concentration of plant proteins.

1.1 Washing of Cells

Washing of cells is a critical step, where salt ions can be introduced into the sample. PBS is the standard washing solution. If it is not pos-

sible to remove the PBS solution completely, it is better to wash the cells with Tris-buffered sucrose (10 mmol/L Tris, 250 mmol/L sucrose, pH 7), or to use this alternative washing solution as a last washing step.

Tris-buffered sucrose washing solution: 1.21 g Tris, 85.6 g sucrose, fill up to 1 L with H_2O_{dist}, titrate to pH 7 with 4 mol/L HCl.

First the default procedure and lysis solution is described (see Tables 1.2, 1.3, 1.4). Alternative or additional reagents should only be used, when the results are not sufficiently good.

Pharmalytes and IPG buffers are amphoteric and acquire a net charge of zero during IEF.

The first dimension, isoelectric focusing, is relatively sensitive to salts and other ionic contaminations. Thus the use of ionic buffers must be avoided as much as possible.

The detection methods listed in Table 1.1 describe categories of sample loads. For facts and comments on detection methods see page 50 and Step 4: Staining of gels.

Tab. 1.1: Protein loads (on large gels, using 18 cm and 24 cm strips).

Radiolabeled	1 µg – 50 µg	"Analytical"
Silver stain	20 µg – 200 µg	"Analytical"
CyDye labeled (lysine)	50 µg to 450 mg	"Analytical to preparative"
CyDye labeled (cysteine)	500 ng – 300µg	"Analytical to preparative"
Deep Purple stain	100 µg – 700 µg	"Analytical"
Zinc imidazol negative stain	200 µg – 500 µg	"Analytical"
Coomassie blue stain	500 µg – 2 mg	"Preparative"
Coomassie blue stain	1 mg – 10 mg	"Preparative" on narrow pH intervals

The maximum applicable sample loads depend on the length of the IPG DryStrip and the kind of pH gradient.

Note: ***A sample for 2-D electrophoresis is very precious, only high purity reagents should be used.***

1.2 Stock Solutions

Disposable gloves must be worn during each step to avoid contamination of the sample.

For the sake of reproducibility it is proposed to use exact concentrations of Bromophenol blue.

1% (w/v) Bromophenol blue solution:

1% Bromophenol blue does not go into solution without Tris

Bromophenol blue	1% (w/v)	100 mg
Tris-base		60 mg
Water, deionized	Dissolve	10 mL

Tab. 1.2: Standard solubilization cocktail "lysis solution, lysis buffer" (10 mL).

9 mol/L urea	5.4 g
4% (w/v) CHAPS	400 mg
1% (w/v) DTT (= 65 mmol/L)	100 mg
2% (v/v) Pharmalytes pH 3 to 10 *)	200 µL
0.002% Bromophenol blue **)	10 µL
Water, deionized, make up to	10 mL

*) or IPG buffer of respective pH interval

**) from 1% Bromophenol blue solution

Important: ***Prepare the solution freshly, shake to dissolve the urea, do not warm it higher than 30 °C to avoid carbamylation.***

Removal of isocyanate

If the purity of the urea is in doubt:

- Dissolve 5.7 g urea in deionized water, fill up to 10 mL.
- Warm the tube to get the urea completely in solution: Do not exceed 37 °C.
- Add 100 mg mixed bed ion exchanger Amberlite IRN-150. Stir for 10 min.
- Filter through paper.
- Add the other additives according to Table 1.2.

1.2.1 Optional Additives

Protease inhibition Add protease inhibitor cocktail in lysis solution until 10 µL/mL is reached.

The inhibitor cocktail inhibits most proteases during extraction; irreversible inhibition is only obtained upon precipitation of the sample.

Phosphatase inhibition Add Na-vanadate and NaF until 1 mmol/L until 1 mmol/L of each is reached.

Repeated freeze thawing must be avoided. Add DTT shortly before use.

The lysis solution can be produced in larger quantities – without DTT – and stored frozen in aliquots at a temperature deeper than –60 °C.

Tab. 1.3: Standard rehydration solution.

8 mol/L urea	4.8 g
0.5% (w/v) CHAPS	50 mg
0.28% (w/v) DTT	28 mg
10% (v/v) glycerol	1.2 mL
1.25% (v/v) Pharmalytes pH 3–10 *)	125 µL
0.002% Bromophenol blue **)	10 µL
Water, deionized, make up to	10 mL

*) or IPG buffer of respective pH interval
**) from 1% Bromophenol blue solution

Removal of isocyanate If the purity of the urea is in doubt:

- Dissolve 4.8 g urea in deionized water containing 10% (v/v) glycerol, fill up to 10 mL.
- Warm the tube to get the urea completely in solution: Do not exceed 37 °C.
- Add 100 mg mixed bed ion exchanger Amberlite IRN-150. Stir for 10 minutes.
- Filter through paper.
- Add the other additives according to Table 1.3.

Repeated freeze thawing must be avoided. Add DTT shortly before use.

The rehydration solution can be produced in larger quantities – without DTT – and stored frozen in aliquots at a temperature at –20 °C or colder.

1.2.2 Liquid Samples

To analyze protein solutions such as serum, plasma etc. the solubilizing mixture is diluted to the desired protein concentration with rehydration solution.

Very diluted samples like cell culture supernatant need to be precipitated, usually with 100% (w/v) TCA.

Diluted samples In general, the lower additive concentration like in the rehydration solution should be adequate. However, some samples might need higher concentrations, indicated on the right half of Table 1.4.

Tab. 1.4: Amounts of additives to 1 mL sample.

Sample	1 mL	Sample	1 mL
8 mol/L urea	0.75 g	9 mol/L urea	0.94 g
0.5% (w/v) CHAPS	8 mg	2% (w/v) CHAPS	36 mg
0.28% (w/v) DTT	5 mg	1% (w/v) DTT	18 mg
1.25% (v/v) Pharmalytes 3–10*)	20 µL	2% (v/v) Pharmalytes 3–10*)	36 µL
0.002% Bromophenol blue**)	2 µL	0.002% Bromophenol blue**)	2 µL
Total volume	**1.6 mL**		**1.85 mL**

*) or IPG buffer of respective pH interval

**) from 1% Bromophenol blue solution

1.3 Examples

The volumes are given for 24 cm IPG strips only. For paper bridge loading 18 cm strips are recommended. The sample would be diluted in 450 µL for anodal and 350 µL for cathodal loading.

Example 1: *Escherichia coli* extract (ideal as test sample) Sample: lyophilized cells of *E. coli* strain B (ATCC 11303), Sigma EC-11303

Lyophilized *E. coli*	30 mg	Measured protein content
Lysis solution	1 mL	~12 mg protein/mL

Freeze (–20 °C) and thaw two times in a microcentrifuge tube. Centrifuge for 10 min with 13,000 rpm.

24 cm IPG strip	Sample supernatant	Rehydration solution
Analytical run: (~180 µg protein)		
Rehydration loading	15 µL mix with	435 µL
Cup loading	15 µL*) at anodal side	450 µL (>6 h pre-rehydration)
Preparative run: (~1 mg protein)		
Rehydration loading	83 µL mix with	367 µL
Cup loading	83 µL at anodal side	450 µL (>6 h pre-rehydration)

*) Mix with 50 µL rehydration solution before you apply it.

Figure 1.1 shows a typical 2-D electrophoresis pattern of an *E. coli* extract applied with rehydration loading.

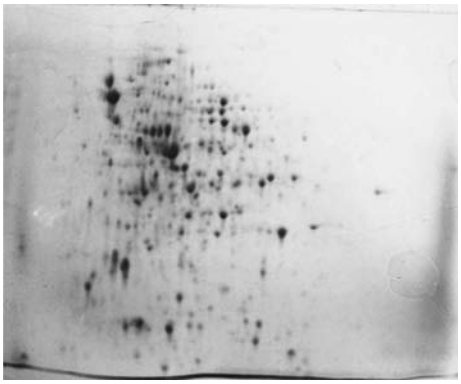


Fig. 1.1: *E. coli* extract (~600 µg protein), IPG DryStrip, pH 3–10, Ettan DALT gel, fast Coomassie staining.

Example 2: *Saccharomyces cerevisiae* (yeast cell lysate)

Lyophilized yeast	300 mg
lysis solution	2.5 mL

Sonicate for 10 min at 0 °C.
Centrifuge for 10 min at 10 °C with 42,000 *g*.

24 cm IPG strip	Sample supernatant	rehydration solution
Analytical run:		
Rehydration loading	30 µL mix with	420 µL
Cup loading	30 µL*) at anodal side	450 µL (>6 h pre-rehydration)
Preparative run:		
Rehydration loading	300 µL mix with	150 µL
Cup loading	100 µL at anodal side	450 µL (>6 h pre-rehydration)

*) Mix with 50 µL rehydration solution before you apply it.

Tissue The tissue of interest is sliced with a scalpel to obtain an appropriately sized piece. It can be frozen with liquid nitrogen and broken into small fragments in a mortar and pestle.

Example 3: calf liver Liver acetone powder, calf, Sigma L-7876

Acetone powder	10 mg
Lysis solution	1 mL

For small tissue amounts a disposable grinding kit is very useful (see Figure 1.2).

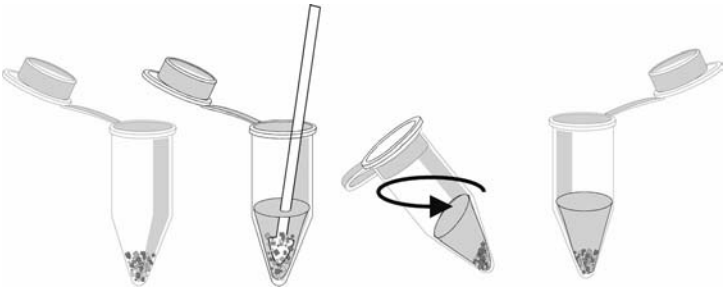


Fig. 1.2: Grinding of tissue material.
Typically 5 mg of the acetone powder or 20–50 mg of animal or plant tissue are ground in 500 µL of lysis solution.

24 cm IPG strip	Sample supernatant	Rehydration solution
Analytical run:		
Rehydration loading	30 µL mix with	420 µL
Cup loading	30 µL *) at anodal side	450 µL (>6 h pre-rehydration)
Preparative run:		
Rehydration loading	300 µL mix with	150 µL
Cup loading	100 µL at anodal side	450 µL (>6 h pre-rehydration)

*) Mix with 50 µL rehydration solution before you apply it.

1.4 Microdialysis

Dialysis of the sample is usually not recommended, because large membrane areas can cause adsorption of proteins. Less protein gets lost with specially designed minidialysis tubes. The fastest removal of small ions is achieved with an 8 kDa cut-off membrane.

In the Tris-glycine buffer system peptides smaller than 10 kDa anyhow co-migrate with the front; they are not displayed in the gel.

Salt and buffer ions can be removed efficiently by dialyzing 2-D samples against at least 40× sample volume of 8 mol/L urea / 1% DTT solution for 2 hours to overnight. The 2-D sample should be prepared in lysis buffer. Other, more expensive additives such as CHAPS

and carrier ampholytes do not need to be included in the dialysis solution. These components are afterwards added back, in the original concentrations.

1.5 Precipitation

For precipitation of proteins from a cell culture supernatant often a 100% (w/v) TCA solution is employed.

A crude extract can contain contaminations with phospholipids and nucleic acids, which are visualized with silver staining as horizontal streaks in the acidic part of the gel. Protein precipitation is therefore often employed to remove the contaminating substances. Protein precipitation is also used to concentrate proteins from samples that are too dilute for effective 2-D analysis.

1.5.1 TCA Acetone

Damerval C, DeVienne D, Zivy M, Thiellement H. Electrophoresis 7 (1986) 53–54.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. Electrophoresis 21 (2000) 1037–1053.

Sometimes the TCA and/or the salt from the sample are not completely removed, which can be observed by horizontal streaking in the 2-D gel. Practice has shown that washing with ice-cold 10% water / 90% acetone instead of pure acetone removes ionic components very efficiently without any negative effects. Therefore the original protocol of Damerval *et al.* (1986) has been modified here. Furthermore the modifications according to Görg *et al.* (2000) are implemented here.

- Freeze plant material – or other material – in liquid nitrogen.
- Grind it with a pestle in a pre-frozen mortar.
- To 30 mg add 5 mL of 20% TCA in cold acetone (–20 °C) containing 0.2% (w/v) DTT.
- Precipitate overnight in a freezer.
- Centrifuge at +20 °C with 13,000 rpm for 10 minutes.
- wash with 90% acetone / 10% water (–20 °C) containing 0.2% (w/v) DTT.
- Centrifuge again.
- Resuspend the pellet in lysis buffer.

Avoid heating!

- Carefully sonicate.
- Centrifuge at +20 °C with 13,000 rpm for 10 minutes.

For protein precipitation from a liquid the precipitation fluid must be at least three times larger than the volume of the sample.

The disadvantage is, however, that some acidic proteins get lost with this procedure, because they are not precipitated into the pellet.

1.5.2 Ettan™ 2-D Cleanup Kit

This procedure has the highest yield of precipitated proteins and proteins coming back into solution.

The Ettan 2-D cleanup kit procedure uses a combination of a unique precipitant and co-precipitant to quantitatively precipitate the sample proteins. The proteins are pelleted by centrifugation and the precipitate is washed to further remove non-protein contaminants. The mixture is centrifuged again and the resultant pellet is resuspended into the lysis buffer.

The sample can contain 1 µg to 1 mg protein in a volume of 1 to 100 µL. Protein can be processed from larger volumes by scaling up the procedure.

1.5.3 Resolubilization

In practice the 2-D cleanup kit and the method according to Wessels and Flügge hold the highest yield among the precipitation methods. Nevertheless, there are sometimes difficulties reported to get the pellet back into solution. Here are a few important hints how to get the pellet completely redissolved:

- Take care that the pellet does not become completely dry!
- Pipette repeatedly lysis solution over the pellet, but do not vortex!

Vortexing can cause oxidation of some proteins, and then they will not dissolve anymore.

- Resolubilization can take several hours or overnight at room temperature. Proteases have been completely inactivated by precipitation.

Do not vortex!

- If this does not work, carefully sonicate with the sample being placed on ice, with short bursts and breaks in between.

Avoid heating of sample

- Or use PlusOne Molecular grinding kit to increase the surface of the pellet

Particularly useful, when several mg of proteins have been precipitated.

- Or freeze pellet with lysis solution at –20 °C for 15 minutes.

Urea crystals will develop, which break up the pellet

- If none of the above measures work, use SDS solution (2% SDS, hot) and then dilute with 9 mol/L urea / 4% CHAPS to an SDS concentration below 0.1%. Also, as a rule of thumb, the CHAPS to SDS ratio must be 8:1 in the sample before application on the IPG strip.

See also "SDS procedure" below

1.6 Very Hydrophobic Proteins

1.6.1 Thiourea Procedure

Always treat thiourea-containing solutions with mixed bed ion exchanger.

For *membrane* proteins and other very hydrophobic proteins a combination *urea/thiourea* in the solubilization solution can be very helpful to get more proteins into solution (Rabilloud, 1998). The modified lysis solution is then composed as follows:

7 mol/L urea	4.2 g
2 mol/L thiourea	1.5 g
With H_2O_{dist} fill up to	9.5 mL

Removal of isocyanate:

- Warm the tube to get the urea and thiourea completely in solution: Do not exceed 37 °C.
- Add 100 mg mixed bed ion exchanger Amberlite IRN-150. Stir for 10 min.
- Filter through paper.
- Add the other additives:

4% CHAPS	400 mg
1% (w/v) DTT	100 mg
2% (v/v) Pharmalytes pH 3 to 10*)	500 µL
0.002% Bromophenol blue**)	10 µL

*) or IPG buffer

**) from 1% Bromophenol blue solution

Important: ***Prepare the solution freshly, shake to dissolve the urea, do not warm it higher than 30 °C to avoid carbamylation.***

Repeated freeze-thawing must be avoided. Add DTT shortly before use.

This lysis solution can also be produced in larger quantities – without DTT – and stored frozen in aliquots at a temperature lower than –60 °C.

1.6.2
SDS Procedure

- Formation of oligomers can be prevented.
- Organisms with tough cell walls sometimes require boiling for 5 minutes in 1–2% SDS before they are diluted with lysis solution.
- Very hydrophobic proteins might require extraction with high percentage of SDS.

Note: ***The SDS-containing sample solution must be diluted with lysis solution (9 mol/L urea / 4% CHAPS) to an SDS concentration below 0.1%. Also, as a rule of thumb, the CHAPS:SDS ratio must be 8:1 in the sample before application on the IPG strip.***

Example: human plasma According to Sanchez *et al.* (1995):

Sanchez J-C, Appel RD, Golaz O, Pasquali C, Ravier F, Bairoch A, Hochstrasser DF. Electrophoresis. 16 (1995) 1131–1151.

10 µL human plasma	Mix with 6.25 µL 10% SDS, 2.3% DTT
Heat for 5 min at 95 °C	
Dilute with	500 µL lysis solution

Note: ***SDS does not always completely separate from the proteins during IEF, even under high field strength. SDS can also be removed by precipitation with the cleanup kit.***

1.7 Quantification

The most reliable protein quantification for 2-D IEF samples is obtained with the Ettan™ 2-D Quant kit, which is based on precipitation. This procedure is compatible with such common sample preparation reagents as 2% SDS, 1% DTT, 8 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 2% Pharmalytes and 2% IPG Buffer. The precipitated proteins are resuspended in a copper-containing solution; the unbound copper is measured with a colorimetric agent. The color density is inversely related to the protein concentration. The volume range of the assay is 1–50 µL and the linear range for quantification is 0–50 µg. The assay has a sensitivity threshold of 0.5 µg.

1.8
SDS Samples for 1-D SDS Electrophoresis

SDS sample buffer:

2% (w/v) SDS		2 g
25% Glycerol (v/v)	85% solution	30 mL
50 mmol/L Tris HCl, pH 8.8	1.5 mol/L stock solution	3.5 mL
0.01% Bromophenol blue		10 mg
Water, deionized	Make up to	100 mL

The glycerol is added to increase the density to prevent mixing with upper buffer.

Store at room temperature.

- Dissolve 50 mg of DTT in 100 µL deionized water.
- Dissolve 20 mg of iodoacetamide in 100 µL deionized water.
- Add 10 µL DTT solution to 100 µL SDS sample buffer.
- Mix 25 µL sample extract with 25µL SDS sample buffer.

Do not heat it, when the sample contains urea!

- Heat at 60 °C for 5 min.
- Add 10 µL iodoacetamide solution to the 50 µL SDS sample solution. Leave it at room temperature for 30 min.
- Apply 20 µL of this solution on the SDS gel.

Step 2: Fluorescence Difference Gel Electrophoresis

2.1 Experimental Design

The experiment setup should be properly designed: Biological replicates ensure that induced biological changes are not mixed up with inherent biological differences. Gel replicates are only necessary when an experiment consists of a low number of samples, like two to six samples. In this case one-time gel replicates are run, which contain inverse labeled samples. Experiments with higher numbers of samples do not require gel replicates.

The pooled internal standard is always labeled with one and the same CyDye: Cy2 for 3-color experiments and Cy3 for 2-color experiments.

Optimally all samples of an experiment should be run together in the first and second dimension to minimize methodical variation. This means that an experiment should not consist of more than 12 gels, if possible.

Running gels of one experiment in different chambers makes spot matching more difficult.

- Prepare a chart for planned randomization of the samples and the internal standard among the gels, like the example in Table 2.1.

Tab. 2.1: Example for "randomized" sample labeling and application for 12 samples.

Gel	Cy2 standard	Cy3	Cy5
1	All 12 samples	Control 1	Treated 3
2	All 12 samples	Control 2	Treated 1
3	All 12 samples	Control 3	Treated 2
4	All 12 samples	Treated 4	Control 5
5	All 12 samples	Treated 5	Control 6
6	All 12 samples	Treated 6	Control 4

Disposable gloves must be worn during each step to avoid contamination of the sample.

2.2 Sample Preparation

In the standard labeling protocol, proteins are first denatured in a lysis buffer; that will eliminate all secondary and tertiary structures of proteins. The protein concentration should then be determined using the Ettan 2-D Quant kit procedure. The fluorescent properties of Cy2, Cy3 and Cy5 can be adversely affected by exposure to light: keep the exposure of protein labeled with CyDye to all light sources to a minimum.

These compounds are added after labeling had been completed by adding a 2× sample solution.

During labeling the samples must not contain primary amines (IPG buffers, carrier ampholytes) and reductants.

Appropriate sample preparation is crucial for good results. Practice has shown that labeling efficiency is much higher after sample cleanup based on precipitation of the proteins. This measure will not only remove disturbing contaminants like lipids, polysaccharides, salt ions, and nucleic acids, but also endogeneous peptides from the sample. Peptides contain also lysines and cysteines, which would be labeled with a CyDye fluor as well. In a standard buffer system the small peptides migrate in the front of the second dimension, and would not be resolved.

2.2.1 Stock Solutions

Sample lysis solutions for DIGE:

Lysis solution 1: 9 mol/L urea, 4% (w/v) CHAPS, 30 mmol/L Tris.
Lysis solution 2: 2 mol/L thiourea, 7 mol/L urea, 4% (w/v) CHAPS, 30 mmol/L Tris.

For pH adjustment: 50 mmol/L NaOH solution

- Solubilize the protein samples either in sample lysis solution 1 or 2.
- Check the protein concentration in the samples: optimally it would be between 5 mg/mL and 10 mg/mL. But 1 mg/mL to 20 mg/mL are tolerable for successfully labeling.
- Check the pH carefully: pipette 2 µL sample on a pH indicator paper. Read out the pH value immediately, because the color will shift with time.

- If necessary adjust the pH value with adding 50 mmol/L NaOH solution.

Note: ***Proteins have some inherent buffering capacity and may have decreased the pH value of the lysis solution below pH 8. Samples which have been cleaned up with TCA acetone or the 2-D cleanup kit can be quite acidic.***

2.3 Reconstitution of the CyDyes

The quality of the DMF is the major source of error for inadequate labeling.

Use 99.8% anhydrous Dimethylformamide (DMF) less than 3 months old from day of opening. The quality of the DMF is critical to ensure that the protein labeling is successful. The DMF must be anhydrous and every effort should be used to ensure it is not contaminated with water. DMF after opening, over a period of time, will degrade with amine compounds being produced. Amines will react with the NHS ester CyDye reducing the concentration of dye available for protein labeling. Adding a 4 Å molecular sieve to DMF during storage is a good measure to prolong the useful lifetime of DMF.

2.3.1 For Minimal Labeling of Lysines

CyDye fluors for minimal labeling are available in different package sizes. Figure 2.1 shows a schematic overview over dye reconstitution and labeling for the smallest package size (5 nmol).

The solutions are stable at –20 °C for several months.

Dye stock solution: CyDye DIGE Fluor minimal dyes solid compounds are reconstituted in Dimethylformamide (DMF) giving a concentration of 1 nmol/µL (e.g. 25 µL DMF to 25 nmol/L of dye). The stock solution of Cy2 will have a deep yellow, Cy3 a deep red, and Cy5 a deep blue color.

- Take a small volume of DMF from its original container and dispense into a microcentrifuge tube.
- Take the CyDye from the –20 °C freezer and leave to warm for 5 minutes at room temperature
- After 5 minutes add 25 µL of the DMF to each new vial of CyDye.
- Replace the cap on the dye microcentrifuge tube and vortex vigorously for 30 seconds.

- Centrifuge the microcentrifuge tube for 30 seconds at 12,000 *g* in a benchtop microcentrifuge.
- The dye can now be used.

After reconstitution CyDye is only stable and useable until the expiry date listed on the tube.

Note: ***When dyes are not being used they should be returned to the –20 °C freezer as soon as possible and stored in the dark.***

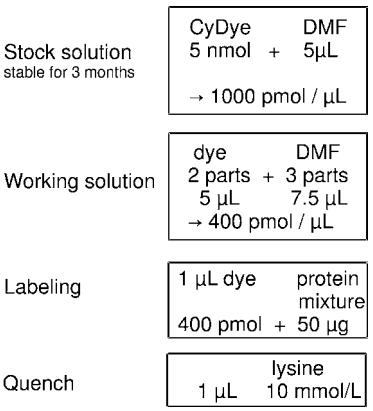


Fig. 2.1: Schematic overview over CyDye reconstitution and labeling for 5 nmol packages for minimal labeling.

Dye working solution: Dilute 1 volume of the stock CyDye in 1.5× volumes of high grade DMF.

- Briefly spin down dye stock solution in a microcentrifuge.
- Add 7.5 µL of DMF to a sterile microcentrifuge tube.
- Add 5 µL of the stock dye solution.

Now there are 5,000 pmol CyDye in 12.5 µL; therefore 1 µL contains 400 pmol.

Note: ***CyDye in the diluted form is only stable for 2 weeks at –20 °C.***

10 mmol/L lysine solution:

Reagent	Quantity	Final concentration
L-Lysine (MW 182.6)	18 mg	10 mmol/L
	Make up to 10 ml with distilled water	

Store at –20 °C. Stable for 6 months.

Note: ***The pipetting error should be kept to a minimum. Therefore, depending on the number of samples to be labeled, the dyes can be directly reconstituted to a working solution.***

For the example in Figure 2.1 the dye would be directly reconstituted with 12.5 µL DMF.

2.3.2 For Saturation Labeling of Cysteines

Dye solution: In principle the procedure is like with minimal dyes, just with different amounts.

- CyDye DIGE Fluor saturation dyes solid compounds are reconstituted in dimethylformamide giving a concentration of 2 mmol/L (50 µL DMF to 100 nmol of dye). The stock solution of Cy3 has a deep red and Cy5 a deep blue color. It is stable at –20 °C for two months maximum. Here the solution is not further diluted for labeling.

The stock solution is used as working solution.

2 mmol/L TCEP (Tris carboxyethyl phosphine):

Reagent	Quantity	Final concentration
TCEP (MW 286.7)	5.7 mg	2 mmol/L
	Make up to 10 mL with distilled water	

2.4 Minimal Labeling of Lysines

Conditions:

- For efficient labeling the cell lysate (protein sample) should have optimally pH 8.5.
- Labeling temperature is 0 °C.
- The protein concentration should be between 5 mg/mL and 10 mg/mL.
- Sample must be free of IPG buffers or carrier ampholytes and reductants.

It must be above pH 8.0.

It must be between 1 mg/ml and 20 mg/ml.

Preparing the pooled internal standard: Take the same amount of aliquot from each sample and mix them together. A minimum of an equivalent of 50 µg protein internal standard per gel is required.

If mass spectrometry analysis requires a higher protein load than 150 µg in a gel, a higher volume of standard is collected and divided

into two portions: one is labeled with Cy2; the other portion is left unlabeled. The required amount of unlabeled standard is later on spiked into the mixture of labeled samples and standard before application on the IPG strip.

Vortexing is not recommended.

Samples and dyes must be mixed well by rigorously pipetting. Insufficient mixing could lead to preferential labeling of some proteins. The sample must not get frozen, because this would cause preferential labeling of some proteins. Since cell lysates are viscous, it is important to mix samples thoroughly in all mixing steps to avoid non-uniform labeling.

Internal standard labeling with Cy2:

n is the number of gels in the experiment.

- Add a volume of internal standard equivalent to $n\times$ 50 µg protein to a sterile microcentrifuge tube.
- Add n µL of diluted Cy2 to the microcentrifuge tube containing the pooled standard (i.e. 300 µg of protein is labeled with 2,400 pmol of dye).
- Mix and centrifuge briefly in a microcentrifuge. Leave on ice for 30 minutes in the dark.
- Add n µL of 10 mmol/L lysine to stop the reaction. Mix and spin briefly in a microcentrifuge. Leave for 10 minutes on ice in the dark.
- Labeling is now finished.
- Standards can now be stored for at least three months at –70 °C in the dark.

Sample labeling with Cy3 and Cy5:

- Add a volume of sample equivalent to 50 µg protein to a sterile microcentrifuge tube.
- Add 1 µL of diluted CyDye to the microcentrifuge tube containing the protein sample (i.e. 50 µg of protein is labeled with 400 pmol of dye).
- Mix and centrifuge briefly in a microcentrifuge. Leave on ice for 30 minutes in the dark.
- Add 1 µL of 10 mmol/L lysine to stop the reaction. Mix and spin briefly in a microcentrifuge. Leave for 10 minutes on ice in the dark.
- Labeling is now finished.
- Samples can now be stored for at least 3 months at –70 °C in the dark.

2.5
Saturation Labeling of Cysteines

The complete procedure takes about 2 hours.

Conditions:

- For efficient labeling the cell lysate (protein sample) must have pH 8.0.
- Labeling temperature is 37 °C.
- The reductant TCEP: dye concentration ratio is always kept at a 1:2 ratio to ensure efficient labeling.
- The protein concentration should be 0.55–10 mg/mL.
- For samples containing proteins with high cysteines content, more TCEP and dye are needed.

Mostly 5 μg sample proteins are labeled and analyzed as proposed below. However in the paper by Sitek *et al.* (2005) labeling of 2.5 μg protein – corresponding to 1,000 pancreatic tissue cells – with the recommended amounts of reductant–dye has been described.

Preparing the pooled internal standard: Take the same amount of aliquot from each sample and mix them together. For cysteine labeling mostly an equivalent of 5 μg protein internal standard per gel is used.

For the internal standard as well as for the preparative gel Cy3 is preferred to Cy5, because Cy3 shows lower self-quenching effects in the high molecular weight region than Cy5. Therefore the image of the preparative gel will be more similar to the image of the pooled standard for optimal spot picking.

