Two-Dimensional Electrophoresis with Immobilized pH Gradients for Proteome Analysis

by

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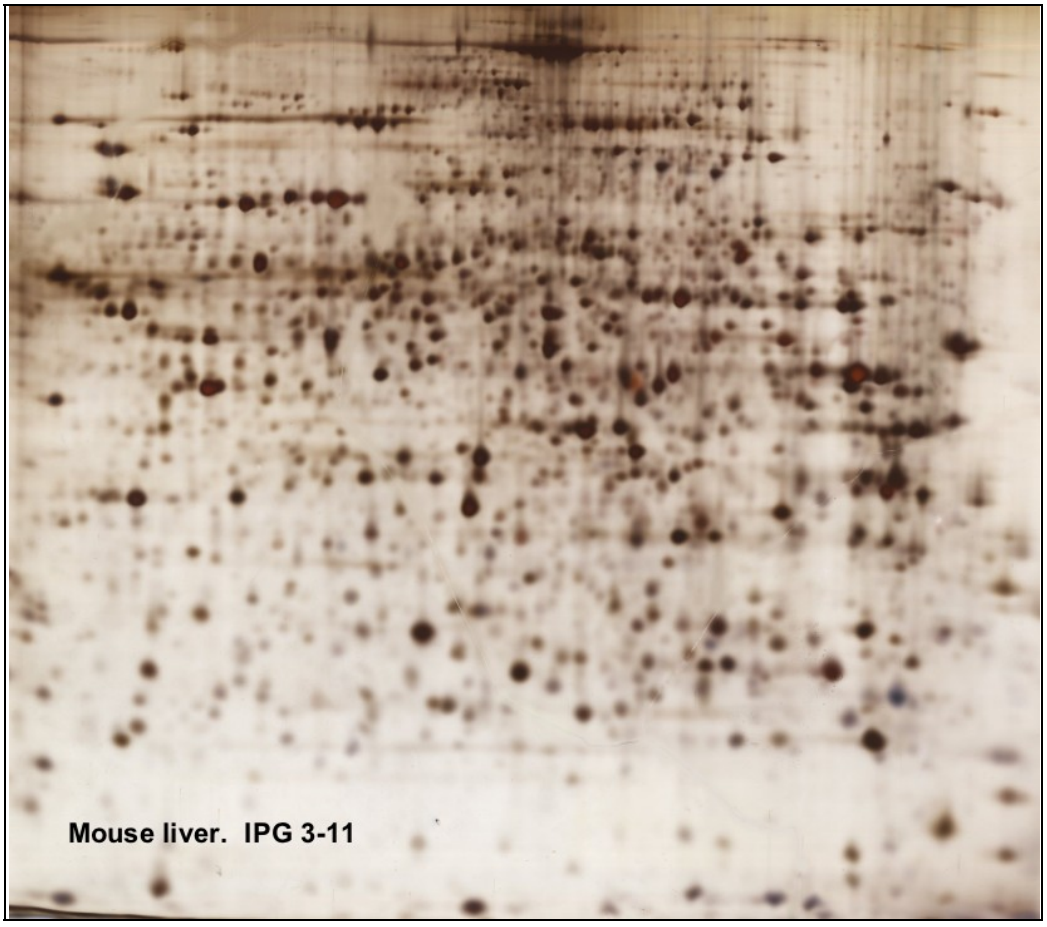

A LABORATORY MANUAL

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Preface

The current laboratory manual of two-dimensional electrophoresis with immobilized pH gradients (IPG-Dalt) was originally prepared for our own use and for the use by those who came to our laboratory to learn the technique we originally described in *Electrophoresis* 1988, 9, 531-546 and updated in *Electrophoresis* 2000, *21*, 1037-1053 and *Proteomics* 2004, *4*, 3665-3685.

Moreover, this manual became the standard protocol of our one-week courses (GDCh 1990-2006, FEBS 1997, etc.), where the participants not only run their own experiments but also their own samples.

Special thanks to Walter Weiss, Günther Boguth, Burghardt Scheibe, Robert Wildgruber, Oliver Drews, and Carsten Lück for contributing current practice not only to the manual but also to all the courses and work shops we run together all over the world.

September 14th, 2007 Prof. Dr. Angelika Görg

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1 INTRODUCTION

High-resolution two-dimensional electrophoresis (2-D PAGE) for the separation of complex protein mixtures was introduced by O'Farrell in 1975 by combining isoelectric focusing (IEF) in the first dimension in presence of urea, detergents and DTT, with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Proteins are separated according to isoelectric point (pI) and molecular mass (Mr), and quantified according to relative abundance. Depending on the gel size and pH gradient used, 2-D PAGE can resolve more than 5,000 proteins simultaneously (~2,000 proteins routinely), and can detect <1 ng of protein per spot. Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modifications. This is in contrast to LC-MS/MS based methods, which perform analysis on peptides, where Mr and pI information is lost, and where stable isotope labelling is required for quantitative analysis. An additional strength of 2-D PAGE is its capability to study proteins that have undergone some form of post-translational modification (such as phosphorylation, glycosylation or limited proteolysis) and which can –in many instances- be readily located in 2-D gels as they appear as distinct 'spot trains' in the horizontal and /or vertical axis of the 2-D gel. In addition, 2-D PAGE not only provides information on protein modifications and/or changes in their expression levels, but also permits the isolation of proteins for further structural analyses by MALDI-ToF-MS, ESI-MS or Edman microsequencing.