Samples and dyes must be mixed well by rigorously pipetting. Insufficient mixing could lead to preferential labeling of some proteins. The sample must not get frozen, because this would cause preferential labeling of some proteins. Since cell lysates are viscous, it is important to mix samples thoroughly by snipping at the tube in all mixing steps to avoid non-uniform labeling.

Vortexing is not recommended.

Internal standard labeling with Cy3:

- Add a volume of internal standard equivalent to $n\times$ 5 μg to a sterile microcentrifuge tube.
- Make up to $n\times$ 9 μL with lysis solution.
- Add $n\times$ 1 μL of 2 mmol/L TCEP.

n is the number of gels in the experiment.

Assuming a cysteine content of 2%.

- Mix vigorously by pipetting and spin.
- Incubate at 37 °C for 1 hour in the dark.

Corresponding to n× 4 nmol/L.

- Add *n*× 2 µL of 2 mmol/L Cy3 saturation dye solution.
- Mix vigorously by pipetting and spin.
- Incubate at 37 °C for 30 minutes in the dark.
- Stop the reaction by adding an equal volume of 2× sample solution.
- Mix vigorously by pipetting and spin.
- Labeling is now finished.
- The labeled samples can be stored for 1 month at –70 °C in the dark.

Sample labeling with Cy5:

- Add a volume of protein sample equivalent to 5 µg to a microcentrifuge tube.
- Make up to 9 µL with lysis solution.

Assuming a cysteine content of 2%.

- Add 1 µL of 2 mmol/L TCEP.
- Mix vigorously by pipetting and spin.
- Incubate at 37 °C for 1 hour in the dark.

Corresponding to 4 nmol/L.

- Add 2 µL of 2 mmol/L Cy5 saturation dye solution.
- Mix vigorously by pipetting and spin.
- Incubate at 37 °C for 30 minutes in the dark.
- Stop the reaction by adding an equal volume of 2× sample solution.
- Mix vigorously by pipetting and spin.
- Labeling is now finished.
- The labeled samples can be stored for 1 month at –70 °C in the dark.

Preparative sample labeling with Cy3: The protein amount of the reference proteome applied on the preparative gel is usually 300 µg.

- Add a volume of protein sample equivalent to 300 µg to a microcentrifuge tube.

Assuming a cysteine content of 2%.

- Add 60 µL of 2 mmol/L TCEP.
- Mix vigorously by pipetting and spin.

Note: ***Cell lysates are viscous. Therefore it is important to mix samples thoroughly in this and all following mixing steps to avoid non-uniform labeling.***

- Incubate at 37 °C for 1 hour in the dark.

- Add 120 µL of 2 mmol/L Cy3 saturation dye solution.
- Mix vigorously by pipetting and spin.
- Incubate at 37 °C for 30 minutes in the dark.
- Stop the reaction by adding an equal volume of 2× sample solution.
- Mix vigorously by pipetting and spin.
- Labeling is now finished.
- The labeled samples can be stored for 1 month at –70 °C in the dark.

Saturation labeling optimization: Because it is impossible to predict the cysteines content of the protein mixture contained in a sample type, it may be necessary to optimize the amount of reductant–dye per protein. In a "same/same experiment" aliquots of the pooled samples are labeled with the same amounts of the different CyDyes, combined, and separated with a 2-D gel. After scanning the images of the Cy3 and Cy5 channel, the patterns are overlaid with ImageQuant™ or PaintShop Pro™ and inspected in a false color image:

When the sample is very limited, a reference proteome has to be found for performing the optimization procedure.

- When the spots are matching perfectly, the amount of reductant–dye is correct for this particular sample type.
- When red spots are visible, which are horizontally offset from some of the green spots, overlabeling has occurred: there is some unspecific labeling of lysines which alter the isoelectric point.

The little size differences are not resolved.

- When there are vertical spot trains and/or streaks, some proteins have been underlabeled: partially labeled proteins migrate faster in SDS electrophoresis than more or fully labeled proteins.

The pI is not affected.

The reductant–dye amount needs to be corrected accordingly. When sufficient pooled sample material is available, a dilution series experiment can be performed, usually in the range from 1 nmol reductant for 2 nmol dye to 4 nmol/L reductant for 8 nmol dye.

2.6 Preparation for Loading the Samples onto IPG Strips

2× Sample solutions: The Pharmalytes 3–10 work very well for wide pH gradients and the pH gradient 4–7. For basic gradients or narrow

gradients the Pharmalytes 3–10 are replaced by the related IPG buffers.

2× Sample solution 1: 9 mol/L urea, 2% (w/v) CHAPS, 1% (v/v) Pharmalytes pH 3–10 (or IPG buffer respective to the pH gradient), 2% (w/v) dithiothreitol (DTT), 0.01% (w/v) Bromophenol blue.

2× Sample solution 2: 2 mol/L thiourea, 7 mol/L urea, 2% (w/v) CHAPS, 1% (v/v) Pharmalytes pH 3-10 (or IPG buffer respective to the pH gradient), 2% (w/v) dithiothreitol (DTT), 0.01% (w/v) Bromophenol blue.

- After the protein samples have been CyDye-labeled, add an equal amount of 2× sample solution and leave on ice for 10 minutes.
- Combine the labeled samples and pooled internal standards according to the experimental design.
- Apply them onto the IPG strip as explained in the next Chapter.

Step 3: Isoelectric Focusing

Disposable gloves must be worn during each step to avoid contamination of the sample.

Note: ***It is very important that each sample is centrifuged before application onto the IPG strip!***

We assume that the reader of this book is starting with 2-D electrophoresis or looking for some advice to improve the quality of the 2-D patterns.

Choose 18 cm IPG strips instead of 24 cm strips, when molecular weight markers and 1-D samples should be applied onto the SDS gel. The "worst case" conditions are given here for running the strip. These "worst case" conditions are applied when a novel sample type has to be separated, whose behavior in the electric field is unknown.

IPG strips with printed serial numbers are very useful for tracing the samples through the 2-D electrophoresis procedure.

- Generally, create a table with a list of all IPG strip numbers used, and fill in which sample will be applied on which IPG strip, as shown in Table 3.1.

Tab. 3.1: IPG strip numbers and sample trace chart (example).

IPG strip number	Sample with Cy3 label	Sample with Cy5 label	Internal standard with Cy2 label
197 514	Control 1	Treated 6	All 24 samples
197 515	Control 2	Treated 5	All 24 samples
197 516	Control 3	Treated 4	All 24 samples
197 517	Control 4	Treated 3	All 24 samples
197 518	Control 5	Treated 2	All 24 samples
197 519	Control 6	Treated 1	All 24 samples
197 520	Treated 7	Control 12	All 24 samples
197 521	Treated 8	Control 11	All 24 samples
197 522	Treated 9	Control 10	All 24 samples
197 523	Treated 10	Control 9	All 24 samples
197 524	Treated 11	Control 8	All 24 samples
197 525	Treated 12	Control 7	All 24 samples

3.1
Reswelling Tray

Passive rehydration loading means without applying an electric field.

This device is used for both, "passive" rehydration loading of samples directly into the IPG strips or pre-rehydration of IPG strips for cup loading.

The next three tables list rehydration solutions optimized for different sample and pH gradient types.

Tab. 3.2: Standard rehydration solution.

8 mol/L urea	4.8 g
0.5% (w/v) CHAPS	50 mg
10% (v/v) glycerol	1.2 mL
1.25% (v/v) Pharmalytes 3–10 *)	125 µL
0.002% Bromophenol blue **)	10 µL
Water, deionized, fill up to	10 mL

*) or IPG buffer of respective pH interval
**) from 1% Bromophenol blue solution

Repeated freeze thawing must be avoided.

The rehydration solution can be produced in larger quantities without the DTT and stored frozen in aliquots at a temperature at –20 °C or colder.

The DTT should be added before use: 0.28% (w/v).

Note: ***If the sample was extracted or solubilized in presence of thiourea, the rehydration solution must also contain thiourea.***

Tab. 3.3: Hydrophobic proteins rehydration solution.

7 mol/L urea	4.2 g
2 mol/L thiourea	1.5 g
0.5% (w/v) CHAPS	50 mg
10% (v/v) glycerol	1.2 mL
1.25% (v/v) Pharmalytes 3–10 *)	125 µL
0.002% Bromophenol blue **)	10 µL
Water, deionized, fill up to	10 mL

*) or IPG buffer of respective pH interval
**) from 1% Bromophenol blue solution

The DTT should be added before use: 0.28 % (w/v).

For acidic pH gradients the addition of DeStreak has no value.

For rehydration loading dilute sample accordingly.

As described in Section 1.5.1, *Sample preparation*, on pages 56 and 79 for basic gels, the addition of DeStreak instead of a reductant pre-

vents the streaking caused depletion of the reductant. This measure can also improve wide pH gradient separations.

Tab. 3.4: Basic gradients pre-rehydration solution.

7 mol/L urea	4.2 g
2 mol/L thiourea	1.5 g
15 mg/mL DeStreak reagent	120 µL
0.5% (w/v) CHAPS	50 mg
10% (v/v) glycerol	1.2 mL
1.25% (v/v) IPG buffer according to pH gradient	125 µL
0.002% Bromophenol blue*)	10 µL
Water, deionized, fill up to	10 mL

*) From 1% Bromophenol blue solution

Such a rehydration solution is available as ready-made mixture (DeStreak solution), however, without the IPG buffer added; see page 79

Note: ***Do not mix sample containing reductant and rehydration solution containing DeStreak!***

Be sure that the reswelling tray has been carefully cleaned and dried before use.

- Adjust the feet to level the tray horizontally, using the inbuilt spirit level as a control (see Figure 3.1).
- Pipette rehydration solution containing the sample according to Table 3.5 into the grooves as streaks slightly shorter than the strips to be rehydrated.

Tab. 3.5: Rehydration volumes.

7 cm strip	125 µL
18 cm strip	340 µL
24 cm strip	450 µL

- Remove the cover film from the IPG strip starting at the acidic (+) end.
- Place the strip into the slot with the dried gel side down, avoiding air bubbles.
- Check whether the serial number of the strip can be read correctly.
- Remove air bubbles by lifting the strip up again with a forceps.

Starting at the basic end might damage the – usually softer – basic gel surface.

If the number is mirror-converted, the gel surface is turned upside.

If some of the sample flows onto the back of the film, it will be pushed down around the edge of the strip when the paraffin oil is pipetted onto it.

To avoid urea crystallization and oxygen contact.

- Pipette 2 mL paraffin oil over the strip, starting at both ends of the strip and moving to the center.
- Repeat this procedure for all samples to be analyzed.
- Close the tray with the sliding coverlid.
- Leave to rehydrate at room temperature according to Table 3.6.

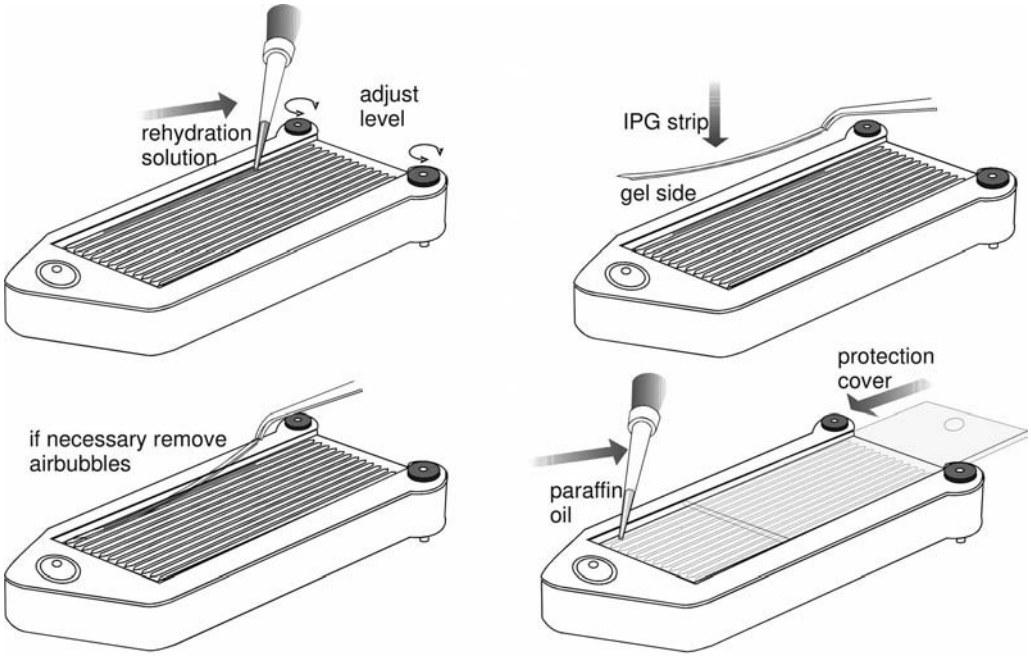


Fig. 3.1: Rehydration of IPG strips in individual grooves in the reswelling tray.

Tab. 3.6: Rehydration times.

Without sample	>6 hours
Including sample	>12 hours or overnight *)

**) The large protein molecules need a long time to diffuse into the strip.*

The grooves are numbered 1–12 to allow sample identification. However, ideally the IPG strips have serial numbers printed on the back of the film support, which are used for tracking the samples.

After rehydration

- Rinse the surface of the strips with distilled water using a squeeze bottle and then place them for a few seconds on their edges on a damp filter paper to drain excess liquid, so that the urea on the surface does not crystallize out.

The strips can be run on the Multiphor flatbed chamber in the *Immobiline DryStrip Kit* (tray for up to 12 strips) or on the IPGphor in the manifold.

Cleaning of the reswelling tray The reswelling tray is thoroughly cleaned with detergent, using a toothbrush or Q-tips for the slots. After rinsing it with deionized water it must be completely dry before the next use.

3.2 Rehydration Loading and IEF in IPGphor Strip Holders

The IPGphor chamber must be horizontally leveled on the bench. Rehydration and IEF separation are carried out at 20 °C.

It is highly recommended to connect the IPGphor to an external computer via the serial port. In this way the electrical conditions can be monitored. This allows one to judge from the shape of the graphs, whether the separation will give good or bad 2-D results.

See Section 1.5.3.4 on page 91 f for further explanations.

- Be sure that the strip holders are carefully cleaned and dried.
- Place strip holders on the cooled electrode contact areas of the power supply with the pointed end on the anodal contact area.
- Pipette rehydration solution mixed with sample into the strip holder as a streak from electrode contact to electrode contact (see Figure 3.2).
- Remove the cover film from the IPG-strip starting at the acidic (+) end.
- Starting at the anodal side, place the IPG strip with the acidic end into the strip holder – dried gel side down. Slowly lower the basic end into the strip holder.

Never use new strip holders without cleaning them before the first run.

Starting at the basic end might damage the – usually softer – basic gel surface.

The distance from the platinum contact to the end of the tray is shorter at the anodal side.

Should an air bubble be caught, lift the strip up with forceps and slowly lower it down again. You might need to repeat this procedure.

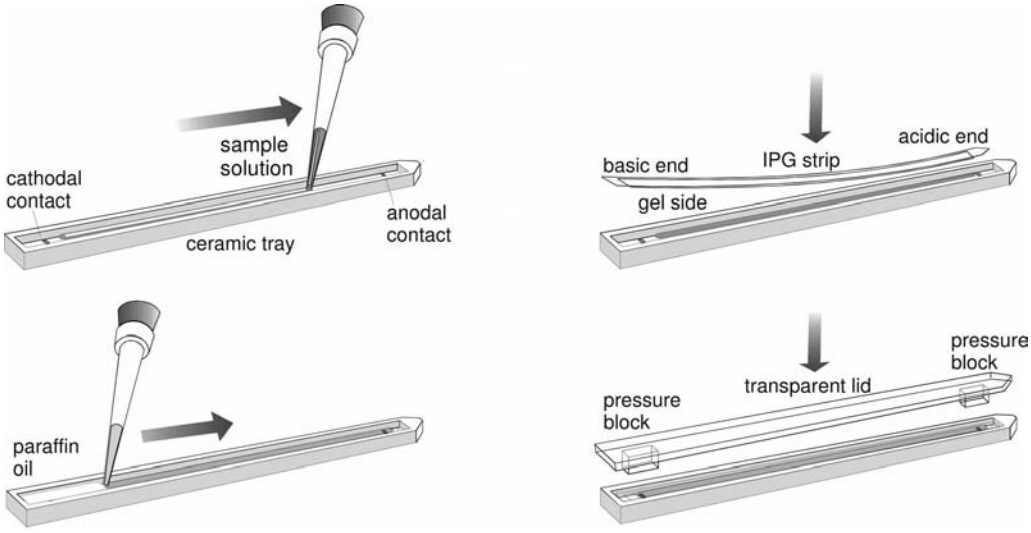


Fig. 3.2: Rehydration loading of the sample solution in the strip holder of the IPGphor.

When some of the sample flows onto the back of the film, it will be pushed down around the edge of the strip when the paraffin oil is pipetted on it.

The cover fluid prevents urea crystallization and oxygen contact.

- Pipette 2 mL paraffin oil over the strip by starting on both ends of the strip, moving to the center.
- Place the plastic cover on the strip holder.
- Close the safety lid.
- Enter the running conditions (see Table 3.7).

Tab. 3.7: "Worst case" conditions for active rehydration loading in strip holders.

Rehydration time		0 h			
Temperature		20 °C			
Current per strip		50 µA			
Strip length		18 cm		24 cm	
pH gradient		3–10 L	3–10 NL	3–10 L	3–10 NL
Step 1	60 V	12 h step and hold		12 h step and hold	
Step 2	150 V	3 h step and hold		3 h step and hold	
Step 3	300 V	3 h step and hold		3 h step and hold	
Step 4	1,000 V	6 h gradient		6 h gradient	
Step 5, analytical	10,000 V	20 kVh 2 h 20 min gradient	20 kVh 2 h 20 min gradient	40 kVh 4 h gradient	40 kVh 4 h gradient
Total time		16 h 20 min	16 h 20 min	18 h	18 h

When a new sample type has to be analyzed or the 2-D patterns are not optimal, it is recommended to apply the following "worst conditions". Generally, active rehydration provides the best results. For active rehydration the "rehydration time" in the program is set to "0", it is performed as a voltage step.

Passive rehydration (with no electric field applied during rehydration) can be performed in the reswelling tray.

For preparative sample loads (>1 mg) increase the final focusing step by 15% of the proposed volt hours.

When an overnight run is finished early in the morning, refocus the proteins by applying 8,000 V on the strips for 15 minutes before equilibration, staining, or storage of the strips.

Observations during the run In each IPG strip the Bromophenol blue tracking dye slowly starts to build a band at the cathodal end, which migrates towards the anode. When all samples have the same salt content, they run almost at the same level. There is no reason to worry, when the Bromophenol blue bands of a few samples migrate slower, this can always happen.

During the final few hours you will observe a strong black, sometimes also an additional yellow band at the anode: this is Bromophenol blue collecting here, and at the very end it becomes very acidic, turning the Bromophenol blue to yellow.

- Before the run is finished: place a tray with detergent solution next to the instrument for the strip holders.

Cleaning of the strip holders The strip holders must be carefully cleaned after each IEF. The solutions must never dry in the strip holder. Cleaning is very effective, if the strip holders are first soaked a few hours in a solution of 2–5% of the specially supplied (non-alkali) detergent in hot water.

The strip holder slot should be vigorously brushed with a toothbrush using a few drops of undiluted IPGphor Strip Holder Cleaning Solution. Then it is rinsed with deionized water.

Clean strip holders should be handled with gloves to avoid contamination.

Sometimes protein deposits are left on the bottom of the strip holder after IEF. This happens when highly abundant proteins have been squeezed out of the gel surface at their isoelectric point (see Figure 1.32 on page 95). It is not always easy to remove these proteins, particularly when they are sticky like serum albumin. In this case the strip holders should be boiled in 1% (w/w) SDS with 1% (w/v) DTT for 30 minutes before the slot is cleaned with the toothbrush.

SDS solution in absence of buffer has a neutral pH.

Important: ***Strip holders may be baked, boiled or autoclaved. But, because of the specially treated surface they must not be exposed to strong acids or basis, including alkaline detergents.***

Note: ***The strip holder must be completely dry before use.***

3.3 IEF of Rehydration Loaded Strips in the Manifold

The IPGphor chamber must be horizontally leveled on the bench. IEF separation is carried out at 20 °C.

See Section 1.5.3.4 on page 91 f for further explanations.

It is highly recommended to connect the IPGphor to an external computer via the serial port. In this way the electrical conditions can be monitored. This allows one to judge from the shape of the graphs, whether the separation will give good or bad 2-D results.

- Be sure that the manifold is carefully cleaned and dried.
- Place manifold on the cooled electrode contact areas of the power supply.

Aligner protrusions along the grooves inside the manifold align the rehydrated IPG strips, keeping them straight and centered when placed inside the manifold.

- Starting at the basic side, place the IPG strip – gel side facing up – into the manifold with the acidic end towards the anode side (see Figure 3.3).
- Soak electrode pads with deionized water. Blot them on filter paper and place them on top of the ends of the strip. The pads should sit completely on the gel surface. If longer strips are required for removal of salt, there must be an overlapping of at least 5 mm.

I there are no ready-cut electrode pads available, cut 5 mm long pads from IEF electrode strips. The pads must be damp, not wet.

- Pour 100 mL Drystrip cover fluid (paraffin oil) over the strips and electrode pads.

The cover fluid prevents urea crystallization and oxygen contact.

- The electrode assembly has electrode teeth on one side and hold-down teeth (for paper bridge-loading) on the other side. It is important to choose the correct orientation, to get contact with the electrode pads.
- Place the electrode assemblies on the pads. Secure them in place with the cams.
- Close the safety lid.
- Enter the running conditions (see Table 3.8).

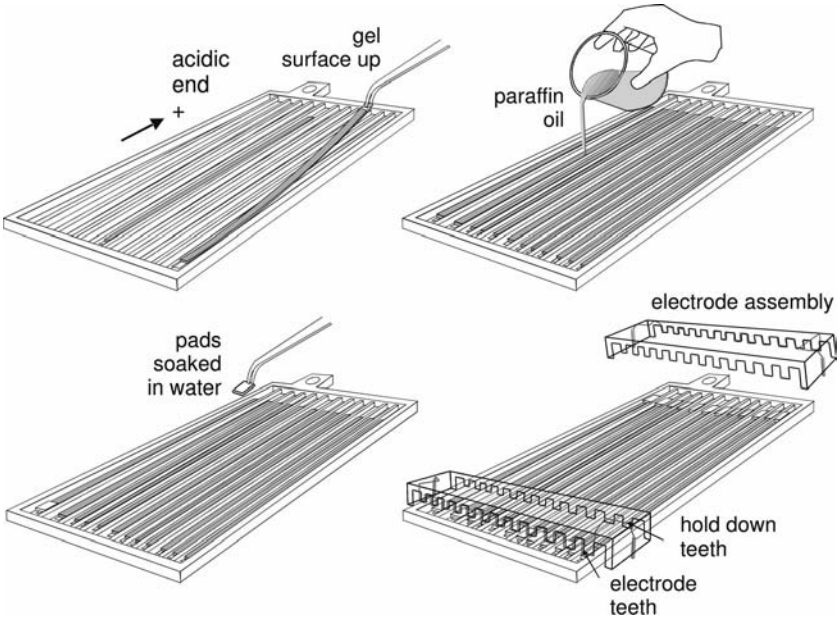


Fig. 3.3: Using the manifold for running rehydration-loaded IPG strips.

Tab. 3.8: "Worst case" conditions for the manifold (cup, paper bridge, rehydration loading).

Rehydration time		0 h			
Temperature		20 °C			
Current per strip		up to 100 µA			
Strip length		18 cm		24 cm	
pH gradient		3–10 L	3–10 NL	3–10 L	3–10 NL
Step 1 Step and hold	150 V	3 h		3 h	
Step 2 Step and hold	300 V	3 h		3 h	
Step 3 Gradient	1,000 V	6 h		6 h	
Step 4 Gradient	10,000 V	1 h		1 h	
Step 5 Step and hold	10,000 V	16 kVh for 2 h	16 kVh for 2 h	24 kVh for 3 h	24 kVh for 3 h
Total time		14 h	14 h	15 h	15 h

For preparative sample loads (>1 mg) increase the final focusing step by 15% of the proposed volt hours.

When an overnight run is finished early in the morning, refocus the proteins by applying 10,000 V on the strips for 15 minutes before equilibration, staining, or storage of the strips.

3.4
Cup Loading IEF

See Section 1.5.3.4 on page 91 f for further explanations.

It is highly recommended to connect the IPGphor to an external computer via the serial port. In this way the electrical conditions can be monitored. This allows you to judge from the shape of the graphs as to whether the separation will give good or bad 2-D results.

For the course: use analytical protein load here.

The IPGphor chamber must be horizontally leveled on the bench. IEF separation is carried out at 20 °C.

Figure 3.4 shows all the parts needed and the proper placements of strips, pads, sample cups, and electrodes.

Never use new manifold without cleaning them before the first run.

- Be sure that the manifold is carefully cleaned and dried.
- Place the manifold on the cooled electrode contact areas of the power supply.

Aligner protrusions along the grooves inside the manifold align the rehydrated IPG strips, keeping them straight and centered when placed inside the manifold.

- Starting at the basic side, place the IPG strip – gel side facing up – into the strip holder with the acidic end towards the anode side. Be sure that the protruding film at the basic end touches the end of the groove.

Mostly anodal sample application is employed.

- Apply the loading cups at correct side of the strip. Press them down to prevent leakage.

Note: ***The cup can straddle on the alignment protrusions, if necessary.***

If there are no ready-cut electrode pads available, cut 5 mm long pads from IEF electrode strips. The pads must be damp, not wet.

- Soak electrode pads with deionized water. Blot them on filter paper and place them on top of the ends of the strip. The pads should sit completely on the gel surface. If longer pads are required for removal of salt, there must be an overlapping of at least 5 mm.

The cover fluid prevents urea crystallization and oxygen contact.

- Pour 100 mL Drystrip cover fluid (paraffin oil) over the strips, around the cups. With this measure leakages are detected, because oil would flow into a cup.

In higher concentrated samples more proteins would tend to aggregate and precipitate.

- Dilute samples with rehydration solution to 50–100 µL for optimum protein entry.
- Pipette samples into the cups.
- Pipette 20 µL paraffin oil on each sample.
- The electrode assembly has electrode teeth on one side and hold-down teeth (for paper bridge-loading) on the other side. It is important to

choose the correct orientation, to get contact with the electrode pads.
- Place the electrode assemblies on the pads. Secure them on place with the cams.
- Close the safety lid.
- Enter the running conditions (see Table 3.8).

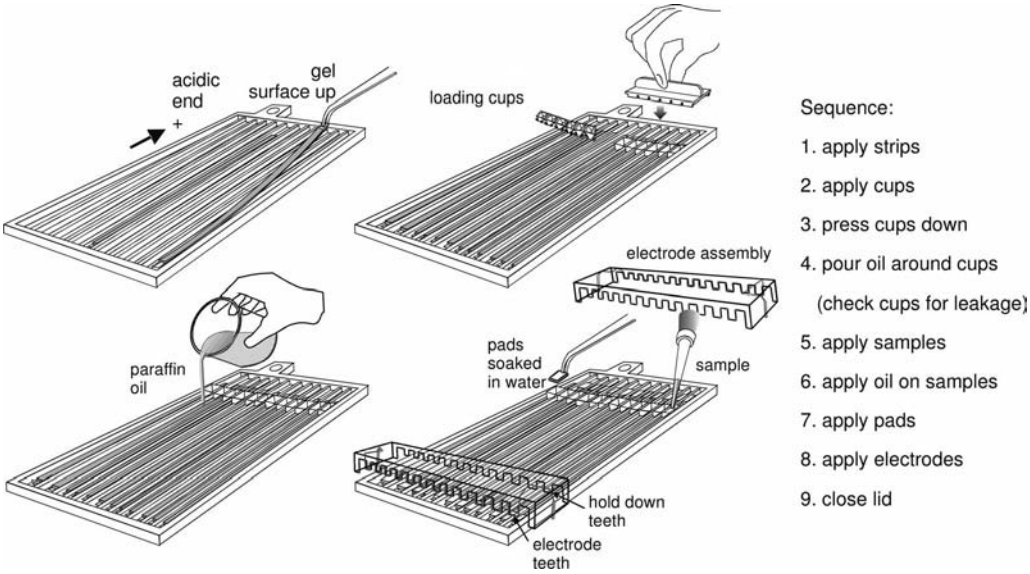


Fig. 3.4: Using the manifold for cup loading.

3.5 Paper Bridge Loading

There are pre-cut paper bridges on the market. If there are no ready-cut bridges available, cut 5 cm long and 0.8 mm wide pieces from very clean filter card board material. They should have pointed 3 mm wide ends as shown in Figure 3.5. The electrode assembly has electrode teeth on one side and hold-down teeth for paper bridge loading on the other side. Therefore the length of the paper bridge is important.

The procedure works in principle as described for rehydration loaded IPG strips. The only difference: the paper bridges, soaked in 500 µL sample solution are placed between IPG strip end and the electrode pad, as shown in Figure 3.5. If this would give a better separation, the paper bridge can also be placed at the cathodal side. As described in the paper by Kane *et al.* (2006) the paper bridge can be sequentially applied on different narrow pH gradients.

Kane LA, Yung CK, Agnetti G, Neverova I, Van Eyk JE. Proteomics 6 (2006) 5683–5687.

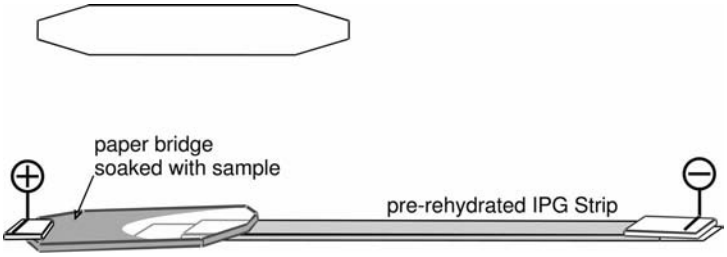


Fig. 3.5: Schematic drawing of the shape of a paper bridge and paper bridge loading for the example of anodal sample application. Note: The paper bridge is not shown to scale! Use the dimensions given in the text.

For running conditions choose the settings in Table 3.5.

Cleaning of the manifold is easier than the regular strip holder.

Cleaning of the manifold The manifold must be carefully cleaned after each IEF. The solutions must never dry in the manifold. Cleaning is very effective, if the manifold is first soaked for a few hours in a solution of 2–5% of the specially supplied detergent in hot water.

The clean manifold should be handled with gloves to avoid contamination.

The manifold grooves should be vigorously brushed with a toothbrush using a few drops of undiluted IPGphor Strip Holder Cleaning Solution. Then it is rinsed with deionized water.

Important: ***The manifold may be baked, boiled or autoclaved. But, because of the specially treated surface they must not be exposed to strong acids or basis, including alkaline detergents.***

Note: ***The manifold must be completely dry before use.***

The IPG strips are directly transferred to equilibration in SDS buffer for the second dimension run or stored in a deep freezer at –60 °C to –80 °C.

See Section 1.5.3.4 on page 91 f for further explanations.

When the IPGphor is connected to an external computer via the serial port, the electrical conditions are monitored. The shapes of the graphs indicate whether the separation will give good or bad 2-D results.

Alternatively some of the strips can be stained for inspection as to whether the IEF step was successful.

3.6
Staining of IPG Strips

3.6.1
Acid Violet 17 Staining of IPG Strips

3% (v/v) phosphoric acid	21 mL	85% H_3PO_4	Make up to 1 L with H_2O_{deion}
11% (v/v) phosphoric acid	76.1 mL	85% H_3PO_4	Make up to 1 L with H_2O_{deion}
1% (w/v) stock dye solution	1 g	Acid violet 17	In 100 mL H_2O_{deion} Heat to 50 °C on magnetic stirrer
0.1% (w/v) staining solution	1 mL	1% (w/v) stock solution	Plus 9 mL of 11% phosphoric acid

Staining procedure

- Fix for 20 minutes in 20% (w/v) TCA.
- Wash for 1 minutes in 3% (v/v) phosphoric acid.
- Stain for 10 minutes in 0.1% (w/v) Acid violet 17 staining solution.
- Destain with 3% (v/v) phosphoric acid until background is clear.
- Wash three times (1 minute each time) with H_2O_{deion}.
- Impregnate with 5% (v/v) glycerol, air dry.

Note: ***This staining is not reversible!***

Step 4: SDS Polyacrylamide Gel Electrophoresis

This chapter describes casting and running of large vertical gels. Small vertical gels and horizontal flatbed gels on film supports are described in detail in "*Electrophoresis in Practice*".

Westermeier R. Electrophoresis in Practice. Fourth Edition. WILEY-VCH, Weinheim (2004).

Wear disposable gloves during the entire procedure.

4.1 Casting of SDS Polyacrylamide Gels

Laboratory-made gels are cast in glass cassettes using relatively simply designed equipment. Casting gels on support films requires more sophisticated equipment. Film-backed gels are therefore only prepared in industrial scale. The preparation of gels with two different gel casters is described, and how to cast gradient gels without a pump. The following protocols are limited to the preparation of 1 mm thick gels, for 1.5 mm gels one-third more monomer volume is used; there is space for a lower number of cassettes in the casters.

1 mm gels are anyhow advantageous for downstream analysis with mass spectrometry, because the yield for the tryptic digestion is higher.

The large cassettes are built from 3 mm thin glass plates. It is important to prepare such gels in multicasters, because this prevents the cassettes from bulging, which would lead to uneven gel thickness. However, as the polymerization process is an exothermal reaction, care must be taken to prevent overheating in multicasters.

Casting single gels requires a support by thick glass or plastic plates.

4.1.1 Stock Solutions

Use a ready-made acrylamide, Bis stock solution containing 40% *T*, 3% *C*.

Much safer than weighing acrylamide and Bis powder.

Or prepare:

Acrylamide, Bis solution (40% *T*, 3% *C*):

40% Acrylamide PAGE solution	40% *T*	1 L
Bis	3% *C*	12 g
Mix thoroughly in the supplied bottle		

Acrylamide powder could also be used, but is not recommended because of health hazards during weighing.

Store in the refrigerator.

Caution! ***Acrylamide and Bis are toxic in the monomer form. Avoid skin contact and dispose of the remains ecologically. Polymerize the remains with an excess of ammonium persulfate.***

1.5 mol/L gel buffer Tris-Cl pH 8.8 (4× concentrated):

Tris base	1.5 mol/L	181.8 g
SDS	0.4% (w/v)	4 g
Water, deionized	dissolve	800 mL
4 mol/L HCl	titrate to	pH 8.8
Water, deionized	make up to	1 L

Store in the refrigerator.

A 10% solution is mixing more homogeneously with the monomer solution than a 40% solution.

Ammonium persulfate solution (APS):

Ammonium persulfate	10% (w/v)	1 g
Water, deionized	Dissolve	10 mL

Prepare fresh.

TEMED is used undiluted as 100%, because it is added to the monomer solution at the beginning.

1% Bromophenol blue does not go completely into solution without Tris

1 % (w/v) Bromophenol blue solution:

Bromophenol blue	1% (w/v)	100 mg
Tris base		60 mg
Water, deionized	dissolve	10 mL

Displacing solution:

Tris-Cl (1.5 mol/L, pH 8.8), SDS	0.375 mol/L	60 mL
85%Glycerol (v/v)	50% (v/v)	142 mL
1% Bromophenol blue (w/v))	0.002%	480 µL
Water, deionized	Make up to	240 mL

Prepare fresh.

Overlay spraying solution:

SDS	0.1% (w/v)	1 g
Water, deionized	Dissolve	1 L

Gel storage solution:

Tris-Cl (1.5 mol/L, pH 8.8), SDS	0.375 mol/L	500 mL
Water, deionized	Make up to	2 L

Store in the refrigerator.

4.1.2 Cassettes for Laboratory-made Gels

It is very important to have absolutely clean glass plates and cassettes at hand. Therefore the tips for cleaning the glass plates at the end of this chapter are very important. To be on the safe side and to remove any dust particles, clean glass plates again before use with distilled water and lint-free tissue.

Inspect the cassettes carefully for any dirt or dust particles.

Hinged cassettes The standard cassettes are hinged on one side for easy handling. Note that one of the class plates are offset to make application of the IPG strip easier.

Separate and low fluorescence glass plates For spot picking, the gels need to be covalently bound to one glass plate. In this case hinged cassettes are not useful, and separate glass plates are used – one with spacers and one without.

For pre-labeling fluorescence detection methods like DIGE, special borosilicate glass plates are needed, which are usually not hinged.

Treating glass plates with Bind-Silane The gel will be bound to the glass plate without spacers; this one has to be treated with Bind-Silane.

Note: ***It is important that glass plates are properly clean.***

If Decon 90 is not available, just use 5% (w/v) potassium hydroxide.

Before re-use, the plates are placed in a 5% (v/v) Decon™ 90 solution overnight. Do not leave plates standing in this solution for a longer time, because this will cause etching due to the alkali nature of Decon.

- Thoroughly wash the plate to be treated. Any gel fragments from previous gels must be removed.

The careful cleaning of the glass plates before casting is important, to ensure a uniform coating with the Bind-Silane and to avoid keratin contamination.

- Thoroughly rinse the plates with deionized water to remove Decon.
- Dry the plate using a lint-free tissue or leave them to air dry.

The IR markers must also be coated with Bind-Silane.

- Apply two internal reference (IR) markers (self-adhesive, fluorescent) onto the middle of the glass plate, each approximately 3.5 cm away from the lateral edges.

Bind-Silane working solution:

Ethanol	8 mL
Acetic acid	200 µL
Bind-Silane	10 µL
Water, deionized	1.8 mL

- Pipette 4 mL of the Bind-Silane solution onto the plate and distribute equally over the plate with a lint-free tissue. Cover the plate to prevent dust contamination and leave to air dry on the bench for 1 hour.

Note that a too thick layer of Bind-Silane can create a high background in fluorescent detection.

- Polish the plate with a lint-free tissue, moistened with a small amount of deionized water or ethanol.
- The gels will stay attached to the glass during electrophoresis, staining procedures, scanning, spot picking, and storage.

This is not necessary for DIGE runs, when IPG strips with printed numbers are used: the strip is scanned together with the SDS gel in the cassette.

Prepare the cassettes with gel labels cut from printed paper or overhead film placed at the corner on the opposite of the filling side (see Figure 4.1).

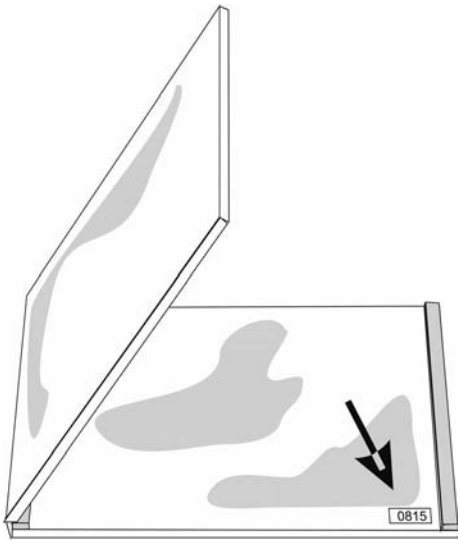


Fig. 4.1: Hinged cassette with gel label made from paper or overhead film (the label is not needed when IPG strips with printed numbers and the DIGE method are employed).

4.1.3 Multiple Gel Caster (Up to 14 Gels)

4.1.3.1 Preparation of the Gel Caster

The gel caster should be set up in a tray, just in case any liquid overflows. Never forget to place the plastic separator sheets between the cassettes; they would firmly stick to each other after gel polymerization. When less than 14 gels are being prepared, block the non-used space with blank cassettes, placing separator sheets between each plate.

Be sure that gel caster, separator sheets, and cassettes are perfectly clean and dry.

Dirty cassettes cause vertical streaking in silver-stained gels.

- Place the gel caster in a tray. Tip it back, so it rests on the support legs.

According to the instruction manual the hinged cassettes should be placed into the caster with the opening edges to the filling side, some proteomics teams have experienced to get a straighter gel surface, when they are inserted with the hinged edge to the filling side.

For non-hinged cassettes the orientation does not matter.

- Start with a separator sheet.
- Assemble non-hinged cassettes outside of the caster before you place them into the caster box.

- Place the cassettes alternating with separator sheets into the caster as shown in Figure 4.2, with the offset edges of the cassettes up.

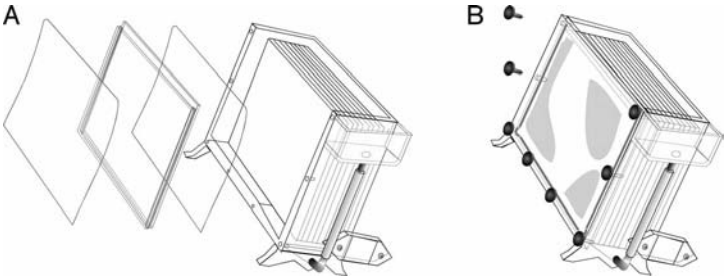


Fig. 4.2: A. Placing cassettes and separator sheets into the caster. Here the hinged edges are oriented towards the filling side. B. Closing the gel caster.

- Press strongly on the spacers areas on both sides to ensure that the spacers and the glass plates stick well together.

Finally a separator sheet and a filling plate are inserted to complete the stack up to the edge of the caster. Do not overfill it. This would cause pressure on the gels during polymerization, when the solution starts to get warm.

Always take the foam gasket out of the front plate after gel casting.

- Place the foam gasket into the groove of the front plate. Do not cover it with grease.
- Turn the first four screws into the bottom holes and place the front plate on the caster with the bottom slots on the screws. Apply the rest of the screws and tighten them evenly. Do not use too much force; the sealing gasket should be compressed evenly to prevent leakage.
- Tip the caster to the front.
- Level the gel caster horizontally.
- Prepare 120 mL of displacing solution.

When the room temperature is higher than 20 °C, the caster should – like the monomer solution(s) – be pre-cooled in a cold room or a refrigerator.

4.1.3.2 **Homogeneous Gels**

Very good results are usually obtained with homogeneous gels (e.g. monomer solutions). Gradient gels need to be prepared in special cases.

For monomer solutions the ammonium persulfate should not be added before everything is in place, because there are only about 10 minutes left to pour the gel until it starts to gel.

Monomer solutions with selected % *T*, and 3% *C* for 14 gels with 1 mm thickness, 900 mL:

	7.5% *T*	**10% *T***	**12.5% *T***	**15% *T***
Acrylamide, Bis solution (40% *T*, 3% *C*)	169 mL	225 mL	281 mL	338 mL
1.5 mol/L Tris-Cl, pH 8.8, 0.4% SDS	225 mL	225 mL	225 mL	225 mL
TEMED (100%)	450 µL	450 µL	450 µL	450 µL
Water, deionized, make up to	900 mL	900 mL	900 mL	900 mL
Mix and degas with a water jet pump				
10% ammonium persulfate	3.6 mL	3.6 mL	3.6 mL	3.6 mL

Add ammonium persulfate shortly before gel casting.

It is important to use only high-quality TEMED and ammonium persulfate.

The amounts of TEMED and ammonium persulfate are based on the author's experience. In order to get reproducible gels with a straight edge, the concentration of TEMED is higher and the concentration of ammonium persulfate is lower than in the instrument instruction. Furthermore, at basic pH, ammonium persulfate can react with the Tris; this effect is minimized by adding more TEMED and reducing the ammonium persulfate content.

- Degas and precool the monomer solution.
- Add the ammonium persulfate to the monomer solution and mix.
- Pour the gel solution directly into the balance chamber, avoiding air bubbles (see Figure 4.3A).
- Stop pouring when the level reaches 3 cm below the upper edges of the casting cassettes.
- Pour the dense displacing solution to fill the V-chamber and the sloped bottom of the caster. The gel solution level will rise to 1 cm below the cassette edges. A thin blue layer should be visible at the bottom of the cassettes.

And it prevents a too strong contraction of the gel during polymerization, which can cause a gap between gel and spacer on the side of the gels where the solution is introduced from the bottom.

Should the volume of the displacing solution in the balance chamber not be sufficient to produce the thin blue layer in the entire area of the caster bottom, top more displacing solution into the balance chamber until the blue layer has distributed over the entire bottom area. This saves you from cutting off protruding gel pieces at the bottom edge of each cassette.

Spraying with SDS solution is better than pipetting water-saturated butanol on the edge.

- Immediately spray 0.1% SDS solution overlay solution over the cassettes (see Figure 4.3C).
- Cover the caster with a cling film.

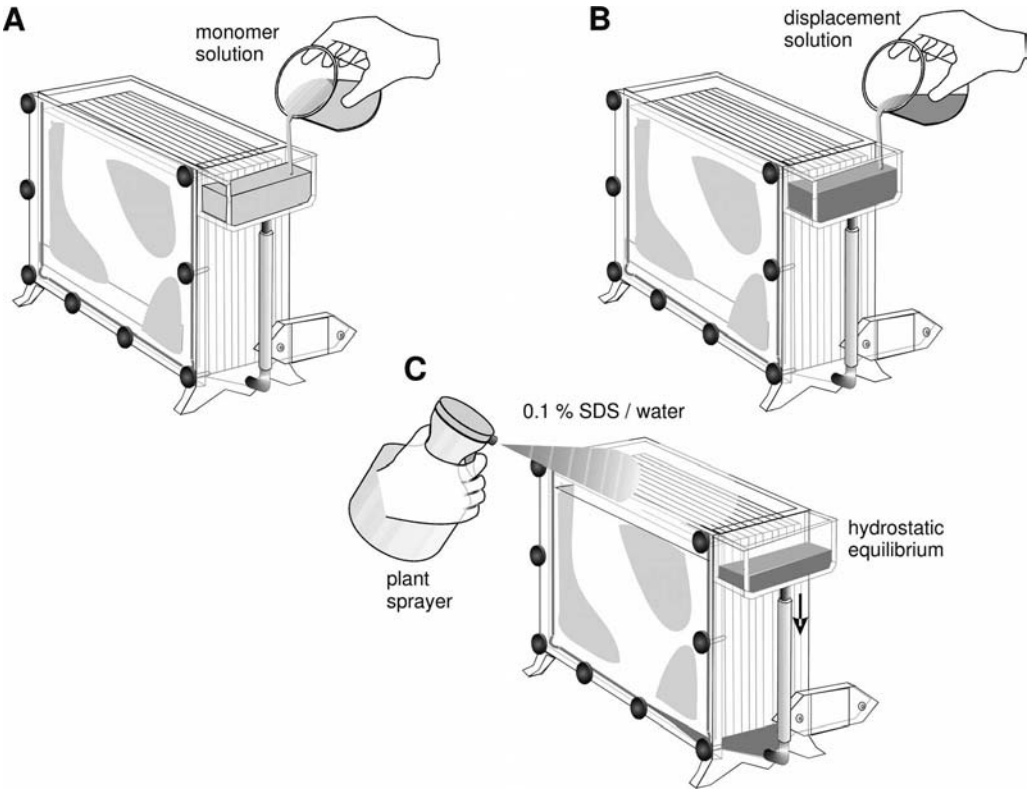


Fig. 4.3: Casting homogeneous gels in a 14-gel caster.
A. Pouring the monomer solution into the balance chamber.
B. Pouring the displacement solution into the balance chamber.
C. Overlaying the gel solution by spraying 0.1% SDS solution.

About 50 mL monomer solution should be left over. Let it *polymerize* in a small beaker before you discard it.

Because the silent polymerization must be completed.

- Let the gel *polymerize* overnight at room temperature.
- During unloading rinse the cassettes to remove excess polyacrylamide.

Note: ***When enough displacing solution had been used, there is no need to cut off protruding gel pieces at the bottom edge of each cassette.***

- Inspect each gel cassette for eventual air bubbles. Gels with air bubbles should not be used.
- Store the usable gels in an airtight container with about 100 mL gel storage solution. When

the gels are not used during the next day, place the container with the gels into a cold room or a refrigerator.

- Take the foam gasket out of the front plate and rinse it with deionized water.
- Rinse the gel caster and the separator sheets with mild detergent and then with deionized water.
- Let them dry in the air.

Do not leave it in the groove, because it would lose its sealing property.

4.1.3.3 Gradient Gels

Gradient gels are more complicated to prepare than homogeneous gels. When homogeneous gels are run for a short time only, they achieve comparable resolution. However, for some samples, gradient gels may show a better spot distribution. Most instructions describe how to cast gradient gels with a peristaltic pump. The author has experienced that very reproducible gels are achieved without a pump, which safes a lot of work with calibrating, setting up and cleaning. Instead of a pump, gravitation is used; the flow rate is controled by the level of the laboratory platform carrying the gradient maker and with a pinchcock clamp on the tubing. A pinchcock clamp can close the tubing completely or reduce the opening of the tubing in several steps, thus reducing the flow rate.

Pinchcock clamps are available in different sizes.

Gradient maker set up

- Place the clean and dry gradient maker with a magnetic stirrer on an adjustable laboratory platform or on a shelf board: the level must be at least 10 cm above the edge of the caster.

Note: ***For reproducible gels, use always exactly the same level for the gradient maker position. Use always the same shelf board or measure and note the position height of the gradient maker.***

- Insert the plastic tube into the hole in the hydrostatic balance chamber.
- Place a pinchcock clamp on the flexible connection tubing, and close it completely.
- Connect the plastic tube with the flexible tubing to the gradient maker.
- Fill the hydrostatic balance chamber with 100 mL of the displacing solution.
- Place a magnetic stirring bar – 2 cm to 3 cm long – into the mixing chamber.

The pinchcock clamp is closed as well.

- Close the connecting valve between the reservoir and mixing chamber.

Monomer solutions for gradient gels

- The dense solution contains 20% glycerol to stabilize the gradient.
- The ammonium persulfate should not be added before everything is in place, because there are only about 10 minutes left to pour the gel until it starts to gel.

Monomer solutions with selected % *T* and 3% *C* for 14 gradient gels with 1 mm thickness:

Light solution, 450 mL:

	8% *T*	**10% *T***	**12% *T***	**..... % *T***
Acrylamide, Bis solution (40% *T*, 3% *C*)	90 mL	113 mL	135 mL	..... mL
1.5 mol/L Tris-Cl, pH 8.8, 0.4% SDS	113 mL	113 mL	113 mL	113 mL
TEMED (100%)	225 µL	225 µL	225 µL	225 µL
Water, deionized, make up to	450 mL	450 mL	450 mL	450 mL
Mix and degas with a water jet pump				
10% ammonium persulfate	1.8 mL	1.8 mL	1.8 mL	1.8 mL

Add ammonium persulfate short before gel casting.

Dense solution, 450 mL:

	14 % *T*	**16% *T***	**20% *T***	**..... % *T***
Acrylamide, Bis solution (40%*T*, 3%*C*)	158 mL	180 mL	225 mL	..... mL
1.5 mol/L Tris-Cl, pH 8.8, 0.4% SDS	113 mL	113mL	113 mL	113 mL
Glycerol (85%)	110 mL	110 mL	110 mL	110 mL
TEMED (100%)	225 µL	225 µL	225 µL	225 µL
Water, deionized, make up to	450 mL	450 mL	450 mL	450 mL
Mix and degas with a water jet pump				
10% ammonium persulfate	1.4 mL	1.4 mL	1.4 mL	1.4 mL

Add ammonium persulfate short before gel casting. Note that less APS is added to the dense solution!

For comments on amounts of TEMED and ammonium persulfate see above on page 329.

Control of gel polymerization for gradient gels The start of the polymerization is controled by the amount of ammonium persulfate. The catalyst amount must not be too high in order to avoid overheating in the caster. The dense solution must contain less catalyst, because the polymerization should start at the top and proceed slowly down to the bottom. This is very important for two reasons:

The polymerization is easier controled with varying the amount of APS than with TEMED.

- Thermal convection is prevented, which would distort the gradient.
- During polymerization the gel contracts. If the lower portion would *polymerize* first, it would pull the upper solutions down, resulting in a curved upper edge.

When conditions are correct, the gels pull the displacing solution down.

Figure 4.4A shows the setup for casting gradient gels in a 14-gel caster.

- Degas and precool the monomer solutions.
- Add the ammonium persulfate to the monomer solutions and mix.
- Pour the dense solution into the reservoir.
- Carefully open the slide valve and let just enough solution flow to fill the connector channel. Close the valve. If too much fluid has flown into the mixing chamber, pipette it back to the reservoir.

Check for air bubbles.

- Pour the light solution into the mixing chamber.
- Start the magnetic stirrer.

Do not mix too fast; air bubbles must be avoided.

- Open the pinchcock clamp to the next setting, the one next to close the tubing completely. The light solution should start to flow down the tubing.
- Open the sliding valve of the gradient maker. Now dense solution will flow into the mixing chamber and will be mixed with the light solution.

Adjust the flow rate to a higher speed with the pinchcock clamp. But the speed must not be too high to avoid mixing of solutions in the V-shaped chamber at the bottom. Casting time should be around 10 minutes. If you estimate that it will take longer, open the pinchcock to the next position.

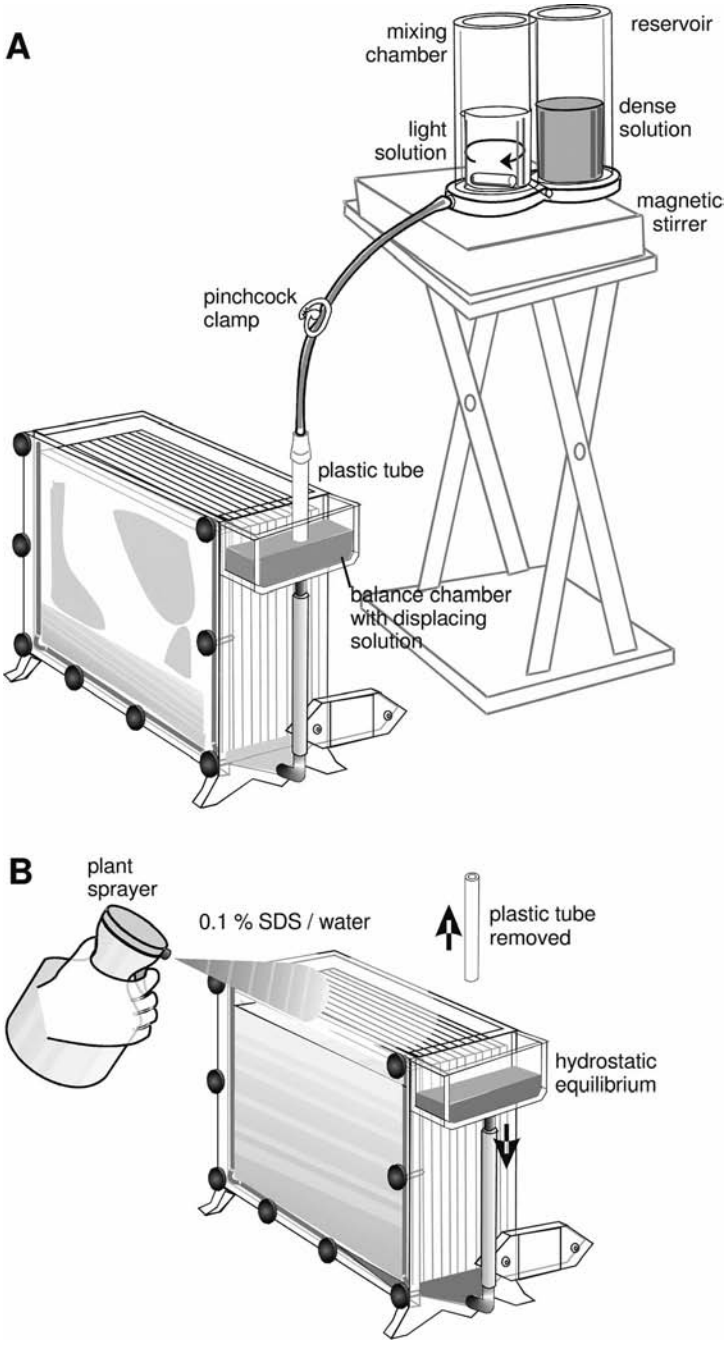


Fig. 4.4: A. Casting gradient gels in a 14-gels caster. B. Removing the tube and overlaying the gel solution by spraying 0.1% SDS solution.

- Close the pinchcock clamp before the connection tubing runs empty, but not later than when the liquid level reaches 3 cm below the upper edges of the cassettes.
- Remove the plastic tube with the connection tubing from the balance chamber and rinse the magnetic stirrer.

If monomer solution is left, fill it in a measuring cylinder; next time, adjust the volumes of the monomers solutions to the appropriate amount. The gels will this time have a slightly flatter gradient slope than intended, but the separation quality will not suffer much.

The dense displacing solution will now flow down the connecting tube and fill the V-chamber and the sloped bottom of the caster. The gel solution level will rise to 1 cm below the cassette edges. A thin blue layer should be visible at the bottom of the cassettes.

Should the volume of the displacing solution in the balance chamber not be sufficient to produce the thin blue layer in the entire area of the caster bottom, top more displacing solution into the balance chamber until the blue layer has distributed over the entire bottom area. This saves you from cutting off protruding gel pieces at the bottom edge of each cassette.

- Immediately spray 0.1% SDS solution overlay solution over the cassettes (see Figure 4.4B).
- Cover the caster with a cling film.
- Let the gel *polymerize* overnight at room temperature.
- During unloading rinse the cassettes to remove excess polyacrylamide.

Spraying with SDS solution is better than pipetting water-saturated butanol on the edge.

Because the silent polymerization must be completed.

Note: ***When enough displacing solution had been used, there is no need to cut off protruding gel pieces at the bottom edge of each cassette.***

- Inspect each gel cassette for eventual air bubbles. Gels with air bubbles should not be used.
- Store the usable gels in an airtight container with about 100 mL gel storage solution. When the gels are not used during the next day, place the container with the gels into a cold room or a refrigerator.
- Take the foam gasket out of the front plate and rinse it with deionized water.
- Rinse the gel caster and the separator sheet with mild detergent and then with deionized water.
- Let them dry in the air.

Do not leave it in the groove, because it would lose its sealing property.

4.1.4 Gel Caster for up to Six Gels

4.1.4.1 Preparation of the Gel Caster

Large gel formats require a casting box, also when only a few gels are prepared. Otherwise the cassettes would bulge; the gels would be thicker in the center. Never forget to place the plastic separator sheets between the cassettes; they would firmly stick to each other after gel polymerization. When less than 6 gels should be prepared, do not block the non-used space with blank cassettes: cast six gels and discard the non-used gels, or store them for another experiment.

Dirty cassettes cause vertical streaking in silver-stained gels.

Be sure that gel caster, separator sheets and cassettes are perfectly clean and dry. The filling channel in the back wall of the caster has originally been designed for casting homogeneous gels. However it is much easier and faster to pour the solution directly onto the cassettes in the caster as shown in Figure 4.5. Gradient gels are poured through the filler port at the bottom of the front plate. For casting homogeneous gels, leave the V-shaped rubber insert in place; for gradient gels, remove it.

- Lay the caster flat on the bench for loading.
- Start loading with a separator sheet.
- Place the cassettes alternating with separator sheets into the caster, with the offset edges of the cassettes up.
- Assemble non-hinged cassettes outside of the caster before you place them into the caster box.
- Press strongly on the spacers areas on both sides to ensure that the spacers and the glass plates stick well together.