Despite these benefits, a major problem of 2-D PAGE has been the exchange of 2-D gel data between laboratories due to spatial irreproducibility between 2-D gels generated by the conventional method of 2-D PAGE using carrier ampholyte (CA) IEF. Equilibrium CA-IEF cannot be achieved because of pH gradient instability with prolonged focusing time, as the pH gradient moves towards the cathode ('cathodic drift') and flattens in the centre ('plateau phenomenon'). Consequently, time-dependent protein patterns are obtained. In addition, reproducibility of pH gradient profiles is limited by the batch-to-batch variability of CA preparations.

The problems of pH gradient instability and irreproducibility were overcome by the introduction of immobilized pH gradients (IPG) for IEF (Bjellqvist *et al.* 1982, Görg *et al.* 1988). IPGs are based on the principle that the pH gradient is generated by a limited number (<10) of well-defined chemicals (the 'Immobilines') which are co-polymerized with the acrylamide matrix. Thus cathodic drift is eliminated, reproducibility enhanced and pattern matching and inter-laboratory comparison simplified. IPGs allow the generation of pH gradients of any desired range (broad, narrow or ultra-narrow) between pH 2.5 and 12. Since sample loading capacity of IPG-IEF is also higher than with CA-IEF, especially in combination with narrow (1 pH unit) or ultra-narrow (0.1 pH unit) IPGs, 2-D PAGE with IPGs is the method of choice for micropreparative separation and spot identification.

1.1 Two-dimensional electrophoresis with IPGs (IPG-Dalt)

The preparation of IPG Strips, as well as the protocol for horizontal and vertical two-dimensional electrophoresis with IPGs in the first dimension (IPG-Dalt) was established in 1988 (Görg *et al.* 1988). Since that time, the protocol has been constantly refined. Compared to classical 2-D electrophoresis with carrier ampholytes (O'Farrell 1975), the employment of IPG-Dalt has produced significant improvements in 2-D electrophoretic separation, permitting higher resolution, especially with narrow-range IPGs, and reproducibility of 2-D patterns both within a laboratory and, more important, between laboratories (Corbett *et al.* 1994; Blomberg *et al.* 1995). Moreover, basic proteins (pI >7.5) normally lost by the cathodic drift of carrier ampholyte focusing or separated by NEPHGE (O'Farrell *et al.* 1977) with limited reproducibility, were perfectly separated under equilibrium conditions using IPGs 4-9, 4-10, and 6-10 for the separation of highly diverse samples such as plant, yeast, mouse liver, human heart, or myeloblast proteins (Görg *et al.* 1988, 1991, 1993). Recently, very alkaline IPGs up to pH 12 were successfully generated for the 2-D electrophoresis of ribosomal proteins and histones (Görg *et al.* 1997, 1998, 1999; Wildgruber *et al*., 2002; Drews *et al.*, 2004).

Due to these features, together with the high loading capacity of IPG-Dalt for micro-preparative runs (up to 10 mg of a crude sample preparation can be applied onto a single 2-D gel) (Hanash *et al.* 1991; Bjellqvist *et al.* 1993; Posch *et al.* 1994), IPG-Dalt combined with protein identification by mass spectrometry (MS) (Aebersold & Mann, 2003) has become the work horse for proteomics. In spite of promising alternative/ complementary technologies (*e.g.,* MudPIT, stable isotope labelling, protein arrays) that have emerged recently, 2-D PAGE is currently the only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures such as whole cell lysates.

1.2 The IPG-Dalt protocol: Principle

The major steps of the 2-D electrophoresis-MS workflow include: (i) Sample preparation and protein solubilization; (ii) Protein separation by 2-D PAGE; (iii) Protein detection and quantitation; (iv) Computer assisted analysis of 2-D patterns; (v) Protein identification and characterization; and (vi) 2-D protein database construction.

The basic protocol of IPG-Dalt is summarized in Figure 1. The first dimension, isoelectric focusing (IEF), is performed in individual IPG gel strips, 3 mm wide and cast on GelBond PAGfilm (either ready-made Immobiline DryStrips^{R} or laboratory-made, obtained from washed and dried immobiline gels cast by the gradient casting technique of Görg *et al.* (1980, 1986). Prior to IEF, IPG dry strips are rehydrated to their original thickness of 0.5 mm with a solution containing 8 M urea, 0.5-2% (non-ionic or zwitterionic) detergent, a

reductant (typically 0.2-0.5% dithiothreitol (DTT)) and 0.5% carrier ampholytes. The rehydrated IPG strips are then placed onto the cooling plate of an electrofocusing chamber and sample cups are placed onto the surface of the gel strips. Sample entry is critical, and best results are obtained using diluted samples dissolved in 9.5 M urea, 2-4% non-ionic or zwitterionic detergent, 1% DTT, and 2% carrier ampholyte (O'Farrell 1975), or -in the case of more hydrophobic proteins- by a mixture of 2 M thiourea and 7 M urea instead of 9.5 M urea and/or other detergents (Rabilloud *et al.* 1997). For better sample entry, a low voltage gradient is applied across the gel for the first hour. Voltage is then increased to 3500 V (Multiphor) (Görg *et al.* 1988), or even up to 8000 V (IPGphor) until the steady state with constant focusing patterns is obtained. As an alternative to cup-loading, samples can also be applied by in-gel rehydration (Rabilloud *et al.* 1994). The latter procedure may be advantageous for high sample loads such as for micropreparative 2D-PAGE. An exciting development for simplification of IPG-IEF is the introduction of an integrated system (IPGphor) where in-gel rehydration and IEF are performed in one step overnight, without personal assistance (Islam *et al.* 1998).