Finally a separator sheet and – if necessary – filling sheets are inserted to complete the stack up to the edge of the caster. Do not overfill it. This would cause pressure on the gels during polymerization, when the solution starts to get warm.

Always take the foam gasket out of the front plate after gel casting.

- Place the foam gasket into the groove of the front plate. Do not cover it with grease.
- Turn the two screws into the bottom holes and place the front plate on the caster with the bottom slots on the screws. Apply the six clamps and tighten the screws evenly. Do not use too much force; the sealing gasket should be compressed evenly to prevent leakage.
- For casting, place the gel caster upright in a tray, for the occasional case that liquid is overflowing.

When the room temperature is higher than 20 °C, the caster should – like the monomer solution(s) – be pre-cooled in a cold room or a refrigerator.

4.1.4.2 Homogeneous Gels

Very good results are usually obtained with homogeneous gels. Gradient gels need to be prepared only in special cases.

- Close the filler port at the bottom of the front plate with the cap or with a piece of tubing and a pinchcock clamp.
- For monomer solutions the ammonium persulfate should not be added before everything is in place, because there are only about 10 minutes left to pour the gel until it starts to gel.

Monomer solutions with selected % *T* and 3% *C* for six gels with 1 mm thickness, 450 mL:

	7.5% *T*	**10% *T***	**12.5% *T***	**15% *T***
Acrylamide, Bis solution (40% *T*, 3% *C*)	84 mL	113 mL	141 mL	169 mL
1.5 mol/L Tris-Cl, pH 8.8, 0.4% SDS	113 mL	113 mL	113 mL	113 mL
TEMED (100%)	225 µL	225 µL	225 µL	225 µL
Water, deionized, make up to	450 mL	450 mL	450 mL	450 mL
Mix and degas with a water jet pump				
10% ammonium persulfate	1.8 mL	1.8 mL	1.8 mL	1.8 mL

Add ammonium persulfate shortly before gel casting.

For comments on amounts of TEMED and ammonium persulfate see above on page 329.

- Degas and precool the monomer solution.
- Add the ammonium persulfate to the monomer solution and mix.
- Pour the gel solution directly into the box, avoiding air bubbles (see Figure 4.5A).
- Stop pouring when the level has reached 3 cm below the upper edges of the casting cassettes.
- Immediately spray 0.1% SDS solution overlay solution over the cassettes (see Figure 4.5B).
- Cover the caster with a cling film.
- Let the gel *polymerize* overnight at room temperature.
- During unloading rinse the cassettes to remove excess polyacrylamide.
- Inspect each gel cassette for eventual air bubbles. Gels with air bubbles should not be used.

Spraying with SDS solution is better than pipetting water-saturated butanol on the edge.

Because the silent polymerization must be completed.

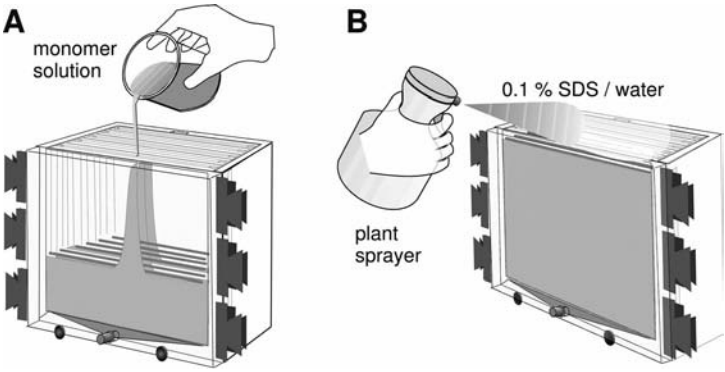


Fig. 4.5: A. Casting homogeneous gels in a six-gel caster.
B. Overlaying the gel solution by spraying 0.1% SDS solution.

- Store the usable gels in an airtight container with about 100 mL gel storage solution. When the gels are not used during the next day, place the container with the gels into a cold room or a refrigerator.

Do not leave it in the groove, because it would lose its sealing property.

- Take the foam gasket out of the front plate and rinse it with deionized water.
- Rinse the gel caster and the separator sheet with mild detergent and then with deionized water.
- Let them dry in the air.

Monomer solution, which has been left over, is *polymerize*d in a small beaker before it is discarded.

4.1.4.3 Gradient Gels

For comments on gradient gels see above on page 331.

For gradient gels the monomer solution has to be introduced through the filling port on the bottom of the front plate. In this case we need to fill the dead volume of the V-shaped chamber in the bottom of the caster with displacing solution.

- Remove the V-shaped rubber insert from the caster.
- Prepare 200 mL of displacing solution.

Gradient maker set up

- Place the clean and dry gradient maker with a magnetic stirrer on an adjustable laboratory platform or on a shelf board: the level must be at least 10 cm above the edge of the caster.

Note: ***For reproducible gels, use always exactly the same level for the gradient maker position. Use always the same shelf board or measure and note the position height of the gradient maker.***

- Place a pinchcock clamp on the flexible connection tubing, and close it completely.
- Connect the filling port with the flexible tubing to the gradient maker.
- Place a magnetic stirring bar – 2 cm to 3 cm long – into the mixing chamber.
- Close the connecting valve between the reservoir and mixing chamber.

The pinchcock clamp is closed as well.

Monomer solutions for gradient gels

- The dense solution contains 20% glycerol to stabilize the gradient.
- The ammonium persulfate should not be added before everything is in place, because there are only about 10 minutes left to pour the gel until it starts to gel.

Monomer solutions with selected % *T* and 3% *C* for 6 gradient gels with 1 mm thickness:

Light solution, 211 mL:

	8% *T*	**10% *T***	**12% *T***	**... % *T***
Acrylamide, Bis solution (40% *T*, 3% *C*)	42 mL	53 mL	63 mL	... mL
1.5 mol/L Tris-Cl, pH 8.8, 0.4% SDS	53 mL	53 mL	53 mL	53 mL
TEMED (100%)	105 µL	105µL	105 µL	105 µL
Water, deionized, make up to	210 mL	210 mL	210 mL	210 mL
Mix and degas with a water jet pump				
10% ammonium persulfate	840 µL	840 µL	840 µL	840 µL

Add ammonium persulfate shortly before gel casting.

Dense solution, 211 mL:

	14% *T*	**16% *T***	**20% *T***	**... % *T***
Acrylamide, Bis solution (40% *T*, 3% *C*)	74 mL	84 mL	105 mL	... mL
1.5 mol/L Tris-Cl, pH 8.8, 0.4% SDS	53 mL	53 mL	53 mL	53 mL
Glycerol (85%)	50 mL	50 mL	50 mL	50 mL
TEMED (100%)	105 µL	105 µL	105 µL	105 µL
Water, deionized, make up to	210 mL	210 mL	210 mL	210 mL
Mix and degas with a water jet pump				
10% ammonium persulfate	670 µL	670 µL	670 µL	670 µL

Add ammonium persulfate short before gel casting. Note that less APS is added to the dense solution!

For comments on amounts of TEMED and ammonium persulfate see above on page 329.

The polymerization is more easily controled with varying the amount of APS than with TEMED.

Control of gel polymerization for gradient gels The start of the polymerization is controled with the amount of ammonium persulfate. The catalyst amount must not be too high in order to avoid overheating in the caster. The dense solution must contain less catalyst, because the polymerization should start at the top and proceed slowly down to the bottom. This is very important for two reasons:

- Thermal convection is prevented, which would distort the gradient.
- During polymerization the gel contracts. If the lower portion would *polymerize* first, it would pull the upper solutions down, resulting in a curved upper edge.

When conditions are correct, the gels pull the displacing solution down.

Figure 4.6 shows the setup for casting a gradient gel in the caster for six gels.

- Degas and precool the monomer solutions.
- Add the ammonium persulfate to the monomer solutions and mix.
- Pour the dense solution into the reservoir.
- Carefully open the slide valve and let just enough solution flow to fill the connector channel. Close the valve. If too much fluid has flown into the mixing chamber, pipette it back to the reservoir.
- Pour the light solution into the mixing chamber.
- Start the magnetic stirrer.
- Open the pinchcock clamp to the next setting, the one next to close the tubing completely. The light solution should start to flow down the tubing.
- Open the sliding valve of the gradient maker. Now dense solution will flow into the mixing chamber and will be mixed with the light solution.

Check for air bubbles.

Do not mix too fast; air bubbles must be avoided.

Adjust the flow rate to a higher speed with the pinchcock clamp. But the speed must not be too high to avoid mixing of solutions in the V-shaped chamber at the bottom. Casting time should be around 10 minutes. If you estimate that it will take longer, open the pinchcock to the next position.

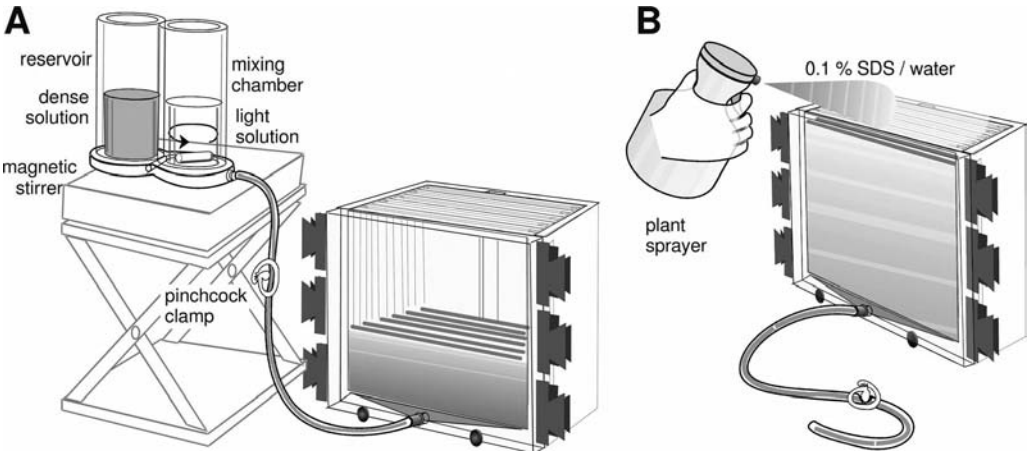


Fig. 4.6: A. Casting gradient gels in a 6-gels caster.
B. Overlaying the gel solution by spraying 0.1% SDS solution.

- Close the pinchcock clamp before the connection tubing runs empty.
- Close the sliding valve between the mixing chamber and the reservoir.
- Pour 200 mL displacing solution into the mixing chamber of the gradient maker.
- Open the pinchcock clamp to the same position like casting the gradient.
- When the monomer solutions have reached the desired level, which is 1 cm below the upper edges of the cassettes, close the pinchcock clamp.

The displacing solution – visible because of the added Bromophenol blue – should fill the V-shaped chamber completely. This saves you from cutting off protruding gel pieces at the bottom edge of each cassette.

- Immediately spray 0.1% SDS solution overlay solution over the cassettes (see Figure 4.6B).
- Cover the caster with a cling film.
- Let the gel *polymerize* overnight at room temperature.
- During unloading rinse the cassettes to remove excess polyacrylamide.
- Inspect each gel cassette for eventual air bubbles. Gels with air bubbles should not be used.

Spraying with SDS solution is better than pipetting water-saturated butanol on the edge.

Because the silent polymerization must be completed.

- Store the usable gels in an airtight container with about 100 mL gel storage solution. When the gels are not used during the next day, place the container with the gels into a cold room or a refrigerator.

Do not leave it in the groove, because it would lose its sealing property.

- Take the foam gasket out of the front plate and rinse it with deionized water.
- Rinse the gel caster and the separator sheet with mild detergent and then with deionized water.
- Let them dry in the air.

4.2
Inserting Ready-made Gels into Cassettes

Ready-made gels on a film support are supplied in airtight aluminum bags and need to be inserted into specially designed reusable cassettes. They are run in a vertical mode in the vertical systems for large gels.

Do not use more than 1 mL, because excess liquid between gel surface and glass plate has to be removed completely with a roller.

- Place the cassette for ready-made gels on the bench top with the hinge down, plastic frame to the left. Clean the inner side of the glass plate thoroughly with a 2% (w/v) SDS water solution, rinse it with water and dry it completely with a lint-free tissue paper. Pipette 1 ml deionized water onto the glass plate as a streak along the spacer of the closing side (see Figure 4.7).

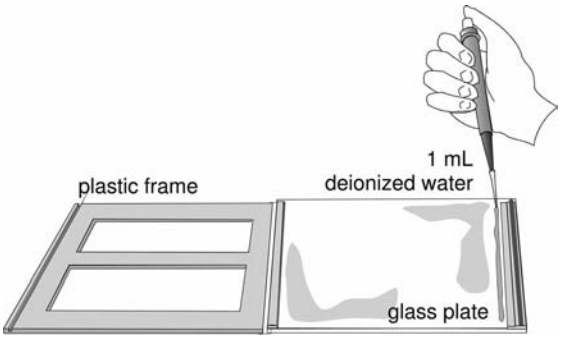


Fig. 4.7: Application of 1 mL deionized water on the glass plate along the spacer of the closing side.

- Open the gel package by cutting around the gel on three sides at about 1 cm from the edge to avoid cutting the gel or the support film. Remove the gel from the package.

The gel is cast onto a plastic support film and does not cover the film entirely. The support film protrudes approximately 15 mm beyond the top (cathodal) edge of the gel and approximately 5 mm at the lateral sides.

- Remove the protective plastic sheet from the gel. Handling the gel only by the side support film margins, hold it, gel-side down, over the glass plate. Align the right edge of the gel with the inner edge of the side spacer next to the opening side, flex the gel downward slightly and lower it slowly toward the glass plate from right to left (see Figure 4.8). Take care that the bottom edge of the gel is flush of the bottom edge of the glass plate. The protruding side support film margins (not the gel) should rest on top of the side spacers.

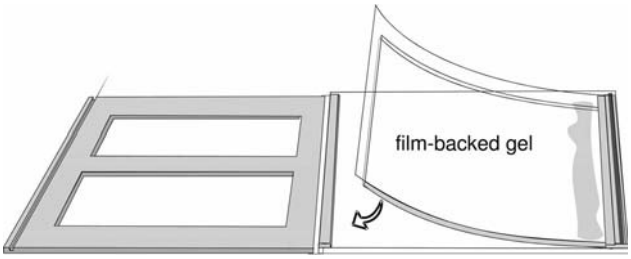


Fig. 4.8: Placing the film-backed gel into the cassette.

With this procedure almost no air bubbles are caught between glass plate and gel surface. The gel edge near to the opening side will take up a few microliters of gel buffer; this prevents eventual current leakage at the closing side, when gel edge should be slightly thinner than the cassette spacer.

- Press out any bubbles or liquid from between the gel and the glass with a roller (Figure 4.9). Press firmly against the plastic support film with the roller and roll over the entire gel.
- Snap the plastic frame to the glass plate and press the edges tightly together. Ensure that the cassette is closed completely: an incompletely closed cassette causes a strongly curved front because of current leakage.
- Keep them in the cassette rack upside down to prevent drying of the upper edge, to which the IPG strip will be applied.

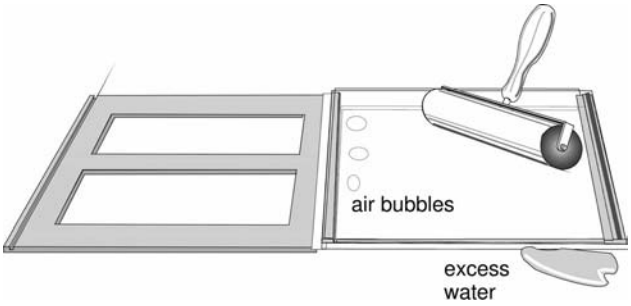


Fig. 4.9: Removing excess water and air bubbles with a roller.

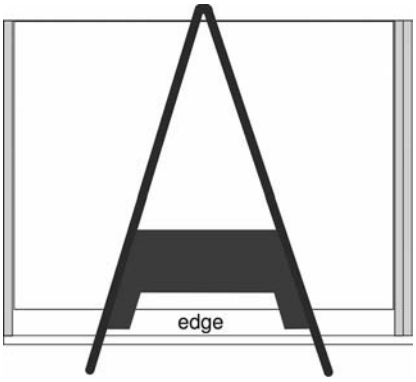


Fig. 4.10: The cassettes are placed into the rack upside down to prevent drying of the surface.

4.3
Preparation of SDS Electrophoresis Equipment

4.3.1
Stock Solutions for Running Buffers

The cathodal buffer is identical for laboratory-made gels containing Tris-Cl pH 8.8 and ready-made gels containing PPA-Cl pH 7.0.

Cathodal buffer (5× to 10× concentration) = "Laemmli buffer":

Tris base	0.25 mol/L	30.4 g
Glycine	1.92 mol/L	144.0 g
SDS	1% (w/v)	10 g
Water, deionized	Make up to	1L

Do not titrate the Tris-glycine buffer!

Store at room temperature.

Note: ***When the instruments are loaded with more than half of the possible gel number, use 2× cathodal buffer instead of 1× buffer to prevent buffer depletion.***

1× cathodal buffer is usually used as anodal buffer for Tris-Cl pH 8.8 gels. When care is taken that anodal and cathodal buffers do not mix during electrophoresis, the anodal buffer concentrate can be composed as follows.

In the standard procedure the same running buffer for the anode and cathode tank is used.

Anodal buffer (10× concentration) for Tris-Cl buffered gels:

This saves costs, particularly when large volumes of anodal buffer are used.

Tris base	0.25 mol/L	30.4 g
SDS	1% (w/v)	10 g
Water, deionized	Make up to	800 mL
4 mol/L HCl	Titrate to	pH 8.4
Water, deionized	Make up to	1 L

Store at room temperature.

When the anodal buffer is mixed with the used cathodal buffer after the run, there will be enough Tris in the buffer for repeated use as anodal buffer. This saves work and reagent costs. Of course, the cathodal buffer must be new for each electrophoretic run.

Also in this case, care must be taken that anodal and cathodal buffers do not mix during the run.

The ready-made gels contain PPA-Cl. For this buffer system, the anodal buffer must be completely free of Tris ions. In a buffer kit 75 mL of the 100× concentrated buffer are supplied, which is enough for one run in the high-throughput instrument and two runs in the instrument for up to six gels.

Anodal buffer (100× concentration) for PPA-Cl buffered gels:

Note, this is a highly viscous fluid.

Diethanolamine	5 mol/L	167 mL
Acetic acid	5 mol/L	100 mL
Water, deionized	Make up to	300 mL

Store at room temperature.

4.3.2
Setting up the Integrated High-throughput Instrument

- Place the separation unit on a leveled bench. Check with a spirit level. If necessary, adjust the level with inserting plastic sheets below some feet of the separation unit.

- Turn the pump valve at the back of the separation unit to "circulate".
- Pour 750 mL Tris-containing anodal buffer or – when running ready-made gels is intended – 75 mL DEA-containing buffer concentrate into the lower buffer tank.
- Fill the tank up to the mark 7.5 L with deionized water; in this way you rinse the buffer concentrate off the buffer seal tubing.

When identical buffers are used for the anode and the cathode, you can pour 950 mL buffer concentrate into the tank and fill up to the mark 9.5 L with deionized water.

- Switch the control unit on, set the temperature to 25 °C, and set the pump to "ON". The pump starts to circulate the liquid, mixes the concentrate with the water and the buffer temperature will be adjusted to 25 °C.

4.3.3

Setting up the Six-gel Instrument

- Take the cassette carrier and the upper buffer chamber out of the instrument.
- Connect the tubing to a circulating thermostat, which has been set to 25 °C.
- Pour 450 mL Tris-containing anodal buffer or – when running ready-made gels is intended – 45 mL DEA-containing buffer concentrate into the lower buffer tank.
- Fill 4 L deionized water into the tank.
- Plug the cable for the pump in. The pump starts to circulate the liquid, mixes the concentrate with the water.

4.4
Equilibration of IPG Strips and transfer to SDS Gels

4.4.1
Equilibration

Equilibration stock solution:

6 mol/L urea		180 g
30% glycerol (v/v)	85% solution	176 mL
50 mmol/L Tris-Cl pH 8.8	1.5 mol/L stock solution	17 mL
0.01% Bromophenol blue		50 mg
Water, deionized	Make up to	500 mL
Dissolve completely		
Then add		
2% (w/v) SDS		10.0 g

Do not add the SDS from the beginning; urea and SDS together would need a long time to become dissolved.

Store in 50 mL aliquots in a freezer at –20 °C or below.

- Equilibrate all strips together in the manifold with 100 mL equilibration buffer for each step.

Or, when the IEF is run in individual strip holders:

- Place each IPG strip into an individual equilibration tube and equilibrate with 10 mL buffer.

Equilibration:

15 min	10 mL equilibration stock solution	Plus 100 mg DTT
15 min	10 mL equilibration stock solution	Plus 250 mg iodoacetamide

Note: ***When samples have been pre-labeled at the cysteines with saturation dyes for a DIGE experiment, the second equilibration step with iodoacetamide is omitted.***

4.4.2
Application of IPG Strips onto SDS Gels

Agarose sealing solution:

0.5% agarose NA	0.5 g
0.01% Bromophenol blue	10 mg
SDS cathode buffer (1× concentration)	100 mL

- Heat on a heating stirrer or in a microwave oven until agarose is completely dissolved. Prepare aliquots for storage; store them at room temperature. Do not melt the agarose repeatedly. For ready-made gels aliquoted sealing solutions are supplied with the buffer kit.

Both types of cassettes (those for lab-cast and for pre-cast gels) have one "longer" glass plate.

This greatly facilitates insertion of the IPG strip into the cassette.

- Pour a few milliliters of deionized water on the upper gel edge using a squeeze bottle.
- Lay the cassette on the bench with the longer glass plate down, the protruding edge oriented towards the operator.
- Place the strip with the acidic end to the left, gel surface up onto the protruding edge of the longer glass plate as shown in Figure 4.11. When the strip is applied gel surface up, with the acidic end to the left side, the gels are assembled correctly in the standard orientation as indicated on page 115.

For film-backed gels it is important to slide the IPG strip into the cassette with its film-side towards the glass and its gel surface towards the film backing. This does not matter for the laboratory-made gel cassettes; however, a standardized orientation should always be applied.

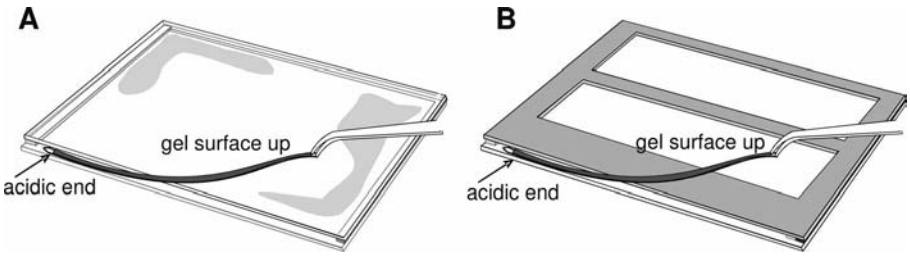


Fig. 4.11: Application of the equilibrated IPG strip into the SDS gel cassette for: A. laboratory-made gels and B. ready-made gels on film support.

- With the forceps move the strip into the cassette slot.
- Place the cassette into the rack, now with the IPG strip-supporting edge upside.
- With a thin plastic ruler, gently push the IPG strip down so that the entire lower edge of the

IPG strip is in contact with the top surface of the SDS gel (see Figure 4.12). Do not push the IPG strip down too hard, it would force a gap between gel surface and glass plate and damage the gel.

- Tilt the cassette by 90 degrees to get rid of the water.

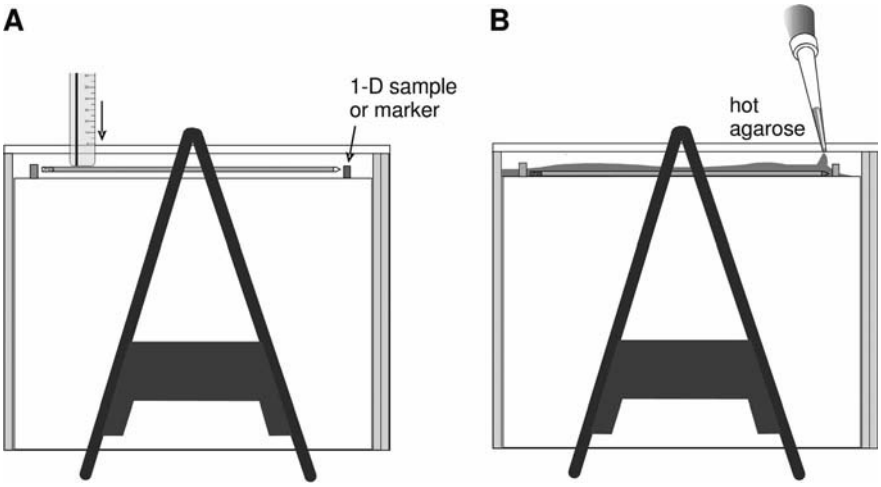


Fig. 4.12: IPG strip application on a two-dimensional gel.
A. Pushing the IPG strip onto the SDS gel with a ruler.
B. Sealing the strip and the 1-D sample pads in place with hot agarose.

4.4.3
Application of Molecular Weight Marker Proteins and 1-D Samples

If molecular weight markers and 1-D samples should be applied, choose 18 cm IPG strips instead of 24 cm strips. They should be placed at least 1.5 cm apart from the spacers to achieve straight bands. The markers should contain 200 ng to 1000 ng of each component for Coomassie staining and about 10 ng to 50 ng of each component for silver or sensitive fluorescent staining.

Close to the spacers there are always some edge effects, which cause bent zones.

- Rehydrate an IPG strip with molecular weight marker protein solution.
- Cut it into small pieces, which are stored in a deep freezer.

Alternatively:

- Apply 15 µL molecular weight marker protein solution on a IEF sample application piece and let it dry.
- For less volume, cut the sample application piece proportionally.
- Pick up the application piece with forceps and apply to the top surface of the gel next to one end of the IPG strip.

4.4.4 Seal the IPG Strip and the SDS Gel

- Melt each aliquot as needed in a 100 °C heating block (each gel will require about 2 mL). Allow the agarose to cool until the beaker or tube can be hold by fingers (60 °C) and then slowly pipette the amount required to seal the IPG strip in place. Pipetting slowly avoids introducing bubbles.

Carbamylated peptides would cause problems for protein identification in mass spectrometry.

For ready-made gels the agarose will also seal the narrow gap between the spacer near the hinge and the gel edge. The agarose must not be too hot, otherwise it will run down through the gap before it gels. Also, in general, carbamylation of proteins must be avoided.

4.5 The SDS Electrophoresis Run

4.5.1 The Integrated High-throughput Instrument

Note: ***Never switch the pump on without liquid in the tank.***

- Check whether the instrument has been prepared as described above in Section 4.3.2.

Spray with the plant sprayer used for overlaying the gel edges.

- Wet the tubing of the buffer seal with 0.1% SDS water.
- Insert the cassettes between the tubing of the buffer seal, starting at the back. Slide them down to the bottom.

Do not force the cassettes down; this could damage the buffer seal. Take care that the silicone tubing are not bent and stretched down; this would cause current leakage and buffer mixing. If you detected bent tubing, move the upper edges of the two neighboring cassettes slightly back and forward, to release the tubing.

- When fewer than 12 gels are run, insert blank cassettes into the free positions. In the front, however, a gel cassette should be inserted: this makes it easier to watch the migration of the Bromophenol blue front during the run.

Turn ready-made gel cassettes with the glass plates to the front, because the plastic frames are not transparent.

When all cassettes are in place, the level of the anodal buffer should be 3 mm to 5 mm below the lower buffer seal edge. If the level is lower, remove one cassette, add some anodal buffer or deionized water (for PPA buffer system for ready-made gels), and slide the cassette in again.

If the level is higher, and you use the identical running buffer, it does not matter. If you use an anodal buffer different from the cathodal buffer: open the draining valve for a very short time and close it again immediately to pump the excess liquid out.

- Make up 2.5 L cathodal buffer by diluting and mixing the concentrate:
  - 1× cathodal buffer for 1–6 gels (250 mL concentrate plus 2.25 L deionized water);
  - 2× cathodal buffer for more than 6 gels, to prevent buffer depletion (500 mL concentrate plus 2 L deionized water).
- Pour the 2.5 L cathodal buffer into the upper tank.
- Close the safety lid, which contains the cathodal electrodes.

The running conditions are the same for both ready-made and laboratory-made SDS gels.

Setting of the programmable power supply (set temperature to 25 °C):

Overnight runs:

Step 1	5 mA per gel	≈0.2 W	2 hours
Step 2	25 mA per gel	1 W	Overnight

Fast runs:

Step 1	5 mA per gel	≈0.2 W	2 hours
Step 2	40 mA per gel	15 W	4–6 hours

In a warm laboratory the buffer temperature can rise to 30 °C. This does not create a problem. When the temperature gets higher, the power setting should be reduced.

Note: ***The running time is also dependent on the water quality. Water with more ions causes slower runs.***

After the run

- Switch off the electric field.
- Open the safety lid.
- Remove the cassettes.

For DIGE DIGE gels are scanned while they are in the low fluorescent glass cassettes. If they cannot be scanned within the next couple of hours, place the cassettes into a refrigerator or into a cold room.

- Do not fix the gels, because acidic fixation would reduce the fluorescent signal considerably.

For staining the gels

If you use metal spatulas or knives, you can damage the glass plates.

- Open the cassettes only with a piece of plastic, like a Wonderwedge™.
- Remove the gels from the cassette; be very careful, when gels are not attached to a glass plate or a support film. Place the gels immediately into fixing solution or the hot Coomassie blue solution (see next chapter).

Or dry them with lint-free tissue paper.

- Rinse the cassettes with mild detergent, tap water and deionized water and let them dry in the air.

Do not walk away during this procedure!

- Pump the used buffer out by opening the valve: Be sure that the end of the tubing is placed into a bucket or a sink. Switch the pump off immediately when the tank is empty.

When the anodal buffer should be re-used (only possible for Tris-Cl gels), pump only 2.5 L out. When the PPA-Cl gels are run, the buffer cannot be re-used. The Tris ions have to be completely removed from the lower buffer tank by filling several times a few liters of water in and pumping it out.

- Rinse the cathode panels on the hinged safety lid with deionized water.
- Wash the lower buffer tank several times with tap water and then with deionized water to remove any disturbing ions.

4.5.2 The Six-gel Instrument with Standard Power Supply

Note: ***Never plug the pump cable in without liquid in the tank.***

When a pump runs dry, it gets destroyed.

- Check whether the instrument has been prepared as described above in Section 4.3.3.
- Wet the buffer seal of the upper buffer chamber with 0.1% SDS water.

Spray with the plant sprayer used for overlaying the gel edges.

- Insert the cassettes into the cassette carrier and place it into the tank.
- When fewer than six gels are run, insert blank cassettes into the free positions. In the front, however, a gel cassette should be inserted: this makes it easier to watch the migration of the Bromophenol blue front during the run.

Turn ready-made gel cassettes with the glass plates to the front, because the plastic frames are not transparent.

When all cassettes are in place, the level of the anodal buffer should have reached the mark "LBC start fill". If necessary, add anodal buffer or deionized water (for PPA buffer system for ready-made gels) to reach the mark. The filling procedure is shown in detail in Figure 4.13.

- Make up 1.2 L cathodal buffer by diluting and mixing the concentrate:
  - 1× cathodal buffer for running 1–3 gels (120 mL concentrate plus 1,080 mL deionized water).
  - 2× cathodal buffer for more than 3 gels, to prevent buffer depletion (240 mL concentrate plus 960 mL deionized water).

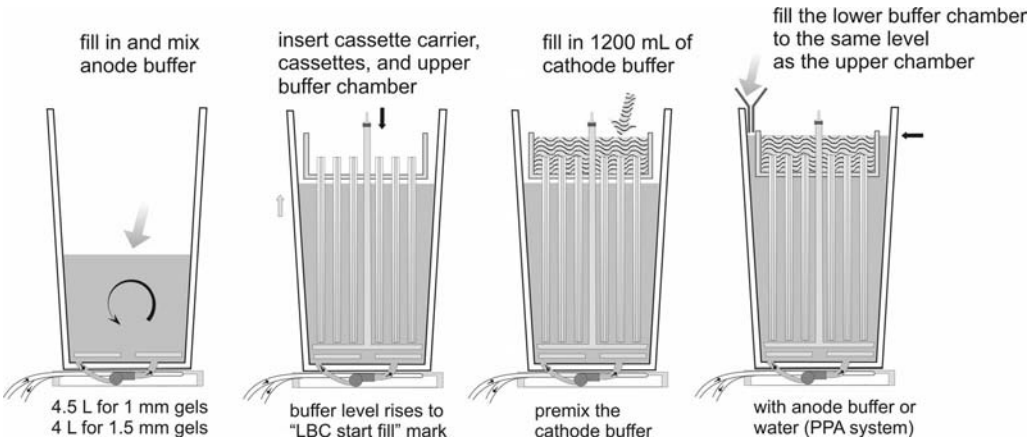


Fig. 4.13: Schematic drawing of the filling steps for the six-gel chamber. It is important to have a hydrostatic balance between upper and lower buffer.

Water is for ready-made gels / buffer, for other gels buffer is poured in.

- Pour 1.2 L cathodal buffer into the upper tank.
- Immediately fill more water or buffer into the lower buffer chamber to adjust the liquid level to the level in the upper buffer. The hydrostatic balance is necessary to prevent leakage of the upper buffer tank and mixing of the buffers (see Figure 4.13).
- Put the safety lid containing the cathodal electrodes in place and connect the cables to the power supply.

The running conditions are the same for both ready-made gels and laboratory-made SDS gels:

Overnight runs:

Step 1	5 mA per gel	≈0.2 W	2 hours
Step 2	25 mA per gel	1 W	Overnight

Fast runs:

Step 1	5 mA per gel	≈0.2 W	2 hours
Step 2	40 mA per gel	15 W	4–6 hours

External thermostat is set to 25 °C.

Note: ***The running time is also dependent on the water quality. Water with more ions causes slower runs.***

After the run

- Switch off the power supply and unplug the connecting cables.
- Unplug the pump cable.
- Remove the safety lid.
- Remove the entire cassette carrier and carry it to a sink. Then remove the upper buffer tank and the cassettes from the carrier.

When the anodal buffer is required for re-use (only possible for Tris-Cl gels), the upper buffer must be collected and poured into the anodal buffer in order to bring the Tris ions back.

For DIGE DIGE gels are scanned while they are in the low fluorescent glass cassettes. If they cannot be scanned within the next couple of hours, place the cassettes into a refrigerator or into a cold room.

- Do not fix the gels, because acidic fixation would reduce the fluorescent signal considerably.

Fixing of gels causes quantitatively and qualitatively modified results.

For staining the gels

- Open the cassettes only with a piece of plastic, like a Wonderwedge™.
- Remove the gels from the cassette; be very careful, when gels are not attached to a glass plate or a support film. Place the gels immediately into fixing solution or the hot Coomassie blue solution (see next Chapter).
- Clean the glass plates as soon as possible (see below)!
- Pour the buffer out and wash the lower buffer tank several times with tap water and then with deionized water to remove any disturbing ions.

If you use metal spatulas or knives, you can damage the glass plates.

When the PPA-Cl gels are run, the buffer cannot be re-used. The Tris ions have to be completely removed from the lower buffer tank by filling several times a few liters of water in and pumping it out.

- Rinse the cathode panels on the safety lid with deionized water.

4.6 Cleaning the Glass Plates

Normal cleaning

- Rinse the cassettes with 0.5% (w/v) SDS solution.
- Wash them with tap water.
- Rinse them with deionized water.
- Let them dry in the air.

Or dry them with lint-free tissue paper.

Removal of Bind-Silane bound gels from glass plates

Longer time can cause etching of the surface.

- Soak the glass plates overnight in a 5% (v/v) Decon 90 solution.
- Remove any gel fragments attached to the plate from previous gels.

If Decon is not available

- Pour a few mL of 0.5 mol/L KOH on the glass plate, distribute the solution evenly over the surface.
- Wait for a few minutes, wipe the gel off with a paper.
- Wash with tap water.
- Rinse with deionized water.
- Store the glass plates and cassettes in a dust-free cabinet.

Step 5: Scanning of Gels Containing Pre-labeled Proteins

Gels containing fluorescence pre-labeled proteins can be scanned while they are inside the cassette. It is important, that cassettes are made from low-fluorescent glass plates. Two types of imagers can be used:

- Laser scanners with photomultiplier tubes;
- White light sources with excitation filters and a scanning CCD camera.

The key point is the prevention of crosstalk between the different dye channels. Therefore the quality of the narrow band pass emission filters is of high importance.

For correct positioning the cassettes and avoiding the development of Newton's rings, the way of cassettes placement is very important. In the Typhoon laser scanner the cassettes are placed on an assembly of alignment guides, which provide an opening of 200 μm between the platen and the cassette glass plate to prevent the formation of Newton rings. The position of the cassettes is easily recognized by the software as a pre-defined tray area. For the scanning CCD camera special carrier cassettes are provided, which have an open bottom for this application.

2-D gels are always scanned in a defined – and the same – orientation, in order to evaluate and display the image with the low isoelectric points on the left hand side and the low molecular weights proteins at the bottom. The print on the IPG strips is very useful to find the correct orientation.

According to a Cartesian coordinate system.

The standard resolution for adequate evaluation with DeCyder software is a pixel size of 100 μm. The scanner computer should contain a software package ImageQuant to overlay the different images for visual control of the separation results and cropping the images.

- Wipe off fluorescent contaminations on the scanner glass platen and glass plates with lint-free tissue paper soaked in 10 % (v/v) H_2O_2 and rinse with double-distilled water.

5.1
Selection of Imager Settings

5.1.1
Laser Scanner

For excitation the appropriate laser is selected together with the related emission filter as shown in Figure 5.1 and Table 5.1.

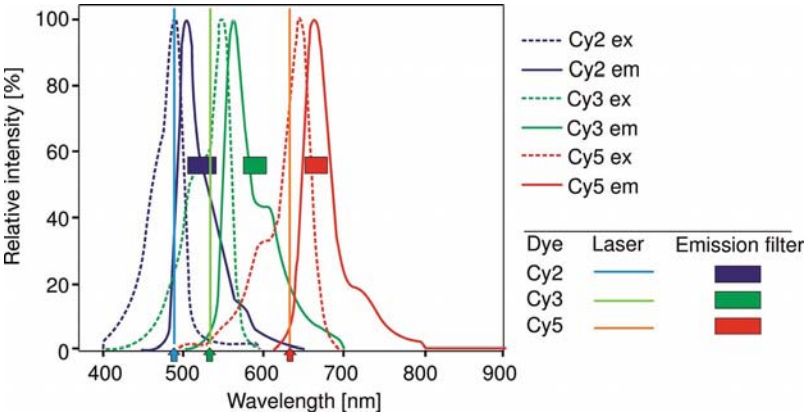


Fig. 5.1: Graph of CyDyes, excitation, and emission filter wavelengths for scanning CyDyes with Typhoon.

Tab. 5.1: Laser and emission filter selection for scanning DIGE gels with Typhoon.

Dye	Laser		Emission filter (nm)
Cy2	Blue	488 nm	520/40
Cy3	Green	532 nm	580/30
Cy5	Red	633 nm	670/30

Saturated spots (>100,000 counts) are indicated in red color.

- Adjust the photomultiplier (PMT) voltage for each wavelength after pre-scanning the entire gel with a low resolution of 1,000 µm. The lowest pixel should have at least ten counts. Saturation must be avoided. The values are usually between 470 V and 550 V.

This is a specialty of confocal optics.

- Select focal plane (+3 mm) for scanning in between the glass plates.

5.1.2
Scanning CCD Camera

For the white light source imager the excitation and emission filters have to be selected as shown in Figure 5.2 and Table 5.2.

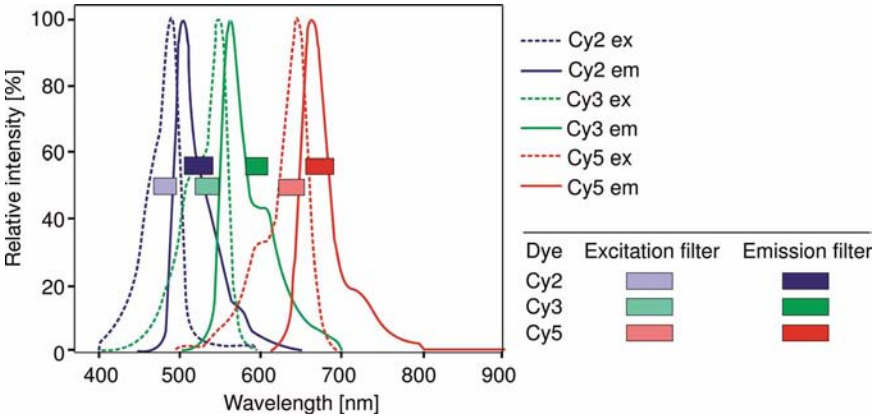


Fig. 5.2: Graph of CyDyes, excitation, and emission filter wavelengths for scanning CyDyes with EDI scanning CCD camera imager.

Tab. 5.2: Excitation and emission filter selection for scanning DIGE gels with EDI scanning CCD camera imager.

Dye	Excitation filter (nm)	Emission filter (nm)
Cy2	480/30	530/40
Cy3	540/25	595/25
Cy5	635/30	680/30

- For the CCD camera adjust the exposure time settings for each wavelength (between 0.15 seconds and 4.0 seconds) after a pre-scan of a small scanning area.

Usually settings are 0.9 seconds or below to keep scanning time down. Weaker signals need longer exposure.

5.2
After Scanning

The files are saved as 16 bit TIFF files. The software saves the different channels in two formats: *.ds and *.gel, to which it adds automatically the suffix Cy2, Cy3, or Cy5, and for the selected internal standard channel it also adds the suffix STANDARD. The *.ds files allow the scanned images to be overlaid in ImageQuant.

If the cassettes have been scanned in the wrong orientation, this can be corrected with the scanner software, which includes tools for rotating and flipping images.

Step 6: Staining of Gels

Only a selection of staining techniques will be described, those which are most useful for fast results, good sensitivity, image analysis, quantification, and mass spectrometry compatibility.

6.1
Visible Stains

6.1.1
Sensitive Stepwise Coomassie Brilliant Blue Colloidal Staining

According to Neuhoff *et al.* (1988).

Neuhoff V, Arold N, Taube D and Ehrhardt W. Electrophoresis 9 (1988) 255-262.

6.1.1.1 Solutions

Stock staining solution A:

- 10% (w/v) ammonium sulfate, 2% (w/v) phosphoric acid in 100 mL deionized water.

Stock staining solution B:

- 5% (w/v) Coomassie brilliant blue G-250 in deionized water.

Staining solution:

- Mix 2 mL of stock solution B with 80 ml of stock solution A. Add 20 mL of methanol into the AB mix.

Note: ***The staining solution should never be filtered because the colloidal dye particles formed are retained on the filter.***

Fixation solution:

- 20% (w/v) ammonium sulfate in water.

Neutralization buffer:

- 0.1 mol/L Tris base, adjust pH to 6.5 with phosphoric acid.

6.1.1.2 Staining Procedure

- Briefly wash the gel with distilled water.
- Stain the gel with the staining solution overnight or one day.
- Transfer gel into neutralization buffer for 1–3 minutes.
- Wash gel with 25% (v/v) methanol for less than 1 minute.
- Transfer gel into fixation solution and fix proteins overnight or for 1 day
- For further staining, just repeat the last four steps. Three times staining should be enough; which takes about 1 week

Gel can stay in stain or fixation solution over a weekend without any effect on the results.

It is said that sensitivity close to a good silver staining procedure can be achieved with repeated staining.

This procedure involves many steps and takes almost a week. However, the sensitivity of the method comes close to the sensitivity of silver staining.

6.1.2 Sensitive "Walk-away" Coomassie Brilliant Blue Colloidal Staining

Anderson NL, Esquer-Blasco R, Hofmann J-P, Anderson NG. Electrophoresis 12 (1991) 907–930.

According to Anderson *et al.* (1991), modified by Sjouke Hoving, Novartis.

- Fix the gel at least 3 hours in 50% ethanol / 3% phosphoric acid (1.5 L for ten gels: 750 mL ethanol, 45 mL of 85% H_3PO_4)
- Wash 3 × 20 minutes in distilled water
- Pre-incubate for 1 hour in 34% (v/v) methanol / 3% (w/v) phosphoric acid / 17% (w/v) ammonium sulfate solution:
  - First dissolve the ammonium sulfate in water/ phosphoric acid.
  - Then add slowly the methanol.
  - For 1.5 L (sufficient for ten gels): 45 mL of 85% H_3PO_4, 860 mL water, 255 g ammonium sulfate; then add 510 mL methanol.

- Add 0.53 g Coomassie brilliant blue G-250 powder per 1.5 L solution and stain for 4–5 days on an orbital shaker.
- Wash the gels a few times in water to remove background stain.

After 1 day the spots are visible, the end-point is reached after 4–5 days

6.1.3 Hot Coomassie Brilliant Blue Staining

Staining Solution: 0.025% (w/v) Coomassie R-350

- Dissolve one PhastGel Blue tablet in 1.6 L of 10% acetic acid.

Staining Procedure

- Place the gel in a stainless steel tray.
- Heat the solution to 90 °C and pour it over the gel in the tray.
- Place the tray on a laboratory shaker for 10 min.

Destaining

- On a rocking table in 10% acetic acid for at least 2 h at room temperature.
- Change solution several times; recycle it by pouring it through a filter filled with activated charcoal.

Placing a paper towel into the destaining solution adsorbs the Coomassie dye.

Staining and destaining solutions can be used repeatedly.

The gels can now be scanned and evaluated. The selected spots can be cut out with a spot picker.

6.1.4 Silver Staining

6.1.4.1 Quality of Reagents

Water For silver staining the water quality must be adequate. Sometimes it is thought, that MilliQ quality is a guarantee for good results. It happens, however, frequently, that the cartridges are not exchanged for a long period of time.

Sometimes, deionized water is better for silver staining than MilliQ water.

There is a simple test: Mix a few droplets of the water with a few droplets of the silver nitrate solution (from a commercial silver staining kit or laboratory-made). When the water contains chloride, the mixture becomes immediately turbid: This water cannot be used.

In case of emergency: buy deionized water from the supermarket.

Ethanol If pure ethanol is not available or too expensive, there are two possibilities:

Also known under the name Vodka.

Do not use otherwise denatured ethanol, do not use methanol.

- Buy a commercial 40% stock solution in a supermarket. For fixing just add the acetic acid.
- Use MEK denatured ethanol (methylethylketone).

Never store the 37% formaldehyde in the refrigerator or in the cold room.

Other reagents The reagent quality is very important for good results. The quality of silver nitrate and formaldehyde is most critical.

Heukeshoven J, Dernick R. Electrophoresis. 6 (1985) 103–112.

The following modification of the silver nitrate technique, described in Table 6.1, is based on the method by Heukeshoven and Dernick (1986), and is probably the most sensitive and reproducible one, when the quality of the chemicals and the water is high, and when the timing of the steps is exactly kept. The sensitivity of this method is of approximately 0.05–0.1 ng/mm^2.

If only a few gels have to be stained, an automated stainer (see Figure 6.1) is very useful. Automatic staining is more reproducible than manual staining.

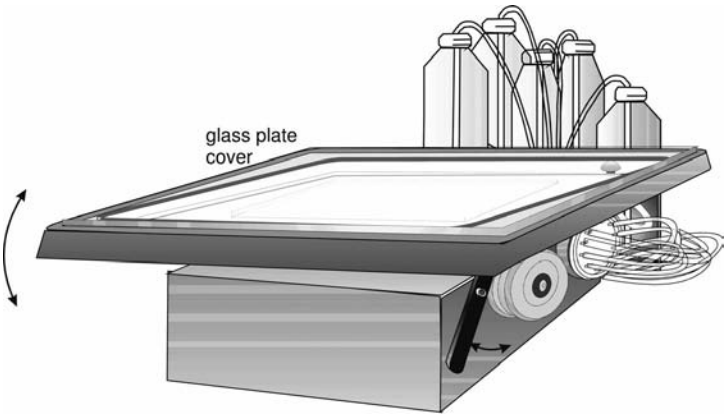


Fig. 6.1: Automated gel stainer.

Tab. 6.1: Silver staining according to Heukeshoven and Dernick (1986).

Step Manual or in automated stainer	Solution Laboratory-made solutions or silver staining kit for proteins	Volume (mL)	Time for gel type 0.5 mm backed or 1 mm unbacked (min)	1 mm on film or glass support or 1.5 mm unbacked (min)
Fixing	10% acetic acid; 40% ethanol; with deionized water to	2× 250	2× 15	2× 60
Sensitizing	75 mL ethanol; 10 mL Na-thiosulfate (5% w/v); 17 g Na-acetate; 1.25 mL glutardialdehyde; with deionized water to	250	30	60
Washing	Deionized water	3× to 5× 250	3× 15	5× 8
Silver	25 mL silver nitrate (2.5%); 100 µL formaldehyde; with deionized water to	250	20	60
Washing	Deionized water	2× to 4× 250	2× 1	4× 1
Developing	6.25 g Na-carbonate; 100 µL formaldehyde; 7 µL Na-thiosulfate (5% w/v); with deionized water to	250	4	6
Stopping	3.65 g EDTA; with deionized water to	250	10	40
Washing	Deionized water	2× to 3× 250	3× 5	2× 30

The addition of a tiny amount of Na-thiosulfate is particularly helpful to reduce the background when thick gels and film-supported 1 mm thick gels are stained.

Note: ***When silver stained spots should be further analyzed with mass spectrometry, some modifications of this protocol have to be made:***

- ***No glutardialdehyde in the sensitizer and no formaldehyde in the silver solution;***
- ***No Na-thiosulfate in the developer.***
- ***The staining tray must be covered with a lid to prevent contamination with keratin.***

6.1.4.2 Staining of Multiple Gels

Up to four gels can be stained at a time with a special set of staining trays on an orbital shaker, as shown in Figure 6.2. One tray is perforated to allow easy changing of staining liquids without breaking the gels. 1.5 L solution is needed for each step.

Fig. 6.2: Staining of multiple large format gels on an orbital shaker. Up to four gels, with or without film-backing can be stained together in one set.

6.1.4.3 Double Staining

By staining gels sequentially in Coomassie blue and silver stain, the sensitivity of detection can be increased. However, the background of the Coomassie blue stain must be completely clear. Because the spots are fixed and the buffer components are already removed, the fixing steps can be omitted, and the procedure starts with the sensitizing step.

6.1.5 Scanner Settings for Visible Dyes

- Activate in the *Scanner Options*: "Always use TWAIN controls."
- Press "Scan", set the scanning parameters in the submenu in the *Scanner Control Window*:
  - If necessary, choose light source color;

For instance: Yellow-brown silver stained spots are best scanned with blue color.

– Transmission mode; *Otherwise you cannot perform proper quantification.*
– Gray scale;
– Bits per pixel as high as possible, e.g. 16 bits; *Important for quantification.*
– Resolution 300 dpi or lower; *To limit the size of the resulting file.*
– Gamma correction. *A tool for intensity adjustment at selected level.*

If the value is set above "1", the intensity resolution will be higher for the strong signals, if it is set below "1"; the weaker signals are displayed with a higher intensity resolution.

- These parameters are saved under "Settings".

6.1.5.1 **Calibration of the Scanner**

At first the measured dimensionless intensity has to be converted into optical density (O.D.) values by calibration of the scanner with a gray step tablet. *Note: It is much easier to calibrate the scanner than calibrating each gel after scanning.*

- Choose "Recalibrate" in the program.
- Set the units, usually: "Optical Density"
- Scan a gray step tablet, available for instance from Kodak, shown in Figure 6.3.

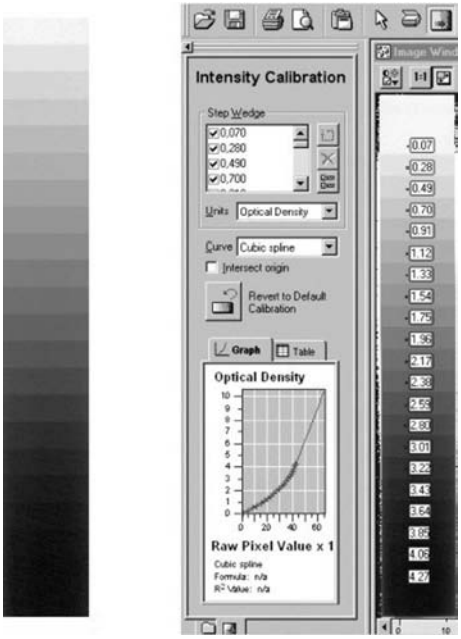


Fig. 6.3: Calibration of the scanner with the help of a gray step tablet.

- Enter the step wedge intensities into the step wedge list.
- Identify the step wedge points, when a value is used, it is indicated in the image window. The software will produce a string of points, which can be stretched to fit the steps.
- Select "Cubic spline". This curve will be saved as the default calibration, which can be loaded for each scan.

6.2 Fluorescent stains

6.2.1 SYPRO Ruby® or RuBPS Staining

Rabilloud T, Strub J-M, Luche S, van Dorsselaer A, Lunardi J. Proteomics 1 (2001) 699–704.

For highest sensitivity with Sypro Ruby or RuBPS the following procedure is recommended by Rabilloud *et al.* (2001).

The volumes of the staining solutions are optimized for a single 1 mm thick Ettan Dalt gel, which has a volume of approximately 50 mL. For 1.5 mm thick gels the volumes should be multiplied by 1.5. The backing must be nonfluorescent glass or film.

Tab. 6.2: SYPRO Ruby®/RuBPS staining.

Step In dark covered plastic tray	Solution Freshly made	Volume (mL)	Time for gel type 1 mm unbacked	 1 mm on film or glass support or 1.5 mm unbacked
Fixation	20% (v/v) ethanol, 7% (v/v) acetic acid in deionized water	500	1 hour	2 hours
Washing	20% (v/v) ethanol	500	4× 30 minutes	4× 45 minutes
Staining	SYPRO Ruby or RuBPS	700	Overnight	Overnight
Washing	Deionized water	500	2× 10 minutes	2× 30 minutes

Stain and store the gels in dark plastic containers. Do not use steel or glass trays.

Tab. 6.3: Laser and emission filter selection for scanning with Typhoon.

Dye	Laser		Emission filter (nm)
SYPRO Ruby or RuBPS	Green	532 nm	620

6.2.2 Deep Purple™ Staining

The volumes of the staining solutions are optimized for one 1 mm thick Ettan Dalt gel, which has a volume of approximately 50 mL. For 1.5 mm thick gels the volumes should be multiplied ×1.5. The backing must be non fluorescent glass or film.

Tab. 6.4: Deep Purple staining.

Step **In dark covered plastic tray**	**Solution** **Freshly made**	**Volume (mL)** **[mL]**	**Time for gel type** **1 mm unbacked**	**1 mm on film or glass support or 1.5 mm unbacked**
Fixation	15% (v/v) ethanol, 1% (w/v) acetic acid (approx. pH 2.3) in deionized water	1,000	1 hour or overnight	2 hours
Staining	0.5% (v/v) Deep Purple in 100 mmol/L sodium borate, pH 10.5–10.8 in deionized water *)	700	1–4 hours **)	1.5–4 hours **)
Washing	15% (v/v) ethanol in deionized water	700	30 minutes	45 minutes
Acidification	15% (v/v) ethanol, 1% (w/v) acetic acid (approx. pH 2.3) in deionized water	250	30 minutes	Repeat or extend up to overnight
Storage	Recycled staining solution, pH adjusted to 2.4 by addition of 5% (w/v) citric acid and filtered	700		

*) Dissolve 6.2g boric acid in 800 mL deionized water and adjust pH to 10.5 with NaOH [approx. 5 mL of a 50% (w/v) solution], then make to 1 L. The volume of staining solution should be 10–20 times that of the gel.

**) Extending the staining time up to 4 hours does not adversely affect results. There is some loss in fluorescence intensity if the staining time is greater than 5 hours.

Stain and store the gels in dark plastic containers at 4 °C in the storage solution. Do not use steel or glass trays.

Prior to imaging rinse the gels 2× 15 minutes in washing solution.

Tab. 6.5: Laser and emission filter selection for scanning with Typhoon.

Dye	Laser		Emission filter
Deep Purple	Green	532 nm	580/30 nm

For scanning Deep Purple-stained DIGE gels it is better to scan with a blue (457 nm) laser with a red (610 nm) band pass filter to avoid cross-talk.

Tab. 6.6: Excitation and emission filter selection for scanning with EDI scanning CCD camera imager.

Dye	Excitation filter		Emission filter	
Deep Purple	540/25nm	Green	595/25 nm	Orange

For scanning Deep Purple-stained DIGE gels it is better to use the violet excitation filter (390/20 nm) with the orange emission filter (595/25 nm) to avoid cross-talk.

6.3 Preserving and Drying of Gels

Film-backed gels can be stored in a sealed sheet protector. The open side is simply closed by sealing it with a kitchen foil welding apparatus.

Gels without film support can be dried between two sheets of cellophane, which are clamped into specially designed frames, as shown in Figure 6.4. The cellophane must be prewetted with 10% glycerol / water without alcohol.

During staining gels tend to swell. When a gel does not fit into such a frame anymore, it has to be re-shrunk to its original size with 30% ethanol / 10% glycerol.

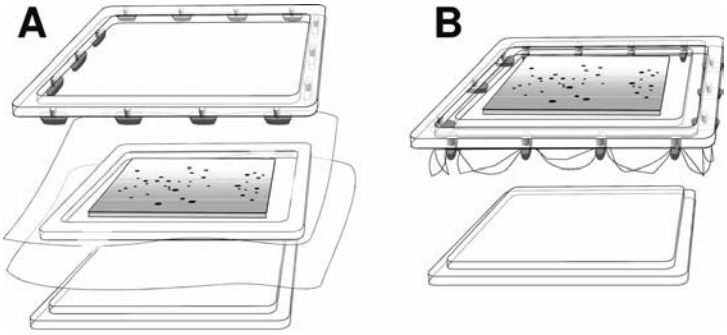


Fig. 6.4: Drying of a polyacrylamide gel between two sheets of cellophane using two plastic frames. A. From the bottom: Loading platform – smaller frame – pre-wetted cellophane – gel – pre-wetted cellophane – larger frame. B. The two clamped frames with the sandwich are removed from the platform; and the screws are turned by 90 degrees.

This drying procedure can also be applied on film-supported gels.

Step 7: Image Analysis and Evaluation of DIGE Gels

As already mentioned above in the overview part in section 1.6.2, image analysis and evaluation is the bottle neck in the traditional 2-D electrophoresis workflow. For the conventional *one sample per gel analysis* and evaluation there are various software packages on the market, which follow different philosophies. The general concept is described in this section.

Also DIGE gels can – in principle – be evaluated with several different algorithms. However, in order to get the full advantage of all features of the DIGE concept, it is highly recommended to apply the DeCyder 2-D software package, which is dedicated to this type of analysis.

The DIGE concept with DeCyder image analysis considerably accelerates and improves the evaluation of 2-D gels.

In the following section the workflow for image analysis of Difference Gel electrophoresis gels with DeCyder™ software is described.