Whatever system is used for isoelectric focusing, after IEF to the steady state, the IPG strips are equilibrated in presence of SDS, DTT, urea, glycerol and iodoacetamide (IAA) (Görg *et al.*, 1987), and then placed onto the surface of a horizontal or on top of a vertical SDS gel (Görg *et al.,* 1988).

For horizontal set-ups, the laboratory-made or ready-made SDS-PAGE gel (ExcelGel SDS), cast on plastic backing, is placed onto the cooling plate of a horizontal electrophoresis system, and the equilibrated IPG gel strip is transferred gel-side-down onto the surface of the the SDS gel alongside the cathodic electrode wick or buffer strip. For vertical setups, the equilibrated IPG gel strips are loaded on top of vertical SDS polyacrylamide gels, with or without embedding in agarose. Vertical setups are especially useful for multiple runs (up to 20 at a time) (Anderson & Anderson, 1978).

Upon completion of electrophoresis, the polypeptides are either stained with silver nitrate or Coomassie Brilliant Blue, or detected by fluorescence staining or autoradiography. For quantitation of differentially regulated proteins, DIGE technology is the method of choice. For specific detection of PTMs such as glycosylation, phosphorylation etc. multiplexed stainings in a singe 2D gel can be used. Alternatively, proteins are transferred („blotted") onto an immobilizing membrane and detected with specific reagents such as antibodies or lectins.

For spot identification, the spots are excised from the gel (or the blotting membrane) and subjected directly or after enzymatic or chemical cleavage to mass spectrometric analysis (MALDI-ToF-MS or ESI-MS).

Legend to Figure 1

(A) Assembly of the polymerisation cassette for the preparation of IPG and SDS gels on plastic backings (Glass plates, GelBond PAGfilm and 0.5 mm thick U-frame)

(B) Casting of IPG- and/or pore gradient gels

(C) Cutting of washed and dried IPG slab gels (or Immobiline DryPlates) into individual IPG strips

(D) Rehydration of individual IPG strips in a vertical rehydration cassette,

(E) in the reswelling tray, or

(F) in the IPG strip holder (IPGphor)

(G) IEF in individual IPG gel strips directly on the cooling plate of the IEF chamber,

(H) in the DryStrip kit, or

(I) on the IPGphor

(K) Storage of IPG strips after IEF

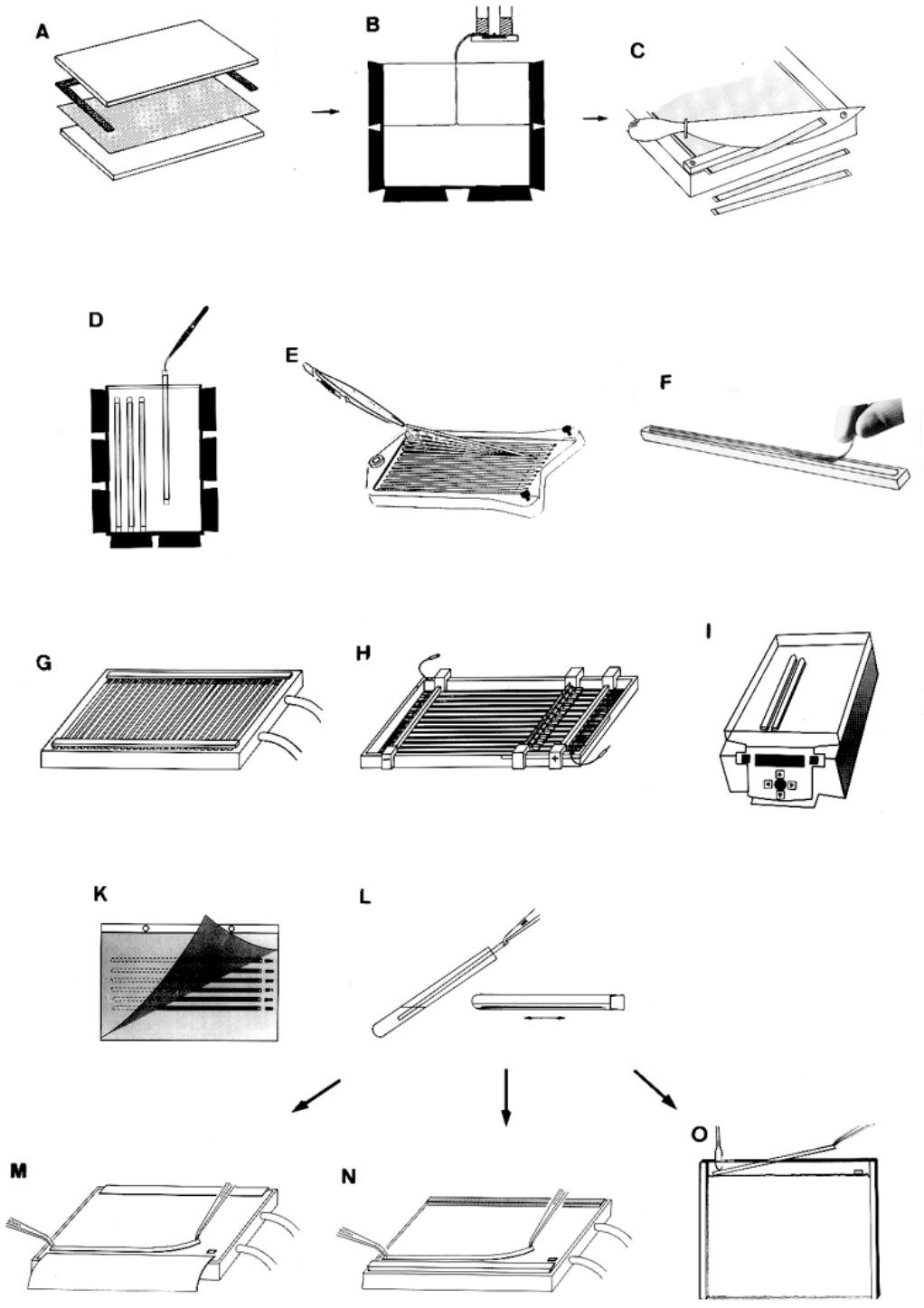
(L) Equilibration of IPG strips prior to SDS-PAGE

(M) Transfer of the equilibrated IPG strip onto the surface of the laboratory-made horizontal SDS gel along the cathodic electrode wick, or

(N) onto the surface of a ready-made horizontal SDS gel along the cathodic buffer strip

(O) Loading of the equilibrated IPG gel strip onto the surface of a vertical SDS gel

Figure 1 Procedure of IPG-Dalt based on the protocol of Görg *et al.* (1988, 2000)



2 SAMPLE PREPARATION

2.1 Sample preparation and protein solubilization

To take advantage of the high resolution of 2-DE, proteins of the sample have to be denatured, disaggregated, reduced and solubilized to achieve complete disruption of molecular interactions and to ensure that each spot represents an individual polypeptide. The major problems concerning the visualization of proteins from total cell or tissue extract lie in the high dynamic range of expression, and the diversity of proteins with respect to molecular weight, isoelectric point and solubility. Although a one-step procedure for protein extraction would be highly desirable with regard to simplicity and reproducibility, there is no single method of sample preparation that can be universally applied to all kinds of samples analyzed by 2-D PAGE. Although a large number of „standard“ protocols has been published, these protocols have to be adapted and further optimized for the type of sample (*e.g.* microbial cells or mammalian tissue) to be analyzed, as well as for the proteins of interest (*e.g.* „soluble“ cytosolic or highly „insoluble“ membrane proteins, respectively). Some general recommendations, however, can be made: (i) Sample preparation should be as simple as possible to increase reproducibility, and (ii) protein modifications during sample preparation must be minimized since they might result in artifactual spots on 2-D gels. In particular, samples containing urea must not be heated in order to avoid charge heterogeneities due to carbamylation of the proteins by isocyanate formed in the decomposition of urea.

The fundamental steps in sample preparation are (i) cell disruption, (ii) inactivation or removal of interfering substances and (iii) subsequent solubilization of the proteins.

Briefly, **cell disruption** can be achieved using osmotic lysis, freeze-thaw cycling, detergent lysis, enzymatic lysis of the cell wall, sonication, grinding with (or without) liquid nitrogen with mortar and pestle, high pressure (*e.g.* French press), homogenization with glass beads and a bead beater, or a rotating blade homogenizator. These methods can be used individually or in combination. All these procedures have their *pros* and *cons*, and the choice will primarily depend upon the type of sample. Typically, microbial cells or plant tissues require rather harsh conditions for the cell lysis due to the robustness of their cell walls, whereas more gentle methods can be applied for mammalian tissues. Gentle cell disruption procedures (*e.g.,* enzymatic lysis) are also required for the preparation of intact organelles (*e.g.,* mitochondriae) for subsequent sub-proteome analysis.

During (or after) cell lysis, **interfering compounds** (*e.g.,* proteolytic enzymes, salts, lipids, polysaccarides, nucleic acids and/or plant phenols) have to be inactivated and/or removed. The two most important parameters are *salt* and *proteolysis*.

Proteases must be inactivated to prevent protein degradation that otherwise may result in artifactual spots and loss of high Mr proteins. To accomplish this goal, protease inhibitors

are usually added, but they may modify proteins and cause charge artifacts. Other remedies are boiling the sample in SDS-buffer (without urea!), or inactivating proteases by low pH (*e.g.*, precipitating with ice-cold trichloroacetic acid (TCA)). However, it should be kept in mind that it may be rather difficult to completely inactivate all proteases. TCA/acetone precipitation is very useful for (i) minimizing protein degradation, for (ii) removing interfering compounds, such as salt, or polyphenols, and (iii) for the enrichment of very alkaline proteins such as ribosomal proteins from total cell lysates (*Görg et al*. 1999). Attention has to be paid, however, to protein losses due to incomplete precipitation and/or resolubilization of proteins. Moreover, a completely different set of proteins may be obtained by extraction with lysis buffer depending on whether or not there was a preceding TCA precipitation step. On the other hand, this effect can be used for the enrichment of very alkaline proteins (such as ribosomal or nuclear proteins) from total cell lysates (Görg *et al*. 1999, 2000).