The scanned images are saved as 16 bit tiff files in two formats: *.ds and *.gel. They carry the suffixes Cy2, Cy3, or Cy5 according to the scanning channel, the internal standard channel additionally the suffix STANDARD. The *.ds files allow the scanned images to be overlaid in ImageQuant.

7.1 Cropping of Images

Cropping the overlay will guarantee identical cropping of all channels.

The images of the different channels are overlaid with ImageQuant or PaintShop Pro™ and inspected in a false color image. It is important to crop the overlay images as *.ds file to exclude the marginal areas of the pattern. The cut-away areas usually show disturbances, the IPG strip and the spacers, and will not contribute any useful information for the 2-D pattern evaluation (see Figure 7.1).

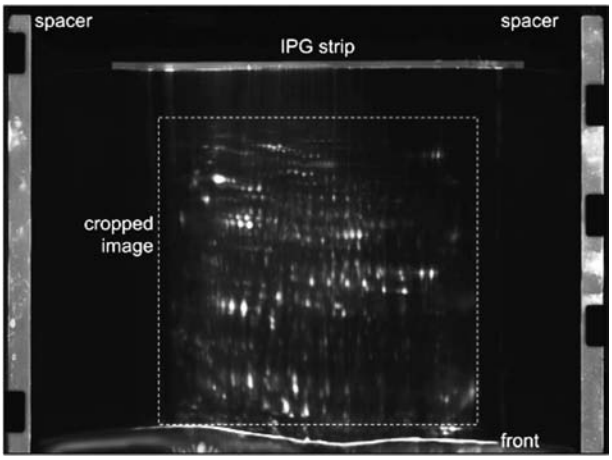


Fig. 7.1: Raw scan image (*.ds file) of a DIGE gel, indicating the cropped area for further evaluation.

The raw images provide some valuable information:

For sample tracing.
- The tracking number of the IPG strip;

Important for methodical optimization
- Whether there are proteins which did not leave the IPG strip;

Important for optimization of sample preparation and labeling
- Quality of the separation.

The images can now be analyzed using appropriate software, which supports spots co-detection and the use of the pooled internal standard.

7.2 Image Loading

Up to 500 gels can be loaded into one experiment.
- Create groups according to the experiment design.

Similar to a Windows explorer system
- Move images into the relevant groups.

7.3 Difference Analysis Within a Gel

This type of analysis can be applied for small sample sets up to three samples, which are run together in one gel. It can easily be employed for a spot volume ratio calculation of up- and down-regulated pro-

teins. With triple spot co-detection information from three channels is acquired together, which improves detection of weak spots and splitting. Corresponding spots have the same boundary. This ensures exact spot volume ratios. In most practical cases a gradual increase or decrease of a protein will happen.

In some situations, however, a protein spot can be completely missing in one of the images. If a protein spot is completely absent in one sample, the volume ratio calculation will always be based on a small volume of the background (> 10 counts).

A division through 0 cannot occur.

However, in most cases difference analysis within one gel serves as a preliminary analysis for setting the correct parameters later on in the batch processor. An experiment will usually consist of more than two samples, therefore in the real analysis several gels will be analyzed together.

In order to obtain usable results, several biological replicates need to be analyzed.

- Enter the number of estimated spots, e.g. 3,000.
- Start calculation "difference in gel analysis (DIA)"of the Cy3 and the Cy5 image.

Spot detection is done fully automatically.

At this stage the spot patterns will be normalized on the basis of total included spots. The logarithms of the spot ratios of the standard and the sample are used. The use of logarithms tightens the data enabling accurate fitting of a Gaussian normal distribution curve. The log ratios follow a normal distribution, assuming that the majority of proteins between two samples remain unchanged. This normalization measure eliminates variations from different total protein loads, scan settings, and dye intensities.

See also the graph area in Figure 7.2

- After the first DIA calculation a divided screen can be selected as displayed in Figure 7.2. All the screen areas are linked together.
- Adjust parameters:
  - Setting the threshold to a higher value reduces the number of up- and down-regulated spots. The software also calculates the two-model standard deviation (2 SD). The value is displayed close to the graph view as indicated in Figure 7.2 with a red ellipse. The value displayed should provide a base for the threshold minimum level. For instance, if a 2 SD of 1.93 is shown, select 2.0 for threshold.
  - Excluding spots with a very small area (number of pixels < 100), small volume (< 30,000), strong slope, and low peak height reduces the number of detected spots.

Small area and high slope indicate a dust particle.

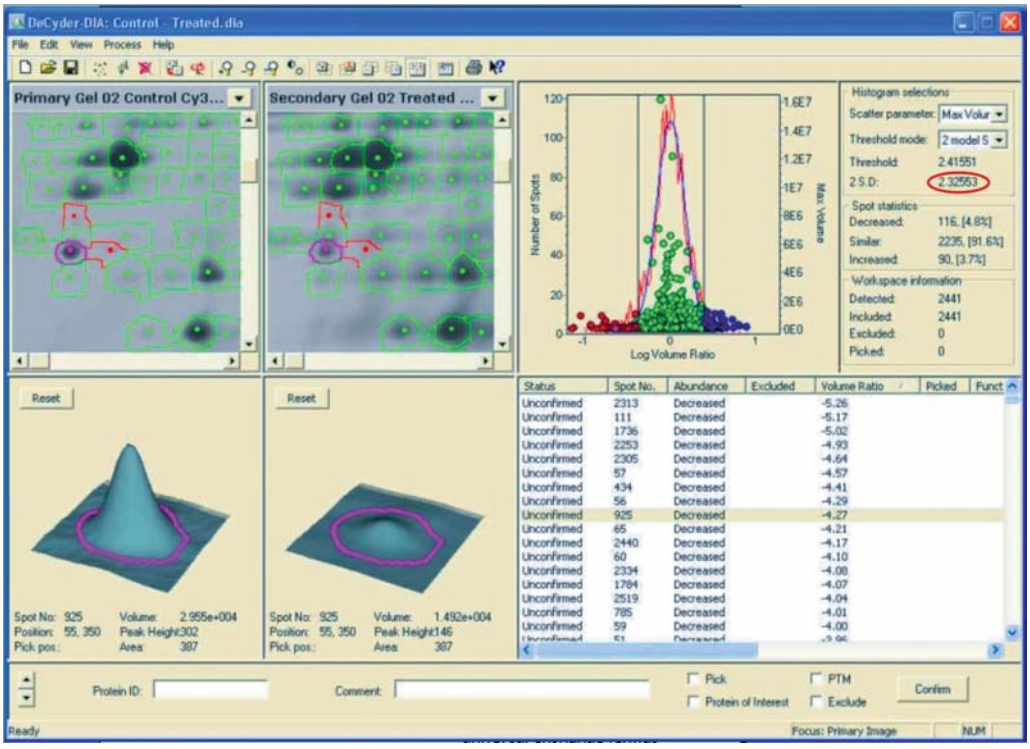


Fig. 7.2: Computer screen of DeCyder difference analysis within a gel. Clockwise, this is divided into: the gel view of both images (enlargement of a certain area), the graph area showing spots plotted over the log volume ratios (green is equal; red is down-, blue is up-regulated), a data table indicating similar and differing spots, and a three-dimensional representation of a selected spot with the spot boundary, which is identical within a gel.

– When the number of spots is modified by such a filtering process, the normalization is newly recalculated and is now based on the new – reduced – spot pattern.

The major applications of the DIA tool are:

For instance for optimization of sample preparation

- Semi-quantitative evaluation of up to three samples;
- Determination of the optimal number of estimated spot;
- Checking of correct cropping;
- Determination of appropriate filter parameters.

However, these filtering functions do not need to be employed too extensively, because dust signals are filtered out during spot matching.

Dust particles will not match, because they are positioned in randomly scattered positions.

7.4 Multi-gel Analysis with a Batch Processor

This is the quantitative evaluation of the images, performed with the batch processor. The internal standard images of the different gels are matched, and statistical analysis of the spots across multiple gels is performed. For this analysis the pooled internal standard plays a very important role for spot matching and spot volume normalization (the second normalization step). This normalization to the internal standard eliminates gel-to-gel variations.

Univariate statistical analysis tools like Student's *t*-test and ANOVA are very important for the automatic selection of spots with significant changes. At the same time the statistical confidence for the changes is given.

When Typhoon or Edi is employed for image acquisition, in the "DIGE naming format" Cy2 should be selected as internal standard, to which the spots of the internal standard images of the other gels will be matched.

- Select the groups to be analyzed.
- Enter the parameters found in the preliminary difference analysis within one gel.
- Select the desired statistical tests.
- Start the automated evaluation of the batch processor.

Figure 7.3 shows the computer screen with the views of two selected gels, a graph indicating the abundances of a selected spot across all the analyzed gels, this spot's data highlighted in the data table and its three-dimensional view. By clicking on another spot, all the views are changed and the data related to this spot is displayed.

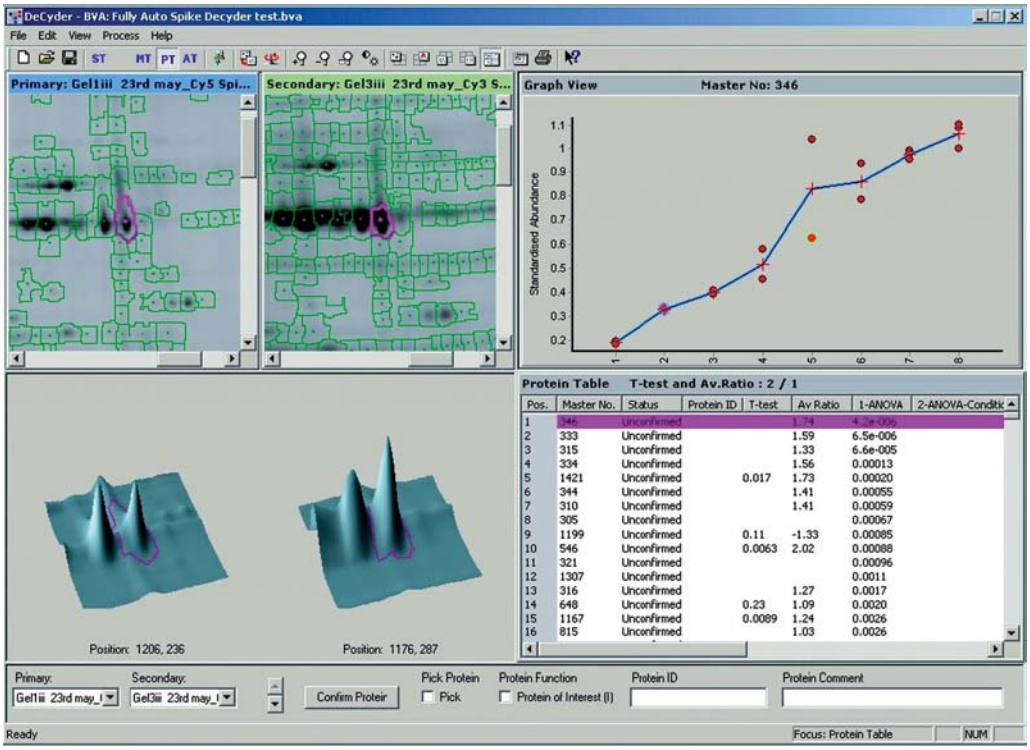


Fig. 7.3: Computer screen of DeCyder biological variation analysis across multiple gels. Clockwise, this is divided into: the gel view of two selected images (enlargement of a certain area), the graph area showing protein abundances of a time course, a data table indicating spot abundance ratios and their statistical values, and a three-dimensional representation of a selected spot with its spot boundary, which varies from gel to gel.

- Check the automatic matches by visual inspection in the MT mode. Matching vectors (lines between spots) indicate which spots are related to the master spots.
- If the matching vectors are crossed over or do not point into similar directions, the matching process can be manually edited. After introducing one or a few landmarks the matching process can be repeated with a result shown in Figure 7.4.

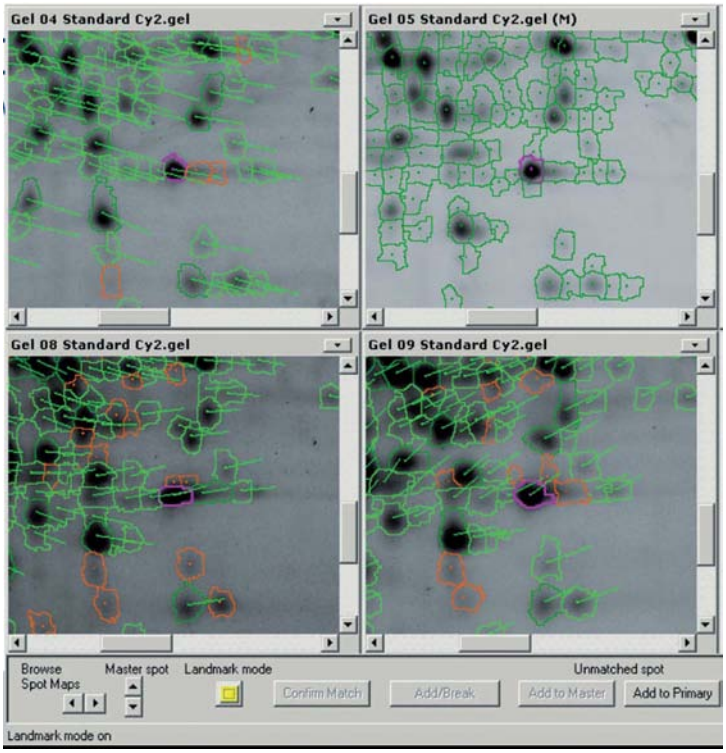


Fig. 7.4: Partial computer screen of DeCyder showing matching vectors across four gels in the gel view. In this case a second matching has been performed after manually introducing a landmark (purple boundary). The broken matches are kept as negative landmarks (orange boundary).

7.5 Preparation for Spot Picking

With a pick filter protein spots for further downstream analysis with mass spectrometry are chosen, see Figure 7.5. The spot coordinates are exported together with the coordinates of the reference markers to a file, which is uploaded to the spot picking computer.

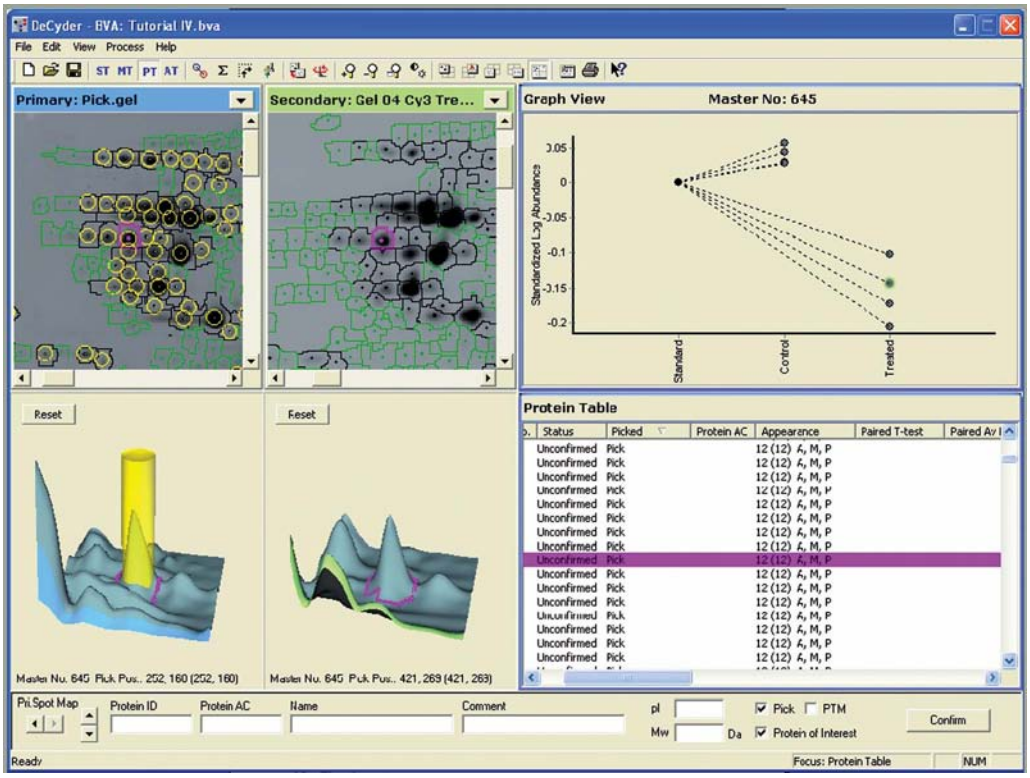


Fig. 7.5: Computer screen of DeCyder for the preparation of spot picking. Clockwise, this is divided into: the gel view of two selected images showing the spots selected for picking in one gel (enlargement of a certain area), the graph view showing protein abundances after normalization against the standard, a data table indicating similar spots selected for picking, and a three-dimensional representation of a selected spot showing the portion to be picked.

7.6 Further Data Analysis

Add-on software packages like DeCyder EDA (Extended data analysis) can be employed to answer remaining questions, for instance:

2-D results are usually quite complex.

- Grouping of results.

When working with a high number of samples the importance of such a control should not be underestimated.

- Controling appropriate sample annotation
- Finding spots which are characteristic for a biological state.

- Finding proteins with similar expression profiles.
- Finding proteins which serve as biomarkers, drug targets, classifiers, etc.
- Finding how many different groups are in an experiment in an unbiased way.
- Linking data from multiple experiments.
- Linking data with data from other experimental techniques.

Those need to be validated subsequently by a different experiment, like Western blotting.

The major multivariate statistical tools employed for these tasks are:

Examples are shown on page 136.

- Principal component analysis (PCA);
- *K*-means;
- Heuristic clustering (supervised and unsupervised);
- Discriminant analysis.

A very useful feature of DeCyder EDA is the direct linkage between the images of the results and the 2-D DIGE experiment: by clicking on a PCA spot or a band on the heat map of heuristic clustering the original experimental data are directly available with a hyperlink. In this way the result can be traced back to the separation and the sample.

Step 8: Spot Excision

Following the selection of the spots of interest, the protein spots have to be excised from the gel. This step is performed manually or automatically with commercially available spot pickers.

The procedure must be performed in clean environment such as a Laminar flow hood in order to minimize or most preferably exclude contaminating proteins such as keratin entering the protein identification workflow at this stage. The keratin(s) will be digested in the same way as the target protein(s), with keratin peptides included in the PMF spectrum, complicating the subsequent dataset. This is particularly problematic for low abundant samples. See Tables 8.1–8.4 for lists of keratin peptides commonly observed in MALDI peptide mass fingerprinting (courtesy of www.matrixscience.com).

Keratin contamination can be introduced into the workflow by poor sample handling at any stage between conclusion of the electrophoresis step and digestion of the target protein. Furthermore, keratin contamination is almost certainly present in the starting acrylamide solutions and solvents; all of which need to be filtered before use. Even the staining unit should be used solely for applications leading to mass spectrometric analysis. All eppendorf tubes to be used should be rinsed with MilliQ water (or at least doubly distilled water), especially if the tubes have been in lying around in drawers before use. This step will remove any residual dust from the tubes, which may contain keratins.

> ***All possible measures need to be taken to avoid keratin contamination.***

Peptides from four common keratins have been reported to contaminate many mass spectra. The commonly observed keratin tryptic peptides from these four keratin proteins are tabulated below.

Tab. 8.1: K2C1 Human: keratin, type II cytoskeletal 1 (K1 skin). Nominal mass (M_r): 65847; calculated pI value: 8.16.

Position in sequence	Experimental mass $[M+H]^+$	Theoretical mass $[M]^+$	Sequence
185–196	1382.68	1383.69	SLNNQFASFIDK
211–222	1474.75	1473.74	WELLQQVDTSTR
417–431	1716.85	1715.84	QISNLQQSISDAEQR
223–238	1993.98	1992.97	THNLEPYFESFINNLR
518–548	2383.95	2382.94	GGGGGGYGSGGSSYGSGGGS-YGSGGGGGGGR
549–587	3312.31	3311.30	GSYGSGGSSYGSGGGSYGSGG-GGGGHGSYGSGSSSGGYR

Tab. 8.2: K2E keratin (Dandruff), 67K type II epidermal – human. Nominal mass (M_r): 65825; calculated pI value: 8.07.

Position in sequence	Experimental mass $[M+H]^+$	Theoretical mass $[M]^+$	Sequence
245–253	1037.50	1036.49	YLDGLTAER
381–390	1193.60	1192.59	YEELQVTVGR
46–61	1320.60	1319.59	HGGGGGGFGGGGFGSR
71–92	1838.90	1837.89	GGGFGGGSGFGGGSGFGGGSGFS-GGGFGGGGFGGGR
93–128	2831.20	2830.19	SISISVAGGGGGFGAAGGFGGR

Tab. 8.3: K.9. keratin 9 (skin), type I, cytoskeletal – human. Nominal mass (M_r): 61950; calculated pI value: 5.14.

Position in sequence	Experimental mass $[M+H]^+$	Theoretical mass $[M]^+$	Sequence
233–239	897.40	896.39	MTLDDFR
224–232	1060.60	1059.59	TLLDIDNTR
242–249	1066.50	1065.49	FEMEQNLR
449–471	2510.10	2509.09 0	EIETYHNLLEGGQEDFESSGAGK
63–94	2705.20	2704.19	GGGSFGYSYGGGSGGGF-SASSLGGGFGGGSR

Tab. 8.4: K10 (dandruff) keratin 10, type I, cytoskeletal – human. Nominal mass: 59492; calculated pI value: 5.17.

Position in sequence	Experimental mass $[M+H]^+$	Theoretical mass $[M]^+$	Sequence
442–450	1165.60	1164.59	LENEIQTYR
323–333	1365.60	1364.59	SQYEQLAEQNR
166–177	1381.64	1380.63	ALEESNYELEGK
41–59	1707.80	1706.79	GSLGGGFSSGGFSGGSFSR
423–439	2024.94	2023.93	AETECQNTEYQQLLDIK
208–228	2366.26	2365.25	NQILNLTTDNANILLQIDNAR

Manual procedure Wearing powder-free gloves, use a clean scalpel blade to cut around the protein spot of interest. Attempt to take as little of the surrounding gel as possible. Using a surgical needle transfer the excised gel piece to a pre-rinsed 500-µL Eppendorf tube.

Step 9: Sample Destaining

Generally three methods are used for gel staining of 2-D gels. Coomassie brilliant blue, silver and Sypro Ruby.

9.1
Coomassie Blue-stained Protein Spots

The CBB will heavily suppress signal in both MALDI and ESI analysis. CBB will be easier to remove before digestion rather than after, because after digestion the CBB will be concentrated along with the peptide digest during the extraction stage. The CBB will be very difficult to remove by a microscale purification step (see Step 10).

Add 25 μL of 75 mmol/L ammonium bicarbonate (40% ethanol) to the excised Coomassie stained gel plug. Vortex and leave to stand. The supernatant will rapidly turn blue, remove supernatant after ten minutes and replace with a further aliquot of the destain solution until the excised spot is destained. The gel plug is now ready for digestion.

The length of time required to destain will be dependant on the intensity of the stain.

9.2
Silver-stained Protein Spots

The silver staining method has to be compatible with MS analysis. Sensitive silver stain methods, in the order of 1–10 ng per protein spot (Rabilloud, 1999), typically required the use of glutardialdehyde as part of the sensitization process. However, glutardialdehyde reacts with the amino groups of proteins, both ε-amino (lysine side chain) and the α-amino, cross-linking the protein to the gel. This step needs to be eliminated for optimal MS analysis (Shevchenko *et al.* 1996; Yan *et al.* 2000; Sinha *et al.* 2001). Further optimization to the procedure was reported by Gharahdaghi *et al.* (1999) by removing the silver ions prior to digestion.

1. Prepare fresh solutions of potassium ferricyanide (30 mmol/L) and sodium thiosulfate (100 mmol/L).

Volume used depends on the size of the excised gel plug.

2. Prepare a 1:1 solution of the above reagents and immerse the excised spots in this newly prepared solution.
3. Once the dark stain has been removed, wash the spot with water.
4. Equilibrate the excised spot in ammonium bicarbonate (200 mmol/L) for 15 minutes, remove supernatant and replace with a second aliquot of ammonium bicarbonate and leave for 15 minutes.
5. The gel plug is now ready for digestion.

Step 10: Protein Digestion

10.1
In-gel Digestion

Perform the reduction and alkylation step prior to digestion. Remember to use the same alkylation reagent as was used between the first and second dimensions of 2-D electrophoresis.

1. Reduction. Add 10 μL of dithiothreitol solution (5 mmol/L in 25 mmol/L ammonium bicarbonate) to the gel plug (or sufficient to cover the gel piece) and incubate for 30 minutes at 60 °C.
2. Alkylation. Add 10 μL of iodoacetamide (55 mmol/L in 25mmol/L ammonium bicarbonate) to the gel plug and stand at room temperature for 30 minutes in the dark.
3. Remove the supernatant, wash with ammonium bicarbonate (25 mmol/L), remove supernatant and wash with acetonitrile.
4. Take the excised gel plug and cut into smaller pieces with the scalpel (2–4 mm^2). Press down on the gel plug with the surgical needle holding the gel plug in place; allowing the cutting of the plug. Transfer the gel plug pieces to the rinsed Eppendorf tube.
5. Dehydration step. Dehydrate gel plug with acetonitrile (3×25 μL, 10 minutes each). The gel plug will begin to look white, after three washes it will be completely white.
6. Dry plug further in a speed vac or drying chamber, until gel plug appears "dust like."
7. Rehydration step. Apply enzyme in buffer (10 μL at 40 ng/μL in 50 mmol/L ammonium bicarbonate) to the dried gel plug and incubate on ice for 45 minutes.

8. Digestion. After 45 minutes remove supernatant and add enough buffer solution (without enzyme) to cover the hydrated gel piece.
9. Incubate at 30 °C, for between 1 hour and overnight (a preliminary peptide mass fingerprint can be obtained after 1 hour by sampling from the digestion mixture).
10. Extraction step. Add 20 µL of 50 mmol/L ammonium bicarbonate and sonicate for 10 minutes. Add 20 µL of acetonitrile : 5% TFA (1:1) and sonicate for 10 minutes. Remove supernatant and dispense into a separate tube.
11. Add 20 µL of acetonitrile : 5% TFA (1:1) and sonicate for 10 minutes. Combine supernatant extracts. Repeat one further time.
12. Add 10 µL acetonitrile and sonicate. Remove and combine with earlier supernatant extracts.

10.2 Non-gel Digestion

Two methods can be considered.

A. Split the sample in to two fractions, 4% CHAPS-soluble and 4% CHAPS insoluble. The samples can then prepared according to the following procedure:

1. Dissolve approximately 1 mg of protein in 1 mL of 9 M urea with 50 mmol/L DTT, and then incubate for 60 min at 20 °C.
2. Add 1 mL of 8 M urea, 250 mM TrisHCl, pH 8.8, and 125 mM iodoacetamide, and then incubate for 60 min at 20 °C.
3. Buffer exchange 1 mL of the mixture with 20 mmol/L ammonium bicarbonate, pH 7.8, on a NAP-10 desalting column.
4. Digest the desalted protein sample with trypsin (concentration ratio of 50:1) for 2 h at 37 °C.
5. Inactivate the trypsin by adding formic acid to the sample.

B. Alternatively:

1. Denature sample by dissolve it in 0.5 mL 8 mol/L (GUA-HCl) 50 mM ammonium bicarbonate,
2. Reduce by adding 20 µL 0.8 M PlusOne DTT (10 min at 37 °C) and alkylate with 25 µL 0.5 mol/L iodoacetic acid (10 min at 37 °C).
3. Exchange the buffer to 50 mM ammonium bicarbonate on NAP-5 desalting columns and digest using Ettan Trypsin sequencing grade, at pH 8.4 for12 h at 37 °C.

Step 11: Microscale Desalting and Concentrating of Sample

The sample preparation for MALDI (and ESI) is a crucial step. The analyte must be incorporated into the matrix crystals, a process that is significantly upset by the presence of contaminating salts and buffers (see table 1 for MALDI salt/buffer compatibility). Sample preparation can be tailored to a particular matrix. For instance α-cyano-4-hydroxycinnamic acid is insoluble in water. Sampling directly from the digestion mixture, the analyte solution can be spotted onto a preformed thin layer of matrix on the MALDI target (thin film method). Once the dried spot has formed, the spot can undergo significant washing with 0.1% TFA solution on the target surface, removing the salt contamination. As the analyte has been incorporated into the matrix crystal, it is preferably bound during the washing step (Vorm *et al.* 1994). Addition of nitrocellulose to the matrix solution allows for improved desalting and improved binding on the MALDI target (Shevchenko *et al.* 1996).

In contrast, 2,5-dihydroxybenzoic acid is a water soluble matrix. It is possible to sample directly from the digest mixture without any sample clean up (on target washing is not applicable) as the matrix excludes the contaminants from the crystallization process

However if sampling from the digest mixture is not applicable (or if this approach is not attractive) then the peptides are extracted as detailed in the section above. However, the combined extract volume (~100 μL) may be too dilute for successful analysis of the peptide mixture; in these instances the sample must be concentrated prior to analysis. Discussed earlier, simply concentrating the sample will concentrate all the contaminants as well, hence a clean up step is recommended. Kussman *et al.* (1997) described the use of microscale purification columns using RP resin.

Kussman et al. J Mass Spectrom 32 (1997) 593-601.

Tab. 11.1: Salt/buffer compatibility with MALDI analysis.

Type of impurity	Concentration
Phosphate buffers	<20 mmol/L
Tris buffer	<50 mmol/L
Detergents	<0.1%
Alkali metal salts	<1 mol/L
Guanidine	<1 mol/L
Ammonium bicarbonate	<30 mmol/L
Glycerol	To be avoided
SDS	To be avoided
Sodium azide	To be avoided

Microscale Desalting and Concentration

Prevents the packing from being eluted during preparation and use.