Salt ions may interfere with electrophoretic separation and should be removed if their concentration is too high (>100 mM); otherwise proteins may precipitate at the site of sample application, giving rise to horizontal and/or vertical streaks. Salt also increases the conductivity of the IEF gel, thereby prolonging the time required to reach the steady-state. In extreme cases, IEF may virtually stop due to salt fronts. Salt removal can be achieved by (spin)dialysis, or precipitation of proteins with TCA or organic solvents (*e.g.*, cold acetone). One alternative is the use of 2-D clean-up kits (*e.g.,* GE Healthcare Lifesciences). Another is dilution of the sample below a critical salt concentration followed by application of a larger sample volume onto the IPG gel. The sample is “desalted” in the gel by applying low voltages (100 V) at the beginning of the run for up to several hours and replacing the filter paper pads beneath the electrodes (where the salt ions have collected) several times (Görg *et al.*, 2000)

High amounts of *lipids* may interact with membrane proteins and “consume“ detergents. Delipidation of lipid-rich biological material (*e.g.,* brain tissues) can be accomplished by extraction with organic solvents (*e.g.,* cold ethanol or acetone). However, severe losses in proteins may be experienced, either because certain proteins are soluble in organic solvent, or because the precipitated proteins do not always resolubilize. Alternatively, high-speed centrifugation and subsequent removal of the lipid-layer has been recommended.

Polysaccarides (especially the charged ones) and *nucleic acids* can interact with carrier ampholytes and proteins, and give rise to streaky 2-D patterns. Moreover, these macromolecules may also increase the viscosity of the solutions and clog the pores of the polyacrylamide gels. Unless present at low concentrations, polysaccharides and nucleic acids have to be removed. A common method is precipitation of proteins with TCA/acetone, but losses in proteins may be experienced due to unsufficient resolubilization of proteins. Other recommendations for the removal of nucleic acids are

digestion by a mixture of protease-free (!) RNAses and DNAses, or by ultra-centrifugation and addition of a basic polyamine (*e.g.,* spermine) (Rabilloud 1999).

Phenols present in plant material (in particular in plant leaves) may interact with proteins and lead to horizontal streaks in 2-D gel patterns. It has been recommended to remove polyphenolic compounds either by binding to (insoluble) polyvinylpolypyrrolidone (PVPP), or by protein precipitation with TCA and subsequent extraction with ice-cold acetone (Granier, 1988; Flengsrud, 1989; Mechin *et al.*, 2003).

Sometimes, *highly abundant proteins* present a problem since they impair separation and detection of lower abundance proteins by limiting the amount of these proteins to be loaded onto the 2-D gel and/or by masking them on the 2-D pattern. Albumin, in particular, which constitutes up to 60 % bulk protein in plasma, is a major problem. There are several albumin removel kits on the market, but due to non-specific binding, one has to be aware that most of these kits remove proteins other than albumin, too (reviewed by Simpson, 2004).

Protein solubilization

After cell disruption and/or removal of interfering compounds, the individual polypeptides must be denatured and reduced to disrupt intra- and intermolecular interactions, and solubilized while maintaining the inherent charge properties. Sample solubilization is usually carried out in in a buffer containing *chaotropes* (*e.g.*, urea and/or thiourea), nonionic and/or zwitterionic *detergents* (*e.g.*, Triton X-100 or CHAPS), *reducing agents*, carrier ampholytes and, depending on the type of sample, protease inhibitors. The most popular sample solubilization buffer is based on O'Farrell's lysis buffer and modifications thereof (9 M urea, 2–4% CHAPS, 1% dithiothreitol, and 2% (v/v) carrier ampholytes) (O'Farrell, 1975). Unfortunately, urea lysis buffer is not ideal for the solubilization of all protein classes, particularly for membrane or other highly hydrophobic proteins. Improvement in the solubilization of hydrophobic proteins has come with the use of thiourea (Rabilloud, 1998) and new zwitterionic detergents such as sulfobetaines (Chevallet *et al*., 1998).

Chaotropes

Urea is quite efficient in disrupting hydrogen bonds, leading to protein unfolding and and denaturation. In contrast, thiourea, which has been introduced by Rabilloud (1998), is better suited for breaking hydrophobic interactions, but its usefulness is somewhat limited due to its poor solubility in water. However, it is better soluble in concentrated urea solutions. Currently the best solution for solubilization of hydrophobic proteins is a combination of 5-7 M urea and 2 M thiourea, in conjunction with appropriate detergents.

The major problem associated with urea in aqueous solutions is that urea exists in

equilibrium with ammonium (iso)cyanate, which can react with the α-amino groups of the N-terminus and the ε-amino groups of lysine residues, thereby forming artefacts such as blocking the N-terminus and introducing charge heterogeneities (altered pIs). To prevent this carbamylation reaction, temperatures above 37°C have to be avoided under all circumstances, and carrier ampholytes (2% v/v), which act as cyanate scavengers, should be included in the urea solution. Given that these precautions have been complied with, it has been demonstrated that protein carbamylation is negligible for a period at least 24 hours, which is sufficiently long for most protein extraction and solubilization protocols (Thoenes *et al.*, 2003). Similar experiments have shown that carbamylation is not a problem during electrophoresis in the presence of urea, even with prolonged run-times, since the urea break-down products are electrophoretically removed (McCarthy *et al.*, 2003).

Detergents (surfactants)

Detergents are utilized to prevent hydrophobic interactions between the hydrophobic protein domains to avoid loss of proteins due to aggregation and precipitation. Since the anionic detergent SDS is one of the most efficient surfactants, solubilization of proteins in (boiling) SDS solution has been recommended for protein solubilization (for details see Boucherie *et al.*, 1995; Harder *et al.*, 1999). However, horizontal streaks in the 2-D pattern are observed if samples initially solubilized in 1% SDS are not diluted with at least four-fold excess of (thiourea/urea) lysis buffer, to displace the anionic detergent SDS from the proteins and to replace it with a non-ionic or zwitterionic detergent to decrease the amount of SDS below a critical concentration (0.2%). Additionally, obtaining sufficient dilution may constitute a major problem when micropreparative protein loads in 2-DE are employed, since the amount of sample volume that can be applied onto an IPG strip is limited. Therefore, non-ionic or zwitterionic detergents are currently favoured for protein solubilization. The most popular non-ionic detergents are NP-40, Triton X-100 and dodecyl maltoside. Regrettably, NP-40 and Triton X-100 are not very effective in solubilizing very hydrophobic membrane proteins. In contrast, zwitterionic detergents such as CHAPS, and sulfobetaines (*e.g.*, SB 3-10 or ASB 14) perform better, and have been shown to solubilize -in combination with urea and thiourea chaotropes- at least several integral membrane proteins (Santoni *et al.*, 2000; Molloy, 2000). More detailed investigations revealed that the efficiency of zwitterionic detergents for solubilizing hydrophobic proteins not only depends on the nature of the protein itself, but also on the presence and nature of other compounds, in particular on the lipid content of the sample (Santoni *et al.*, 1999). Besides these chaotropes and detergents, organic solvents have also been successfully applied for solubilization and IEF of very hydophobic proteins (Molloy *et al.*, 2003; Deshusses *et al.*, 2003).

Regardless all recent advances, these studies demonstrated that there does not exist a single solution for the complex solubility problem of membrane proteins. Most membrane

proteins cannot be solubilized adequately with a single non-ionic or zwitterionic detergent, and empirically testing and optimizing the composition of sample solubilization buffer to improve the solubility of of membrane proteins still remains important (Luche *et al.*, 2003).

Reducing agents

Reduction and prevention of re-oxidation of disulfide bonds is also a critical step of the sample preparation procedure. Reducing agents are necessary for cleavage of intra- and intermolecular disulfide bonds to achieve complete protein unfolding. The most commonly used reductants are dithiothreitol (DTT) or dithioerythritol (DTE) which are applied in excess, *i.e.*, in concentrations up to 100 mM. Unfortunately, these agents are weak acids with pK values between 8.5 and 9, which means that they will ionize at basic pH, and therefore, run short in the alkaline gel area due to migration to the anode during IEF. Moreover, DTT and DTE are not well suited for the reduction and solubilization of proteins which contain a high cysteine content, such as wool keratins. Herbert *et al.* (1998) have proposed tributylphosphine (TBP) as an alternative to DTT. TPB is applied in quite low concentrations (2 mM) due to its stoichiometric reaction. However, this reagent has also several disadvantages, the major of which are its low solubility in water and its short half-life. Moreover, TBP (and its solvent dimethyl-formamide, respectively) is toxic, volatile, and has a rather irritating odor. Alternatively, tris(2-carboxyethyl)phosphine (TCEP) is used in the saturation labelling procedure in fluorescent difference gel electrophoresis (DIGE). In conclusion, in certain cases TBP and TCEP have several advantages over DTT and DTE, but the choice of reductant is predominantly sample specific. For additional information see also the section on 2-D PAGE of alkaline proteins.

Protein concentration

Protein extracts should not be too diluted to avoid loss of protein due to adsorption to the wall of the vessel (glass or plastic). The optimal protein concentration is 5-10 mg/ml, whereas the minimum protein concentration should not fall short 0.1 mg/ml. If samples are rather diluted and contain relatively high concentrations of salts which can interfere with IEF, samples may be desalted (see above). Alternatively, proteins can be precipitated with ice-cold TCA / acetone to remove salts. Diluted samples with a *low* salt concentration may also be applied directly without further treatment, if the dried IPG strips are reswollen in sample solution. In this case, solid urea, CHAPS and DTT are added to the sample until the desired concentation is obtained (Rabilloud *et al.* 1994).

Short-time storage (several hours to overnight) of extracts is often possible in the refrigerator (4°C). For a longer time, storage in a freezer at -70°C, on dry-ice (-78°C) or in liquid nitrogen (-196°C) is mandatory. Repeated freezing and thawing of the sample must be avoided. Make small aliquots (100–200 µl) and thaw only once!

2.2 Sample prefractionation procedures

Since there is no amplification step for proteins analogous to the polymerase chain reaction method for amplifying nucleic acids, and due to the high dynamic range and diversity of expressed proteins, particularly in eukaryotic tissues, it is often preferable to carry out a prefractionation step to reduce the complexity of the sample, to enrich for certain proteins such as low-copy number proteins or alkaline proteins, and to get some information on the topology of the proteins. This can be accomplished by:

- Isolation of specific cell types from a tissue, *e.g.*, fluorescence activated cell sorting (FACS), or laser capture micro dissection (LCM)
- Isolation of cell compartments and/or organelles, *e.g.*, by sucrose gradient centrifugation, or free flow electrophoresis
- Selective precipitation of certain protein classes (*e.g.*, TCA/acetone precipitation for ribosomal proteins)
- Sequential extraction procedures with increasingly powerful solubilizing buffers, for example, aqueous buffers, organic solvents (*e.g.*, ethanol or chloroform/ methanol), and detergent-based extraction solutions
- chromatographic or electrokinetic separation methods, such as column chromatography, affinity purification, electrophoresis in the liquid phase and/or IEF in granulated gels.