1. Take a gel-loader pipette tip and very carefully pinch the tapered lower end of the tip with a pair of flat armed tweezers.
2. Prepare a suspension of reversed phase resin in methanol.
3. Add 50 µL of methanol to the pipette tip, followed by 2–3 µL of the suspension.
4. A pipette can then be used to gently push the methanol though the column. The RP resin forms a small column at the end of the tip.
5. Equilibrate the column with 0.1% TFA (20 µL).

Complete drying or lyophilization not recommended, extensive sample loss.

6. At this stage ensure the acetonitrile concentration of the peptide extracts is sufficiently low to allow good retention of the peptides on the column. Hence dry the peptide extracts to a volume of approximately 10 µL.

Heptafluorobutyric acid is a very hydrophobic ion-pairing reagent and will make the digested peptides "very sticky" improving there retention on a reversed phase C18 column.

7. Further improve the retention of the peptide mixture by acidifying the mixture with heptafluorobutyric acid (HFBA). Add 9 µL of water and 1 µL of HFBA to the semi-dried peptide extracts, creating a 55% concentration (v:v) of HFBA.
8. Load the acidified peptide extracts onto the column using a pipette. Gently push through the solution with a pipette. Apply the eluate back to the column; repeat five times to improve retention of the peptides.

9. Wash the column with 0.1%TFA (20 µL).
10. Elute peptides with 3–5 µL of MeCN: 0.5% formic acid (1:1, v:v).

Alternatively, these columns are available commercially (ZipTip^{TM}). Follow the manufacturers instructions, which are similar to the above.

Step 12: Chemical Derivatization of the Peptide Digest

12.1
Guanidation and Sulfonation of the Peptide Digest

This method (Keough *et al.* 1999; 2000a 2000b) describes the procedure for the guanidation of lysine residues and the subsequent sulfonation of the α-amino group at the N-terminus of peptides in the digest mixture. The sulfonation reagent, 2-chlorosulfonyl acetylchloride is highly hygroscopic and strenuous precautions must be made to ensure that water (including moisture in the atmosphere) is absent from the reagents and reaction vessel.

1. Take combined peptide extracts and concentrate in a speed vac to a volume of 20 μL.
2. Guanidate the lysine side chain. Mix 2mL *O*-methylisoureahydrogen sulfate solution (86 mg/mL MilliQ H_2O) with 8 mL 0.25 mol/L $NaHCO_3$, pH 11.5 and add to digest. Place it in the oven at 37 °C for 2h. (The reaction can also be performed at room temperature overnight or at 70 °C for 10 minutes).
3. Remove reagents using a reversed phase microscale desalting and concentration column as described in Step 9 above, elute peptide digest and evaporate solvent to dryness.
4. Reconstitute in 10 μL THF:DIEA 19:1.
5. Add 2 μL sulfonation reagent (2 μL neat material in 1 mL THF)
6. React for 1–2 min. at room temperature.
7. Dry completely to remove organics and excess base.
8. Reconstitute in 5–10 μL of 0.1% TFA.
9. Apply to MS target as described in Step 11. 2,5 DHB is the preferred matrix of choice, though α-cyano 4 hydroxy cinnamic acid can be used.

12.2
Sulfonation of the Peptide Digest Using CAF Chemistry

This method describes sulfonation of the α-amino group at the n-terminus of peptides in the digest using a novel, modified water-stable reagent. Steps 2 and 3 can be omitted if guanidation of the lysine groups in the peptide digest is not favored.

1. Apply peptide digest extracts to a reversed phase microscale desalting and concentration column as described in Step 9 and dry down extract to a minimal volume. Alternatively the peptide can be dried down to a minimal volume and proceed from step 2 (or step 4 if the guanidation step is to be omitted).
2. Reconstitute peptide digest in the guanidation reagent [mix 2 μL *O*-methylisoureahydrogen sulfate solution (86 mg/mL MilliQ H_2O) with 8 μL 0.25 mol/L $NaHCO_3$, pH 11.5].
3. Place it in 37 °C for 2 h. (The reaction can also be performed at room temperature overnight or at 70 °C for 10 minutes)
4. Prepare fresh reagent. Dissolve the CAF reagent in 0.25 M $NaHCO_3$, pH 9.4 (10mg/100 μL).
5. Apply 10 μL of the CAF-reagent solution to the sample and leave for 15 minutes.
6. Add 1 μL 50% hydroxylamine solution. Reverses tyrosine modifications at the hydroxyl group.
7. Add 8 μL water and 1 μL HFBA to the digest solution and apply to a reversed phase C18 column as described in section Step 9
8. Elute derivatized peptides in aceonitrile: 0.5% formic acid (1:1, v:v) 1–5 μL
9. Apply to the MALDI target as described in Section 13.1.

Step 13: MS Analysis

13.1
Sample Preparation for MALDI

Three matrices are generally used for peptide and protein analysis. A variety of methods have been described, particularly for the use of α-cyano-4-hydroxy-cinnamic acid for peptide analysis. Notably, Vorm *et al*. described the thin film method and Jensen *et al*. 1996 incorporated nitrocellulose into a similar method for improved performance. This section will describe the dried droplet method, probably first choice for most applications for each of the three matrices concerned. It is important in each case to keep the size of the target spot as small as possible. Remember the width of the laser beam is very narrow (~20 microns) and as such the majority of the sample will not be ablated with the laser from a large sample spot.

Vorm O, Roepstorff P, Mann M. Anal Chem 66 (1994) 3281-3287.

Jensen ON, Podtelejnikov A, Mann M. Rapid Commun Mass Spectrom 10 (1996) 1371–1378.

Do not use non-volatile solvents (e.g. glycerol, polyethyleneglycol, DMSO, Triton X or 2-mercaptoethanol) with this method.

Loading the sample in high organic content (high methanol or acetonitrile causes the droplet to spread uncontrollably across the target surface.

13.2
α-Cyano-4-hydroxy-cinnamic acid. Analysis of Peptides and Peptide Digests

1. Prepare a fresh solution of α-cyano-4-hydroxy-cinnamic acid in acetonitrile:0.1 %TFA (1:1, v:v; 10 mg/mL). Prepare a fresh solution of matrix wherever possible.
2. Take 0.2–0.5 μL matrix and mix with 0.2–0.5 μL of sample. Mix thoroughly by uptaking and displacing the volume from a pipette several times. For reproducible analysis, thorough mixing of the matrix and analyte is necessary.

It is important to keep the pH of the matrix solution below pH 4, using 0.1% TFA maintains and ensures this.

If the analyte concentration is sufficiently large then 1–2 μL can be mixed with several μL of matrix and subsequently vortexed before applying to the target.

Do not heat the sample target to speed up drying process (place on a hot plate).

3. Apply to the target and leave to dry at room temperature.
4. Insert target into mass spectrometer.

13.3
2,5 Dihydroxybenzoic Acid. Analysis of Peptide Digests

The concentration of acetonitrile can be increased up to 30%.

1. Prepare a solution of 2,5-dihydroxybenzoic acid in 0.1% TFA (10mg/mL).
2. Take 0.2–0.5 μL matrix and mix with 0.2–0.5 μL of sample. Mix thoroughly by uptaking and displacing the volume from a pipette several times. For reproducible analysis, thorough mixing of the matrix and analyte is necessary.
3. Apply to the target and leave to dry.
4. Insert target into mass spectrometer.

2,5-Dihydroxybenzoic acid is commonly used for the analysis of oligosaccharides released from glycoproteins, the above method can be followed by the analysis of oligosaccharides.

Tip – oligosaccharide analysis Since 2,5-DHB forms long needle-like crystals, homogeneity of the sample can be increased by redissolving the dried matrix-sample with ethanol. Apply 0.1 μL of ethanol to the dried spot.

13.4
Sinapinic Acid. Analysis of Proteins

1. Prepare a solution of sinapinic acid in acetonitrile and 0.1% TFA (60:40, v:v; 10mg/mL).
2. Take 0.2–0.5 μL matrix and mix with 0.2–0.5 μL of sample. Mix thoroughly by uptaking and displacing the volume from a pipette several times. For reproducible analysis, thorough mixing of the matrix and analyte is necessary.

Dried droplets are quite stable; they can be kept in a drawer or in vacuum for days.

3. Apply to the target and leave to dry.
4. Insert target into mass spectrometer.

13.5
Sample Preparation for ESI MS

Nanospray In electrospray mass spectrometry the presence of salts, involatile buffers and polymeric material in a sample is often detrimental to the analysis. High amount of salts often result in partial or complete blocking of the needle orifice resulting with, at best, an intermittent and instable spray. Subsequently, sensitivity is reduced and the spectrum can be complicated with salt adducts. The ingredients in a protein digest reaction, salts and possibly detergents, are present in millimolar amounts and they have to be removed before analysis. As with MALDI analysis, a microscale desalting and concentration step often eliminates this problem and at the same time allows a concentration of the analyte(s).

A microscale desalting and concentration may be less successful for removing detergents.

A microscale desalting and purification column is prepared as explained in step 9. Peptides are eluted into the nanospray needle using 50% methanol:0.5% formic acid, or eluted into a sample tube and pipetted into a nanospray needle.

LC-MS/MS Following the in-gel digestion step redissolve the digest extract in 5 µL water. (Optional, 0.5 µL can be used for MALDI MS analysis). The remaining 4.5 µL is mixed with 4.5 µL of a 0.1% aqueous formic acid solution for subsequent ESI LC-MS/MS analysis. The remainder of the digest extract solution can be loaded on an autosampler, from which 5 µL are injected into a nano-HPLC (or alternatively manually injected). Separation is typically performed using a 75 mm × 150 mm silica C18 (3 mm particle size) PepMap column (LCPackings, Amsterdam, The Netherlands) with 0.1% formic acid in water as solvent A and 0.08% formic acid in 80% acetonitrile / 20% water as solvent B by starting after equilibration with 5% solvent B linearly increasing it to 40% within 32 min using a flow rate of 200 nL/min. The nano-HPLC is directly coupled to a tandem mass spectrometer equipped with electrospray ionization. This method was supplied by Dr Rainer Cramer, Ludwig Institute for Cancer Research.

Step 14: Calibration of MALDI-ToF MS

The MALDI peptide mass fingerprint can be calibrated internally or externally.

14.1
Internal Calibration for Peptide Analysis

Trypsin autolysis during the digestion of the target protein(s) can be very useful for calibration purposes. However, it is important to know which species the trypsin is derived from, porcine or bovine. It is fortunate that the two major porcine trypsin autolysis peaks present in the peptide mass fingerprint actually bracket the majority of the mass range of interest. The internal calibration procedure will be dependent on the MS in use, but the suggested two peptides to use are:

Trypsin II 842.509 $[M+H]^+$
Trypsin IV 2211.104 $[M+H]^+$

Both of these molecular weights are the monoisotopic C12 isotope, protonated form of each peptide. Other porcine trypsin autolysis peaks observed in a MALDI PMF include 515.32, 1045.56, 2283.17, and 2299.17 (2283.17 with oxidized Met).

Using an internal calibration, high-mass accuracy is achievable (sub 25 ppm).

The expected peptides from a theoretical trypsin digest of porcine and bovine trypsin are listed in Tables 14.1 and 14.2.

Obtained with permission from: www.matrixscience.com.

Tab. 14.1: Theoretical tryptic peptides of porcine trypsin, TRYP_PIG (protonated monoisotopic ^{12}C mass, unprotonated average mass).

Position in sequence	Monoisotopic mass $[M+H]^+$	Average mass $[M]^+$	Sequence
52–53	262.14	261.28	SR
54–57	515.32	514.63	IQVR
108–115	842.50	842.01	VATVSLPR
209–216	906.50	906.05	NKPGVYTK
148–157	1006.48	1006.15	APVLSDSSCK
98–107	1045.56	1045.16	LSSPATLNSR
134–147	1469.72	1469.68	SSGSSYPSLLQCLK
217–231	1736.84	1736.97	VCNYVNWIQQTIAAN
116–133	1768.79	1768.99	SCAAAGTECLISGWGNTK
158–178	2158.02	2158.48	SSYPGQITGNMICVGFLEGGK
58–77	2211.10	2211.42	LGEHNIDVLEGNEQFINAAK
78–97	2283.17	2283.63	IITHPNFNGNTLDNDIMLIK
179–208	3013.32	3014.33	DSCQGDSGG...SWGYGCAQK
9–51	4475.09	4477.04	IVGGYTCAA...VVSAAHCYK
9–51	4489.11	4491.07	IVGGYTCAA...VVSAAHCYK

Obtained with permission from: www.matrixscience.com.

Tab. 14.2: Theoretical tryptic peptides of bovine trypsin, TRY1_BOVIN (protonated monoisotopic ^{12}C mass, unprotonated average mass).

Position in sequence	Monoisotopic mass $[M+H]^+$	Average mass $[M]^+$	Sequence
110–111	260.19	259.35	LK
157–159	363.20	362.49	CLK
238–243	633.31	632.67	QTIASN
64–69	659.38	658.76	SGIQVR
112–119	805.41	804.86	SAASLNSR
221–228	906.50	906.05	NKPGVYTK
160–169	1020.50	1020.17	APILSDSSCK
229–228	1111.55	1111.33	VCNYVSWIK
146–156	1153.57	1153.25	SSGTSYPDVLK
207–220	1433.71	1433.65	LQGIVSWGSGCAQK
191–206	1495.61	1495.61	DSCQGDSGGPVVCSGK
70–89	2163.05	2163.33	LGEDNINVVEGNEQFISASK
170–190	2193.99	2194.47	SAYPGQITSNMFCAGYLEGGK
90–109	2273.15	2273.60	SIVHPSYNSNTLNNDIMLIK
120–145	2552.24	2552.91	VASISLPTS...LISGWGNTK
21–63	4551.12	4553.14	IVGGYTCGA...VVSAAHCYK

The practice of adding synthetic peptides to the peptide digest to perform an internal calibration is not recommended for two reasons. First, it is difficult to estimate the concentration of calibrants to add; too high concentration may suppress the ionization of the peptides of interest. Second, the calibrants may co-incide with peptides of interest, therefore precluding these peptides from the subsequent database search.

14.2 External Calibration for Peptide Analysis

When one or both of the autolysis peaks are absent from the spectrum, an external calibration has to be performed. This is normally achieved by calibrating a spectrum acquired from the sample spot adjacent to the sample spot whose spectrum needs to be calibrated. Hence it is often useful to have a calibration sample alongside each sample, or at least every two samples. Hence an internal calibration is performed on the calibration mixture, the calibration file saved and then applied to the spectrum of interest (the procedure will depend upon the MS instrument used). This is a useful fallback method in case successive spectra cannot be calibrated internally from the trypsin autolysis peaks.

Obviously a calibration file obtained from the internal calibration using both trypsin autolysis peaks can be applied to the spectrum acquired from an adjacent spot, without having to use a calibration mixture such as this.

As opposed to the internal calibration where only two autolysis peaks act are used as the calibrants, the external calibration mixture can contain several calibrants defining the calibration curve more efficiently. A table of potential calibrants is listed below.

Tab. 14.3: Suggested peptides for use in an external calibration mixture (All molecular weights are ^{12}C monoisotopic values).

Peptide	Monoisotopic mass $[M+H]^+$
Angiotensin I	1296.678
Angiotensin II	1046.535
Substance P	1347.728
[Glu]-fibrinogen b	1570.670
Renin substrate	1759.932
Apamin	2026.887
HACTH clip 18–39	2465.188
HACTH clip 7–38	3657.922

Each combination of instrument mode (detection mode (linear/reflectron), accelerating potential) needs individual calibrations.

Step 15: Preparing for a Database Search

This section describes the function of each category observed on a commonly used search engine web browser for peptide mass fingerprinting and peptide sequence analysis.

15.1
Number of Missed Cleavages

The number to be input is dependent on the efficiency of the digestion. If the digestion is ideal then the enzyme cleaves at the correct site every time, affording the highest discrimination. However, enzymes rarely act in such an ideal fashion and partial peptides can be observed in the peptide mass fingerprint. Typically, one missed cleavage is selected as this. Selecting a higher number than this can reduce discrimination, as the experimental peptide mass list will be searched against more theoretical peptides.

15.2
Choice of Cleavage Agent

Table 1.8 in Section 1.7.2.1 lists a range of enzymes available for proteolytic digestion. However, not all of the listed enzymes are suitable for subsequent protein ID. Enzymes with low specificity, particularly those generating small peptides should be avoided. Trypsin is the most commonly used enzyme as it yields peptides across a suitable mass range, relatively high specificity and locates the basic residues at the c-terminus of the peptide. This is useful for subsequent MS and MS/MS analysis.

15.2.1
Choice of Amino Acid Modification

Proteins can be modified as a result of cellular processes, specifically post-translational modications; a sample preparation, including methionine oxidation and/or directly as part of an analytical method such as the reduction and alkylation of cysteine residues.

These modifications can be regarded as complete or partial for a database search. For instance, if there is uncertainty about the quantitative modification of a particular residue, or whether a modification of a particular residue exists at all then the partial modification option can be used. In this instance, the search engine uses the masses of both the native amino acid and the modified amino acid in the search. For example, when a protein is applied to the second dimension the methionine residues present in the protein are prone to oxidation due to the conditions of electrophoresis. Oxidation of a methionine residue results in a mass increase of 16 Da (nominal mass). Therefore, selecting a partial modification of the methionine enables both forms to be identified if they are present.

Second, if all cysteine disulfide bonds were quantitatively reduced and the resulting cysteine residues alkylated with iodoacetamide prior to the second dimension, and the procedure repeated prior to in gel-digestion then theoretically all cysteine amino acids will now have a mass of 160 Da and not 103 Da (nominal mass). For a database search, the search engine requires this information if it is to correctly identify any cysteine containing peptides.

It is very difficult to predict the presence of post-translational modifications. A particular example is phosphorylation. It would be unwise to simply use a partial phosphorylation calculation on all the potential residues.

15.3
Peptide Mass Fingerprint

This is where the masses from the PMF are manually inputted. A trypsin digestion yields useful peptides in the 800–3500 Da mass range. Select peptides that fall within this mass range. Using masses much below this range is unadvisable as they be matrix related or too small to be significant in the search. Further, Fenyo *et al.* (1998) demonstrated that a high mass peptide was useful for constraining the database. The number of peptides submitted to the search should also be considered. Too many peptides can be as problematic as too few peptides (see Figure 3.16 in Section 3.3.3). Peptide masses which cannot be matched with the correct protein contribute to the num-

bers of random matches, and it is possible that proteins with a number of random matches may achieve a significantly high score.

15.4
Charge State

In the case of MALDI all the peptides are singly charged. The choice is to input the protonated form, the measured mass given in the spectrum, or the deprotonated form of the peptide. It is important that whatever option is chosen, it matches the masses used in the mass list. If these two pieces of information are different then there is a 1-Da discrepancy, and this trivializes the performance of the instrument and significantly reduces the discrimination of the database search.

15.5
Monoisotopic Mass or Average Mass

It is important to select whether the peptide masses are the monoisotopic or average masses. If the incorrect option is selected then the peptide masses have an error of 1 Da, again trivializing the performance of the instrument and significantly reducing the discrimination of the database search.

15.6
Error Tolerance

This option will determine the stringency of the database search and will be dependant on the mass accuracy of masses measured within the peptide mass fingerprint. Choose an error tolerance (ppm value) that allows all the peptides in your peptide mass fingerprint to be included in the search.

If the error tolerance is too stringent then a peptide mass measure to a greater error than the tolerance limit contributes nothing towards the score, even though it may be a peptide from the protein of interest. However, if the error tolerance is too lenient then the number of spurious matches also increases, reducing the specificity.

Hint: ***If a 100 ppm error tolerance is selected then a window of 100 ppm will be applied to all the theoretical masses in the database, only the experimental masses which have an error 100 ppm or less will have be accepted by the search.***

Extra information Any additional information may help to constrain the search, enhancing the discrimination. Thus molecular weight and pI may be important. It may be useful to remember though that many proteins may be post-translationally modified or alternatively spliced, altering the mass and/or the pI.

15.7 Taxonomy

If the origin of the sample is known then the search can be limited to that particular species or groups of species. This is useful for fully sequenced and well characterized genomes. However if the species is not fully sequenced then it may well be useful not to restrict the search engine to just search against this species. For instance if the species of origin is mouse, it may also be advantageous to also search against rat and human; a homologous protein may be matched.

Part III
Trouble Shooting

1
Two-dimensional Electrophoresis

Including images of bad gels.

Additional trouble shooting guides for 2-D electrophoresis are found in: GE Healthcare Handbook: 2-D Electrophoresis. Principles and Methods. GE Healthcare Life Sciences (2005); 80-6429-60.

Trouble shooting guides for one-dimensional electrophoresis and blotting are found in: Westermeier R. Electrophoresis in Practice, 4th edition. Wiley-VCH, Weinheim (2004).

1.1
Sample Preparation

Symptom	Cause	Remedy
Cleanup based on precipitation: pellet cannot be seen after centrifugation.	A small protein pellet is not always easy to see.	Always follow the instruction stringently including the direction of the hinges during centrifugation.
Cleanup based on precipitation: pellet does not go back into solution during resolubilization.	Vortexing has been performed.	Do not vortex! Follow the hints in Part II, Chapter 1: Sample preparation.
Quantification: the signal is drifting.	Color reaction influenced by timing.	Follow a well organized time schedule, as indicated in the instruction.

1.2 Isoelectric focusing in IPG strips

Symptom	Cause	Remedy
Rehydration solution is distributed unevenly within the gel strip.	Some coating in the strip holder or reswelling tray.	Wash strip holder and reswelling tray with detergent, rinse with deionized water.
	Uneven pipetting of the rehydration solution.	Pipette the solution as an even streak.
	Reswelling tray or IPGphor not leveled.	Adjust the level of the reswelling tray or the IPGphor on the bench.
Rehydration liquid is left in the reswelling tray or strip holder.	Rehydration time too short.	Rehydrate at least for 6 hours without and 12 hours with sample.
	Liquid volume too high.	Follow the recommendations on the package.
	IPG strips improperly stored.	Always store IPG strips in the freezer, do not leave them on the bench at room temperature for too long a time.
Basic part of the gel comes off during rehydration.	Surface has been damaged during removal of cover film.	Always start at the acidic side to remove the cover film.
Voltage too low (8 kV or 10 kV not reached).	Short strips (7 cm and 11 cm) are used, 8 kV is reached in those strips only with some samples under exceptional conditions.	Nothing to worry about.
	Poor quality of urea and/or thiourea.	Use high quality urea and thiourea; remove ions with a mixed bed ion exchanger.

Symptom	Cause	Remedy
	Too much salt in the sample.	Remove salts by microdialysis or precipitation; replace PBS for cell washing with something non-ionic like 250 mmol/L sucrose/1 mmol/L Tris.
	TCA left in the sample from precipitation.	Use 10% water/90% acetone for washing instead of pure acetone.
Bromophenol blue band stops and does not migrate completely into the anode.	Too much salt in the sample.	See above.
Strip starts to burn at a certain position.	Too much salt in the sample.	See above.
Visible brown band develops.	Tris-chloride in the sample and rehydration solution.	Run the sample in the Manifold.
Cover fluid (paraffin oil) leaks out of the cup loading strip holder during IEF.	High protein and salt load cause water transport that carries the oil with it.	Reduce the initial voltage and prolong the first low-voltage steps. Or, alternatively use the Manifold, which is more tolerant to high protein and salt concentrations.
The basic part of the strip swells during IEF and becomes mechanically unstable.	Many cations in the sample, for instance Tris.	Avoid adding too much Tris-base, replace it by adding 25 mmol/L spermine base, treat the sample with microdialysis; apply IEF strips soaked with deionized water between gel and electrode to accommodate ions.
Urea crystallized and IPG strip dried during IEF.	Not enough cover fluid used.	Use 3 mL cover fluid for 18 cm and 24 cm strip holder and 105 mL for the manifold.
	Cover fluid has been moved around in the strip and leaked out.	Reduce the initial voltage and prolong the first low-voltage steps.

Symptom	Cause	Remedy
	Running temperature was incorrect.	Set the rehydration and separation temperature to 20 °C.

1.3 SDS PAGE

Symptom	Cause	Remedy
Gel casting with 14-gel multicaster: formation of a gap between gel and spacer on the side where the solution is introduced from the bottom.	Contraction of monomer solution during polymerization in the bottom reservoir.	Pour sufficient displacing solution in the balance chamber to produce the thin blue layer in the entire area of the caster bottom.
Unequal gel levels in a multicaster.	Polymerization has started before solutions have been leveled out completely.	Pre-cool the monomer solution in the refrigerator.
Uneven or curved upper edge.	Improper overlay. Affinity of overlay solution to the spacer material.	Spray 0.1% (w/v) SDS over the edges with a plant sprayer instead of overlaying water-saturated butanol or isopropanol.
Casting gradient gels: curved upper edge.	Polymerization started at the bottom, and caused thermal convection, and uneven contraction of the gel.	Make sure that the polymerization starts at the top by adding less catalyst to the dense solution.
The gel becomes opaque during polymerization.	The cross-linking factor is too high.	Check the bisacrylamide content. Use ready-made acrylamide/Bis solution.
Upper buffer tank is leaking.	Hydrostatic balance between the buffers is not achieved.	Fill enough buffer into the lower tank according to the instruction.
Migration of dye front is too slow.	Poor quality of reagents and water.	Make sure that you use only high quality of reagents, check the water.

Symptom	Cause	Remedy
	Upper buffer contains chloride ions.	Do not titrate the running buffer.
	PPA buffer system: upper and lower buffer have mixed because of leakage of upper buffer tank.	Fill enough buffer into the lower tank according to the instruction. Do not overfill lower and upper chamber.
The dye front is not straight in the beginning.	Uneven conductivity across the IPG strips originating from the fixed pH gradient.	Nothing to worry about. The dye front will become straight when it reaches about the middle of the cassette.
The dye front becomes curved during the run.	Current leakage in the ready-made gel cassette.	Remove all excess buffer or water with the roller. Close the cassette properly.
Irregular dye front.	Poorly polymerized gels.	Optimize catalyst amounts and use high quality reagents.
	Smiling effect because of overheating.	Use cooling during the run and/or reduce the power setting.

1.4 Staining

Symptom	Cause	Remedy
Background formed like a “sail” in the basic part of the gel.	Complexes between SDS and basic carrier ampholyte.	Increase fixing time or destaining time. Try alternative IPG buffer or carrier ampholytes.
Silver staining: negative spots.	Dependent on structure of protein.	Stain briefly with Coomassie blue first.
Silver staining: “donut"-shaped spots.	Happens with highly abundant proteins.	Stain briefly with Coomassie blue first.
Silver staining: dark background.	Silver nitrate reduced by sulfuric compounds, like softeners.	Use glass or stainless steel trays only.

Symptom	Cause	Remedy
	Bad water quality.	Check water with a few drops of silver nitrate solution before you use it for silver staining.
Sypro Ruby, RuBPS, or Deep Purple staining: dye precipitates.	Not sufficiently washed gel or wrong tray material.	Wash the gels after staining, use only dark polypropylene containers.

1.5 DIGE Fluorescence Labeling

Symptom	Cause	Remedy
The pH of the protein lysate is less than pH 8 prior to labeling.	The lysis of the cells has caused a drop in the pH. The cell wash buffer was not completely removed prior to addition of the lysis buffer.	1. Increase the buffering capacity of your lysis buffer to 40 mmol/L Tris. 2. Increase the pH of the lysis buffer by the addition of a small volume of 50 mmol/L NaOH. Or add an equal volume of the lysis buffer that is at pH 9.5.
	The samples have been stored or shipped on dry ice, CO_2 has diffused into the samples, created carbonic acid.	Seal reagent tubes very thoroughly with adding Parafilm® between cup and cap, and placing them into several layers of plastic bags in the dry-ice box.
The fluorescent signal is weak when scanned on a 2D gel.	The dyes after reconstitution have a fixed lifetime in DMF that may have been exceeded.	Check the expiry date on CyDye.

Symptom	Cause	Remedy
	The DMF used to reconstitute CyDye was of poor quality or has been opened for longer than 3 months.	Always use the 99.8% anhydrous DMF to reconstitute your CyDye. Breakdown products of DMF include amines which compete with the protein for the CyDye labeling
	CyDye has been exposed to light for long periods of time.	Always store CyDye in the dark.
	CyDye has been left out of the –20 °C freezer for a long period of time.	Always store CyDye at –20° C and only remove them for short periods to remove a small aliquot.
	The pH of the protein lysate is less than pH 8.	Increase the pH of the lysis buffer by the addition of a small volume of 50 mmol/L NaOH. Or add an equal volume of the lysis buffer that is at pH 9.5.
	Insufficient mixing of dye and protein sample.	Click fingers against the reaction tube during labeling from the first moment on.
Problems specifically with minimal labeling (lysine labeling).		
The fluorescent signal is weak when scanned on a 2D gel.	Primary amines such as Pharmalytes or ampholytes are present in the labeling reaction competing with the protein for CyDye.	Omit all exogenous primary amines from the labeling reaction.