The major problem with diseased (*e.g.*, cancerous) mammalian tissues is their heterogenous nature. It is, therefore, of utmost importance to obtain targeted populations (*e.g.*, tumor cells) from tissue specimens (Hanash, 2000). Hence, several microdissection techniques are applied to enrich for targeted cell populations, such as laser capture microdissection (*LCM*) technology that allows the isolation of pure cell populations. Craven & Banks (2001) have demonstrated that samples which had been enrichend with LCM can be analyzed with 2-DE. The drawback of this technology, however, is that it is rather time-consuming because a large number of cells must be dissected to obtain sufficient protein for 2-DE analysis. Another possibility for recovering specific subpopulations of cells from clinical samples is fluorescence-activated cell sorting (*FACS*) of antibody-bound cells (reviewed by Orfao *et al.*, 1996). However, it is not clear to what extent stress is exerted on the cells by this kind of treatment, and whether their protein expression profiles are affected.

Sub-fractionation of cell components, *e.g.*, organelles such as mitochondria by centrifugation in a sucrose density gradient is the most effective method for *organelle* isolation (reviewed by Huber *et al.*, 2003). Other, although less common techniques, are

free flow electrophoresis (FFE) (Hannig *et al.*, 1974; Völkl *et al.*, 1997) or immunoaffinity binding methods. Whereas these procedures can be applied for mammalian cells with relative ease since they do not possess a cell wall, access to organelles is complicated for most microorganisms because in this case a lysis method is required which is both efficient in disrupting the cell wall and gentle enough to guarantee that organelles remain intact. For example, spheroblasts are prepared for the isolation of intact organelles from yeast by digesting the cell wall with polysaccharide-cleaving enzymes prior to liberating the cell content by “gentle“ lysis conditions such as hypotonic solutions and/or mechanical treatment (Zinser & Daum, 1995). The quality of these prepartions is not always sufficient for proteome analysis, but Pfanner and co-workers have established an improved purification protocol for yeast cell mitochondriae (Meisinger *et al.*, 2000) which has been successfully applied for 2-DE/MS analysis of the yeast mitochondrial proteome.

Due to the limitations in sample loading capacity on the first dimension IEF gel, a whole cell lysate may not yield sufficient quantities of lower abundance proteins to be displayed on a 2-DE gel. Precipitation procedures allow increased loading of particular proteins while keeping the total protein load constant. In particular, TCA/acetone precipitation has been found very valuable for the enrichment of alkaline proteins such as ribosomal proteins from total cell lysates (Görg *et al.,* 2000). Additional benefits of TCA/acetone precipitation are inactivation of proteases to minimize protein degradation, and removal of interfering compounds. However, attention has to be paid to protein losses due to incomplete precipitation and/or resolubilization of proteins.

Sequential extraction of proteins from cells or tissues on the basis of their solubility properties is another possibility to enrich for certain protein classes, and to simplify the 2-D pattern for subsequent image analysis and protein identification by MS. *E.g.,* plant proteins from barley and wheat seeds were extracted sequentially with Tris-HCl buffer, aqueous alcohols and urea/NP-40/DTT lysis buffer and then analyzed by 2-D PAGE (Weiss *et al.*, 1992, 1993). In a similar manner, Tris-base was used to solubilize cytosolic *E. coli* proteins (Molloy *et al*, 1998). The resultant pellet was then subjected to conventional solubilizing solutions (urea/CHAPS/DTT), and, finally, the membrane protein rich pellet was partially solubilized using a combination of urea, thiourea, and zwitterionic surfactants. Eleven membrane proteins from this pellet could be identified, including two outer membrane proteins that had previously been known only as an open reading frame in *E. coli*. One approach for improved solubilization of membrane proteins is to treat isolated membrane preparations (*e.g.*, obtained by ultracentrifugation) with sodium carbonate at alkaline pH or chaotropic salts (*e.g.*, potassium bromide) to remove carry over cytoplasmic proteins and only loosely attached peripheral membrane proteins (“membrane washing“, “membrane stipping“). Other procedures to enrich for hydrophobic proteins are based on the differential extraction of membrane proteins by organic solvents, *e.g.*, chloroform/methanol mixtures (Ferro, 2000). The major limitation of these procedures is cross-contamination between the individual fractions.

Several different chromatographic procedures have been used successfully to enrich for low-abundance proteins, including hydrophobic interaction chromatography, hydroxyapatite and heparin chromatography and chromatofocusing (Fountoulakis *et al*., 1999).

Other approaches are based on electrophoretic prefractionation according to isoelectric point in the liquid phase, such as preparative isoelectric focusing (Hochstrasser *et al*., 1991), IEF in a rotating, multi-chamber device (Egen *et al.*, 1988), FFE, or the use of a multifunctional electrokinetic membrane apparatus (Locke *et al.*, 2002) in which samples are separated by charge and/or size. A multicompartment electrolyzer with isoelectric membranes has been developed by Herbert & Righetti (2000). Zuo & Speicher (2001, 2002) have simplified this device for prefractionation of various kinds of samples. These procedures are particularly useful if the prefractionated proteins are then applied onto narrow-range IPG gels (‚zoom gels'). It has been reported that this type of prefractionation allows higher protein load (6 to 30 fold) on narrow IPG gels without protein precipitation and allows detection of low abundance proteins because major interfering proteins such as albumin have been removed. The major drawbacks of most chromatographic and electrophoretic prefractionation procedures in the liquid phase are that (1) sophisticated instrumentation is required, (2) the sample is usually diluted during or after the separation process, and (3) protein precipitation cannot always be avoided.