Symptom	Cause	Remedy
	DTT or other substances such as SDS are present in the labeling reaction at too high a concentration.	Remove the substances from the labeling reaction if not essential. If they are essential test if the reduction in labeling efficiency can be counterbalanced by increasing CyDye concentration.
	The protein lysate concentration is too low, i.e. less than 2 mg/mL.	Make a new batch of protein lysate reducing the volume of lysis buffer to increase the protein concentration. Or precipitate the proteins and resuspend them in a smaller volume of lysis buffer; check the pH and concentration of the new sample before labeling.
Vertical streaks and/or vertical spot arrays mainly in the basic area.	Multiple lysine labels caused by overlabeling, too high CyDye/protein ratio.	Reduce amount of CyDye, increase amount of proteins, and improve protein quantification procedure.
Problems specifically with saturation labeling (cysteine labeling)		
Spots shifting in horizontal direction.	Overlabeling, too high label/cysteine ratio in the particular sample type.	Decrease amount of reductant and CyDye for this particular sample type. Optimize procedure with same–same experiment.
Spots elongated in vertical direction and vertical streaking.	Underlabeling, too low label/cysteine ratio in the particular sample type.	Increase amount of reductant and CyDye for this particular sample type. Optimize procedure with same–same experiment.

Symptom	Cause	Remedy
Dark fluorescent background.	Too much Bind-Silane applied.	Work strictly according to the instruction and remove excess of Bind-Silane with lint-free tissue.
Many dust particles.	Powder from gloves.	Use only powder-free gloves.
Fluorescent streaks and/or areas not correlated to the front.	Contaminations with the ink of a pen.	Do not write on cassettes or casting box with a pen, also not with a waterproof pen.
The IPG strips appear with high background.	The cassette has been scanned with the film-support of the IPG strip down.	Apply the cassette with the correct orientation, in spite of the possibility of correcting the image later by flipping it with the image analysis program.

1.6 Results in 2-D Electrophoresis

Symptom	Cause	Remedy
No or very few spots.	Low protein content.	Check with reliable quantification method; check your entire procedure with a standard, like E. coli lyophylisate; try alternative sample extraction procedure.
	Proteins have formed complexes and did not migrate into the gel.	Check sample preparation procedure, apply cleanup based on precipitation.

Symptom	Cause	Remedy
	Problem(s) with silver staining.	Check, whether all the solutions have been made correctly, the time for each step has been correct, and no step has been forgotten. Do not use plastic trays, but glass or stainless steel.
Horizontal line across the gel a few cm below the upper edge with no or blurred protein spots between the upper edge and the line.	Depletion of glycine.	Use 2× SDS Tris-glycine buffer in the upper buffer tank, fill enough buffer into the upper buffer chamber
Missing protein spots in the high molecular weight area.	High molecular weight proteins did not enter the IPG strip.	Perform active rehydration with 50 V applied to the IPG strip for 12 hours.
	High molecular weight proteins formed aggregates in the first phase of isoelectric focusing.	Apply "worst case conditions" during isoelectric focusing, which means lower starting voltages over longer time periods.
	Equilibration of IPG strip was too short.	Increase equilibration steps to 2× 20 minutes
	Equilibration of IPG strips was not efficient.	Increase SDS concentration in the equilibration buffer to 6% (w/v).
	Poor transfer of proteins from the IPG strips to the SDS gel.	Prolong the protein transfer phase at low power setting before you switch to the separation conditions.
Missing proteins.	Sample application on IPG strips was not optimized.	Try all alternatives, also cup loading on different sides.
	Equilibration was not efficient enough.	Increase SDS content in equilibration buffer from 2% to 6%.

Symptom	Cause	Remedy
Vertical streaking.	Dirty glass cassettes.	Clean glass plates thoroughly, touch them only with gloves.
	Inefficient equilibration of the IPG strip.	Equilibrate at least 2× 15 minutes. Use iodoacetamide in the second equilibration step.
	Electroendosmotic effects during first phase of SDS electrophoresis.	Prolong the protein transfer phase at low power or current setting before you switch to the separation conditions.
	Equilibration under oxidative conditions.	Equilibrate first for 15 minutes in presence of DTT and then 15 minutes in presence of iodoacetamide.
Spots elongated in the vertical direction.	Depletion of the upper buffer, because of insufficient volume.	Fill enough buffer into the upper buffer chamber: 1.2 L into the Ettan DALT 6 and 2.5 L into the Ettan DALT 12.
	Depletion of the upper buffer	Use 2× SDS Tris-glycine buffer in the upper buffer chamber.
	Poor quality of chemicals.	Use only high-quality chemicals. In case of doubt try reagents from an alternative supplier.
	High glycoprotein content.	Use gradient gels and Tris-borate in the cathodal chamber instead of Tris-glycine.
Vertical streak and blurred pattern in the acidic area, when thiourea was used in the first dimension.	Contaminated reagent.	Try another batch of thiourea.

Symptom	Cause	Remedy
	Focusing of thiourea together with some other reagents.	Reduce the volt-hours applied to the IPG strip. Follow the instructions supplied with the IPG strips, sometimes applying lower volt-hours.
Vertical perturbation in the center of the gel, particularly in gels cast close to the back of the six-gel caster.	Heat development in the solution feeding channel during polymerization causes local modifications in gel structure.	Pre-cool the monomer solution in the refrigerator.
Blurred or twinned spots.	Gel strengthener was used, gel composition is modified.	Be careful when using gel strengtheners.
Vertical gap(s).	Air bubbles between first and second dimension gel.	Place the IPG strip carefully on the SDS gel and seal it carefully with agarose.
	Amphoteric buffer in the cell culture, for instance HEPES, occupies a part of the pH gradient.	Avoid amphoteric buffers.
Double spots in vertical direction.	IPG strip not applied in the correct way.	Plastic film has to be in contact with the glass plate.
Multiple spots in vertical direction in preparative gels.	Insufficient DTT amount.	Increase amount of DTT during the first equilibration step.
Clouds of spots in the low M_r area.	Proteins partly digested into peptides.	Add protease inhibitors during protein extraction; use cup loading, paper bridge loading, or active rehydration loading under voltage. Treat the sample with a cleanup procedure based on precipitation.
Horizontal streaking.	Particles in the sample solution.	Always centrifuge the sample before application on the IPG strip. If necessary, prolong centrifugation.

Symptom	Cause	Remedy
	Underfocusing.	Prolong the volt-hours in the last IEF step.
	Overfocusing: labile proteins are degrading at their pI. This happens particularly in the basic range.	Shorten the volt-hours in the last IEF step.
	Urea and/or detergent concentration too low.	Increase concentrations of urea to 9 mol/L and CHAPS to 2% or 4%.
	Wrong precipitation procedure was applied.	Do not apply ammonium sulfate precipitation. Either use cleanup kit based on precipitation or apply the procedure according to Wessels and Flügge (1984) (methanol/chloroform).
	Incomplete rehydration of the IPG strip.	Check the rehydration conditions.
	TCA was not completely removed from pellet after precipitation cleanup.	Add two or three washing steps with ice-cold 10% water/90% acetone.
	Instability of some proteins because of wrong sample application.	Try alternatives, like cup loading at the acidic or basic end of the IPG strip.
	Highly abundant proteins formed ridges and were squeezed out, and then became distributed over the surface.	Run IPG strips containing highly abundant proteins only with gel surface up.
Horizontal streaking in the acidic area.	Nucleic acids in the sample.	Apply a cleanup procedure based on precipitation. Alternatively add benzonase or DNAse and RNAse before adding the urea (and thiourea).

Symptom	Cause	Remedy
Horizontal streaking in the basic area.	DTT depletion in the basic part of the gradient.	Pre-rehydrate the strips with DeStreak solution and apply reduced proteins via cup-loading on the anodal side.
	Samples prepared with DeStreak solution or reagent.	Never treat the sample with DeStreak. Treat samples with a reductant. DeStreak is only used in the IPG strip, not in the sample.
	DeStreak and reductant has been mixed, resulting in the formation of 2-mercaptoethanol.	Never mix DeStreak and a reductant, see above.
Horizontal streaking in the acidic area and the basic areas with good separation in the center.	Salt content is too high.	Treat the sample with microdialysis or cleanup with precipitation.
	Electroendosmosis effects at the ends of IPG strips. IPG strips have not been embedded with agarose.	Always embed IPG strips with agarose; this equalizes the differences of charged groups in the IPG strip.
	Electroendosmosis effects at the ends of narrow interval IPG strips because of accumulated proteins of higher and lower pIs.	Place filter paper pads soaked in deionized water between electrodes and gel during the last IEF step.
Horizontal streaking in the center with good separation in the acidic area and the basic areas.	Uneven rehydration of the IPG strip, incomplete rehydration in the middle.	During rehydration of the strip, take care that the liquid is distributed evenly over the entire strip length. Alternatively, use a vertical rehydration cassette for rehydration of IPG strips.

Symptom	Cause	Remedy
Protein spots arranged like a string of beads in horizontal direction.	Differential carbamylation of some proteins because of presence of isocyanate.	Do not heat sample. Do not store urea and thiourea solutions at room temperature. Use only high quality urea and thiourea. Remove ionic compounds with mixed bed ion exchanger.
	Storage proteins in plant seed extracts, differentially glycosylated.	No artifact. Nothing to worry about.
Cloudy background and/or front.	Micelles between SDS and the nonionic or zwitterionic detergent have formed.	Reduce content of CHAPS or alternative detergent in the rehydration solution for the IPG strip (e.g. maximum 2% CHAPS).
Blurred and streaky spot pattern in preparative gels.	Insufficient IEF conditions.	Prolong the volthours in the last IEF step by 15%.
	DTT depletion in preparative basic gels.	Add 2.5% DTT, 20% isopropanol, 5% glycerol to the rehydration solution, apply paper wick soaked in rehydration solution plus 3.5% DTT at the cathode. Or apply DeStreak concept (see above).
Blurred spot pattern in ready-made gels.	Liquid and/or bubbles between surface of ready-made gel and glass plate.	Use only low amount of gel buffer and roll out excess buffer and air bubbles completely.
Very basic proteins are lost.	Too many volt-hours applied on basic gradients, autohydrolysis of basic buffering groups in the gel.	Shorten the volt-hours in the last IEF step. Apply pH 7–11 NL strip, this one is stabilized against hydrolysis.

Symptom	Cause	Remedy
„Wrong" proteins coming from other organisms, which should not be present, have been identified in the gel.	Contamination with previous sample or proteins from the laboratory environment.	Use only highly pure reagents; clean equipment thoroughly, particularly reswelling tray and strip holders for IPG strips; filter solutions through a membrane filter.

2
Mass Spectrometry

Symptom	Cause	Remedy
No signal in reflectron mode MALDI MS.	No accelerating voltage.	Check read out from electronics gate.
	Laser not firing.	Contact supplier.
	Laser not firing in correct place.	Re-alignment of laser required.
	Laser power far too low.	Increase laser power.
	Potentially a detector or amplifier problem.	Check signal in linear mode.
Poor analyte signal.	Insufficient protein present in gel plug for digestion.	Run a new gel with a higher protein load.
	Trypsin inactivity.	Prepare trypsin fresh on ice. Perform rehydration step on ice. Ensure correct buffer for trypsin activity.
	Sample concentration too low.	Use 0.1–10 pmol/µL final concentration.
	Sample concentration too high.	Sample signal may be suppressed. Dilute sample to 0.1–10 pmol/µL final concentration.
	Poor crystallization, presence of salt/buffer.	Use a higher concentration on TFA (up to 1%) to improve ionization. Avoid phosphorylated/sulfated buffers.

Symptom	Cause	Remedy
	Poor crystallization, presence of salt/buffer.	Microscale purification to remove contaminants.
	Poor crystallization, presence of nonvolatile contaminants.	Eliminate nonvolatile components from sample before analysis. Do not use nonvolatile components to solubilize sample.
	Poor crystallization, presence of visualization agent, i.e. CBB.	Destain gel plug prior to digestion.
	Laser power below required threshold.	Increase laser power.
	Old matrix.	Prepare fresh matrix.
	Unsuitable matrix.	See Table 3.1 for correct matrix selection.
	Known contaminants, keratin, dominate and suppress analyte signal.	Address sample handling prior to digestion. See Step 8, page 179 ff.
	Poor digestion and extraction efficiency.	Improve digestion and extraction procedure.
Poor resolution across mass range of interest.	Pulse time and voltage not optimized for mass range of interest.	See manufacturer's recommendations.
	Laser poor far above threshold level.	Reduce laser power to optimal point.
	Incorrect reflectron voltage.	See manufacturer's recommendations.
Poor mass accuracy across mass range of interest.	Poor resolution, unable to select ^{12}C peak of the isotopic envelope.	Improve resolution, see above.
	Unable to calibrate, one or both internal calibrants absent.	Perform external calibration. In worst case scenario, respot sample with calibrants added to the mixture.
	Incorrectly calibrated.	Check internal calibrants and their masses.

Symptom	Cause	Remedy
Unsuccessful or ambiguous protein ID from PMF.	Insufficient peptides.	Acquire more specific information; combine PMF with composition or partial sequence. Acquire actual sequence.
	Protein does not exist in the database or low homology with other proteins.	Acquire sequence data from product ion MS/MS or PSD experiments.
	Sample is a mixture.	Acquire more specific information.
	Error tolerance too high due to poor mass accuracy.	Constrain the database search by improving mass accuracy.
	Keratin contamination.	Remove known keratin peaks from mass list (see Step 8) and repeat search.
	Incorrect database search settings.	Check settings: enzyme, error tolerance, fixed and potential modifications, taxonomy, charge state and mono-isotopic or average masses.
	^{12}C isotope not selected from mass list.	
Non-quantitative derivatization CAF.	Reagent not freshly prepared.	Always prepare reagent freshly and use immediately.
	Residual ammonium bicarbonate.	Perform a micro-scale purification.
	Incorrect ratio of reagent to sample.	Ensure reaction conditions.
	Reaction pH not optimal.	Ensure correct reaction conditions.
	Reaction pH not optimal.	Ensure correct reaction conditions.

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Glossary of Terms

Term	Definition
General	
Analysis of variance (ANOVA)	Statistical tool to test differences among several group means, whether they are different enough not to have occurred by chance.
Background subtraction	The process in which the background (chemical and detector noise) is subtracted, leaving the peaks above the noise at the base level.
Dalton (Da)	According to the guidelines of the SI, the use of the term Dalton for 1.6601×10^{-27} kg is no longer recommended. However it is still a current unit in biochemistry.
Dendrogram	A tree diagram to display data sets partitioned into clusters.
Expression proteomics	The massive parallel study of highly heterogeneous protein mixtures with high throughput techniques like 2-D electrophoresis and MALDI mass spectrometry or MDLC-ESI MS.
Functional proteomics	This research is only possible with non-denatured cell extracts and requires different tools than 2-D electrophoresis and MALDI MS. A smaller subset of proteins is isolated from the highly heterogeneous protein lysate and analyzed with mild techniques that do not affect protein complexes and three-dimensional structures.
Heat map	A two-dimensional diagram to display data sets partitioned into clusters indicated by false color representation.

Term	Definition
False discovery rate (FDR)	Statistical tool to determine new thresholds for large numbers of Student's *t*-tests, to be 95% sure that a change is real.
K-means	Statistical algorithm for clustering. In proteomics it can be employed to produce clusters of proteins which vary in similar ways over time.
Laboratory workflow system	Database and computer network for the integrated laboratory to control the entire workflow in the proteomics factory.
Molecular mass (M_r)	The relative molecular mass is dimensionless. In practice and in publication the dimension Da (Dalton) is used. Particularly in electrophoresis the term "molecular weight" is frequently used.
Normalization	All peaks are reported with peak heights relative to the highest peak height or area in the spectrum.
Optical Density (O.D.)	The unit O.D. for the optical density is mostly used in biology and biochemistry and is defined as follows: 1 O.D. is the amount of substance, which has an absorption of 1 when dissolved and measured in 1 mL in a cuvette with a thickness of 1 cm.
Post-translational modification (PTM)	Over 800 modifications of proteins have been identified, common examples include phosphorylation and glycosylation. PTM analysis is an integral part of proteomics.
Principal component analysis (PCA)	Statistical tool for visualization of multidimensional data by reducing the dimensionality of the data set.
Proteome	The complete profile of proteins expressed in a given tissue, cell or biological system at a given time.
Proteomics	Systematic analysis of the protein expression of healthy and diseased, or treated and untreated cells, tissues or biological fluids.
Quantification	In many papers the term "quantitation" is used, which is incorrect. In gel electrophoresis only relative quantification is possible, but the adjective "relative" is omitted in most papers on this subject.
Surface plasma resonance	A technique which measures biomolecular binding events in real time without the use of labels and has become an established technique for measuring biomolecular interactions

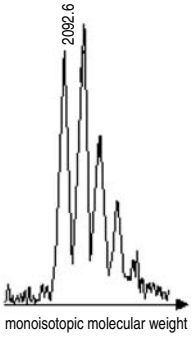
Term	Definition
Student's *t*-test	A statistical tool to check, whether the means of two groups are statistically different from each other.
Electrophoresis	
Analytical 2-D electrophoresis	Proteins are loaded in amounts of 10–100 μg. Mostly broad pH intervals are used in the first dimension.
Electroendosmosis (EEO)	In an electric field, fixed charges on the gel matrix or on a glass surface are attracted by the electrode of opposite sign. As they are fixed, they cannot migrate. This results in a compensation by the counterflow of H_3O^+ ions towards the cathode for negative or OH^- ions towards the anode for positive charges. In gels, this effect is observed as a water flow.
Immobilized pH gradients	Polyacrylamide gels, which contain an in-built pH gradient, created by acrylamide derivatives, which carry acidic and basic buffering groups. Because an immobilized pH gradient is absolutely continuous, narrow pH intervals can be prepared, which allow unlimited resolution.
Isoelectric point (pI)	The pH value where the net charge of an amphoteric substance is zero. Because the pK values of buffering groups are temperature-dependent, this is valid also for the pI. The pI of a protein that can be measured.
Phosphate-buffered saline (PBS)	140 mol/L NaCl, 2.7 mmol/L KCl, 6.5 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, pH 7.4.
Preparative 2-D electrophoresis	Proteins are loaded in the lower mg amounts. Mostly narrow pH intervals are used in the first dimension.
Rehydration	The correct word is "rehydratation". Because this word is a tongue-twister and is used many times for the methodical description of the first dimension separation, the incorrect term "rehydration" is generally used.
Two-dimensional electrophoresis	There are more than one possibility to combine two different electrophoretic separation principles. If not further specified, 2-D electrophoresis means isoelectric focusing under denaturing conditions followed by SDS polyacrylamide gel electrophoresis.

Term	Definition
Western blotting	Electrophoretic transfer of separated proteins from an electrophoresis gel onto the surface of a protein binding membrane for immuno-detection.
Chromatography	
IMAC	IMAC is a technique for the enrichment of phosphopeptides from complex mixtures.
Mass Spectrometry	
Adduct peak	Results from the photochemical breakdown of the matrix into more reactive species, which can add to the polypeptide. Can also result from salt ions, Na^+, etc., that are embedded in the matrix.
Average molecular weight 	The mass of a molecule of a given empirical formula calculated using the average atomic weights for each element. An average mass is obtained in MALDI-TOF-MS when a peak is not isotopically resolved (see mono-isotopic molecular weight).
AQUA	A method for absolute quantitation, employing synthetic peptides containing stable isotopes.
Base peak	The most intense peak in a mass spectrum. A mass spectrum is usually normalized so that this peak has an intensity of 100%.
Bottom-up proteomics	A strategy for protein ID within proteomics. In this approach separated proteins or complex protein mixtures are digested and the resultant peptides analyzed by MS in order to identify the native protein.
Calibrant	A compound used for the calibration of an instrument.
Calibration	A process where known masses are assigned to selected peaks. The purpose is to improve the mass accuracy of an MS instrument.

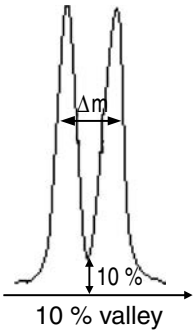
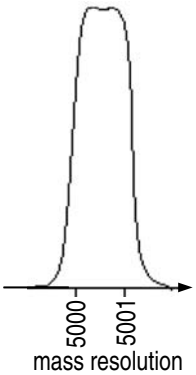
Term	Definition
Centroided mass peak	The centroided mass peak is located at the weighted centre of mass of the profile peak.
Collision induced dissociation (CID)	A process whereby an ion of interest, the precursor ion, is selected, isolated, excited and fragmented by collisions with an inert gas within the mass spectrometer.
Daughter ion (see product or fragment ion)	An ion resulting from CID performed on a precursor ion during a product ion MS/MS spectrum.
Digestion	Cleavage of subject protein by proteolytic enzymes, including trypsin and chymotrypsin.
Dried droplet method	Sample preparation method for MALDI-TOF MS applicable to peptides, protein digests, and full-length proteins.
Electrospray ionization (ESI)	An ionization technique which enables the formation of ions from molecules directly from samples in solution. The ions formed in this process are predominantly multiply charged. Commonly coupled with analyzers capable of tandem mass spectrometry (MS/MS). It is readily coupled with HPLC or capillary electrophoresis.
Electron capture dissociation	An alternative fragmentation method to CID, which generates peptide and protein sequence information, but is restricted to FT-ICR MS
Electron transfer dissociation	A new fragmentation technique, non-ergodic in nature, that fragments peptides by transferring electrons to positively charged peptides and generates peptide sequence information
External calibration	A calibration is performed with a known calibration mixture. The resultant calibration constants (file) are then applied to a separate sample.
Fragment ion (product or daughter ion)	See product ion.
Fragmentation	A physical process of dissociation of molecules into fragments in a mass spectrometer. The resultant spectrum of fragments is unique to the molecule or ion. Fragmentation data can be used to sequence peptides and resultantly provide data for protein identification.

Term	Definition
Full length protein	An intact polypeptide chain, constituting a protein in its native or denatured state. The molecular weight of which can be determined accurately with MALDI and ESI MS.
ICAT	Site specific labelling of cysteine residues with isotope-coded affinity tags for quantitative analysis
In-gel digestion	The embedded protein in the gel is cleaved using enzymes of known specificity. During the process, peptides are formed, which are extracted from the gel for subsequent analysis.
Internal calibration	Calibration where known masses in each spectrum are used to calibrate that spectrum. Greater mass accuracy than an external calibration.
Ion detector	A detector that amplifies and converts ions into an electrical signal.
Ion gate	Typically an electrical deflector that permits certain ions through to later stages of ion optics (open), or deflects unwanted ions out of the way of the later stages of the mass spectrometer. Commonly used in post-source decay (PSD) analysis for the selection of a precursor ion.
Ion source	Region of the mass spectrometer where gas phase ions are produced.
Ion transmission efficiency	Refers to the fraction of the ions produced in the source region that actually reaches the detector.
Ionization	The process of converting a sample molecule into an ion in a mass spectrometer.
Isotope	Atoms of the same element having different mass numbers due to differences in the number of neutrons.
Isotope abundance	The relative amount in nature of certain atomic isotopes.
ITRAQ	labeling of up to primary amino groups with isobaric mass tags for quantitative analysis.
Linear time-of-flight (TOF) mass analyzer	Simplest TOF analyzer, consisting of a flight tube with an ion source at one end and a detector at the other.
Mass accuracy	The ability to assign the actual mass of an ion. This is typically expressed as an error value.

Term	Definition
Mass analyzer	Second part of the mass spectrometer, separating the ions forms in the ion source according to their m/z value. Examples of mass analyzers include ion trap, quadrupole, time-of-flight and magnetic sector.
Mass range	The area of interest to be measured in an experiment. Or the capability of the analyzer.
Mass spectrometer	An instrument that measures the mass to-charge ratio (m/z) of ionized atoms or molecules. Comprises three parts: an ion source, a mass analyzer, and an ion detector.
Mass spectrometry (MS)	A technique for analyzing the molecular weight of molecules based upon the motion of a charged particle in an electric or magnetic field.
Mass spectrum	A plot of ion abundance (y-axis) against mass-to-charge ratio (x-axis).
Mass-to-charge ratio (m/z)	A quantity formed by dividing the mass of an ion (Da) by the number of charges carried by the ion.
Matrix	Necessary for the ionization of sample molecules by MALDI. A small, organic compound which absorbs light at the wavelength of the laser.
Matrix-assisted laser desorption/ionization (MALDI)	Ionization technique, commonly used for the ionization of biological compounds. Sample is incorporated into the crystal structure of the matrix and irradiated with the light from a laser.
Metastable ion	An ion that decomposes into fragment ions and/or neutral species, during its passage through the mass spectrometer.
MS^n or MS/MS/MS	Multistage tandem mass spectrometry where n is greater than 2 and is used for further characterization, such as identifying sites of phosphorylation.

Term	Definition
Monoisotopic molecular weight  monoisotopic molecular weight	The mass of a molecule containing only the most abundant isotopes, calculated with exact atomic weights. With respect to peptide analysis, the mono-isotopic peak is the ^{12}C peak, i.e. the first peak in the peptide isotopic envelope.
Multiple-charged ion	Ion possessing more than a single charge. Characteristic of ESI.
Neutral loss scan	A type of MS/MS experiment. Useful for the indication of individual components in a complex mixture.
N-terminal amino acid	The amino acid residue at the end of a polypeptide chain containing the free amino group.
Parent ion (precursor ion)	Refers to the peak of an ion that will be selected for fragmentation in a product ion MS/MS or PSD spectrum.
Newton's rings	Interference pattern caused by light reflection between a flat surface surface and an adjacent imperfectly flat (slightly spherical) surface. It appears as a series of concentric rings centered at the point of contact.
Peptide-mass fingerprinting	Technique for searching protein databases for protein ID. Subject protein is cleaved and the resultant cleaved peptide masses are used for a database search.
Peak area	The area bounded by the peak and the base line. Can be calculated by integrating the abundances from the peak start to the peak end.
Peak height	The distance between the peak maximum and the baseline.
Peak resolution	The extent to which the peaks of two components overlap or are separated. Compare with FWHM.

Term	Definition
Peak width	The width of a peak at a given height.
Post-source decay (PSD)	A technique describing fragmentation of a precursor ion that occurs in the first field free region of the TOF before the reflectron.
Precursor ion (parent ion)	Ion selected to undergo fragmentation within the mass spectrometer in a product ion MS/MS or PSD spectrum.
Precursor ion scan	A type of MS/MS experiment. Useful for the indication of individual components in a complex mixture.
Product ion (see daughter ion)	An ion resulting from CID performed on a precursor ion during a product ion MS/MS spectrum.
Product ion scan	The principle MS/MS experiment. Involves the selection of a precursor ion to undergo fragmentation within the MS.
Protein characterization	The identification of structural aspects of a protein. Includes amino acid sequence, molecular weight, three-dimensional structure, post-translational modifications and biologic activity of a particular protein.
Protein sequencing	The determination of the order of amino acids in a subject protein or peptide.
Pulsed mass analyzer	Includes time-of-flight, ion cyclotron resonance, and quadrupole ion traps. An entire mass spectrum is collected from a single pulse of ions.
Reflectron	Improves resolution of a mass spectrometer by acting act as an ion mirror. Compensates for the distribution of kinetic energy, ions of the same mass experience in the source.

Term	Definition
Resolution 	Refers to the separation of two ions where resolution, $R = m/m$. For a single peak made up of singly charged ions at mass m in a mass spectrum, the resolution maybe expressed as m/where m is the width of the peak at a height that is a specified fraction of the maximum peak height (for instance full width at half maximum height; FWHM). A second definition for defining resolution is 10% valley. Two peaks of equal height in a mass spectrum at masses m and $m–m$ are separated by a valley that at its lowest point is just 10% of the height of either peak.
Resolving power (mass) 	The ability to distinguish between ions differing slightly in mass-to-charge ratio.
Sample preparation	A crucial stage to achieve efficient, optimal ionization.
SDS electrophoresis	Proteins are separated in a polyacrylamide gel as negatively charged protein–detergent micelles. Secondary, tertiary, quaternary structures and the individual charges of the proteins are cancelled. The migration distances of the resulting zones from the origin correlate roughly to the logarithm of the M_r of the respective protein.
Seeded microcrystalline film method	A sample preparation method. First, a thin layer of small matrix crystals is formed on the sample slide. Then a droplet containing the analyte is placed on top of this layer. This is left to dry. The deposit is washed before the sample slide is inserted into the mass spectrometer. Is a direct replacement of the dried droplet method.

Term	Definition
Signal-to-noise ratio (S/N)	The ratio of the signal height and the noise height. An indication of the sensitivity of an instrument or analysis.
SILAC	Stable isotope labeling with amino acids in cell culture (SILAC) is an approach to in vivo incorporation of a label into proteins for MS-based quantitative proteomics.
Slow crystallization method	A sample preparation method. Here, large matrix crystals are grown. The analyte is added to a saturated matrix solution. Microcrystals are formed. The supernatant is removed and a slurry of crystals is made. The slurry is then applied to the sample slide, allowed to dry and inserted into the mass spectrometer. Can be used when the dried droplet method has failed.
Tandem mass spectrometry (MS/MS)	Used to elucidate structure within the mass spectrometer. Three types of MS/MS experiment can be performed.
Thin layer method	A sample preparation method. Applying the matrix onto the substrate creates a very thin layer of matrix. A droplet of the analyte is then dried onto this layer. Contaminants can now be washed away before introducing the sample into the mass spectrometer. A variant of the dried droplet method.
Threshold fluence	The lowest laser fluence at which analyte can be observed in MALDI, fluence for optimal resolution.
Time-ion extraction (time lag focussing or delayed extraction)	Improved resolution is obtained for a specified mass range by applying a controlled delay between ion formation and acceleration, also called delayed extraction.
Time-of-flight (TOF) analyzer	Separates ions in time as they travel down a flight tube.
Top down proteomics	A strategy for protein ID within proteomics. In this approach, proteins are fragmented directly by MS in order to identify the native protein.
Tuning	The process of optimizing the MALDI instrument's laser power and target position to obtain the best possible sensitivity and signal-to-noise ratio for a specific type of experiment.
Unit resolution	Distinguishes between ions separated by 1 m/z unit.

Term	Definition
Western blotting	Electrophoretic transfer of separated proteins from an electrophoresis gel onto the surface of a protein binding membrane for immuno-detection.

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