Recently, a simple, cheap, and fast prefractionation procedure based on flat-bed IEF in granulated gels has been devised by Görg *et al.* (2002). Flatbed IEF in granulated gels for the separation of enzymes was described by Radola as early as 1973. Görg *et al.* have adapted this method for sample prefractionation before loading on 2-D gels with narrow pH ranges. Complex sample mixtures, *e.g.*, mouse liver proteins, were prefractionated in flat-bed Sephadex gels containing urea, thiourea, zwitterionic detergents, DTT, and carrier ampholytes. After IEF, up to ten Sephadex fractions alongside the pH gradient can be removed with a spatula and directly applied onto the surface of the corresponding narrow-range IPG strips for the first dimension of 2-D-PAGE. Proteins in the Sephadex gel fraction are transported electrophoretically into the IPG gel with high efficiency and without any sample dilution.

In conclusion, prefractionation procedures have many advantages in terms of protein enrichment, and visualization of low-abundance proteins. The major disadvantage of most prefractionation procedures lies in cross-contamination between individual fractions and in the fact that they are either time consuming, complicated to handle, require concentration steps due to elution/dilution precedures, and/or do not allow to process more than a few samples in parallel.

PROTOCOLS

I. Extraction and solubilization of cell and tissue samples

The following section provides a brief description of procedures for the extraction and solubilization of microorganisms, plant seeds and leaves, and mammalian tissue samples. In general, cells or tissues are disrupted (singly, or in combination) by techniques such as grinding in a liquid nitrogen-cooled mortar, shearing, or homogenization. During -or immediately after- cell lysis, interfering substances must to be inactivated or removed. Proteins are then solubilized with sonication in urea lysis buffer, thiourea lysis buffer, or SDS sample buffer. Recommended final protein concentration is between 5 and 10 mg/ml. *E.g.,* myeloblasts ($5x10^8$ cells) or human or animal tissue such as liver or heart (50 mg) are typically homogenized under cooling with liquid nitrogen. The resulting powder is then solubilized in 1.0 ml Lysis buffer. For enrichment of alkaline proteins, e.g. from *mouse liver,* a tissue specimen is ground in a liquid nitrogen-cooled mortar, suspended in ice-cold TCA in acetone, and kept at -20°C overnight for protein precipitation. Following centrifugation, the supernatant is discarded and the protein pellet washed in ice-cold acetone containing 0.2% DTT, dried under vacuum and then solubilized in lysis buffer (Görg *et al.,* 1999).

After protein solubilization, extracts containing urea should be centrifuged at 40,000*g* for one hour to remove any insoluble material (cell debris) at 15°C (to prevent urea crystallization at low (4°C) temperatures or protein carbamylation at higher (37°C) temperatures, respectively). SDS extracts may be centrifuged at any temperature between 4°C and 37°C. Prior to IEF, SDS extracts must diluted with at least a 3- to 5-fold excess of urea lysis buffer or thiourea lysis buffer.

Protein extracts can be used immediately, or stored at – 70°C for several months. For analytical runs, typically twenty µl of sample solution are applied onto a single IPG gel strip, whereas for micropreparative runs up to several hundred microliters can be applied, portion by portion. The amount of protein to be loaded onto a single IPG gel strip (separation distance: 180 mm) varies between 50-100 µg for analytical, and 0.5-10 mg for micro-preparative runs, respectively. Alternatively, the sample can be applied directly by in-gel rehydration (Rabilloud *et al.*, 1994, Sanchez *et al.*, 1997).

MATERIALS

Note: Not all of the materials listed below are required for all samples.

Chemicals

CHAPS (*Roche*), Pharmalyte (pH 3-10) (GE Healthcare Lifesciences), dithiothreitol (DTT) (*Sigma-Aldrich*), Serdolit MB-1 (*Serva*), urea, Pefabloc® (*VWR*), Thiourea (*Fluka*).

Reagents

- Urea lysis buffer [9.5 M urea, 1% (w/v) dithiothreitol (DTT), 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3–10) and 10 mM Pefabloc® proteinase inhibitor].
  To prepare 50 ml of urea lysis buffer, dissolve 30.0 g of urea (*GE Healthcare Lifesciences*) in deionized H_2O and adjust the volume to 50 ml. Add 0.5 g of Serdolit MB-1, stir for 10 minutes, and filter. Add 1.0 g CHAPS, 0.5 g DTT and 1.0 ml of Pharmalyte pH range 3–10 to 48 ml of the filtered urea solution. If necessary, add 50 mg Pefabloc® proteinase inhibitor (VWR) immediately before use.
- Thiourea/urea lysis buffer [2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 1% (w/v) DTT, 2% (v/v) carrier ampholytes (pH 3–10) and 10 mM Pefabloc® proteinase inhibitor]
  To prepare 50 ml of thiourea/urea lysis buffer, dissolve 22.0 g of urea (*GE Healthcare Lifesciences*) in deionized H_2O, add 8.0 g of thiourea (Fluka) and adjust the volume to 50 ml with deionized H_2O. Add 0.5 g of Serdolit MB-1 mixed bed ion exchange resin (Serva), stir for 10 minutes and filter. To 48 ml of the urea solution add 2.0 g CHAPS, 1.0 ml of Pharmalyte (pH 3–10), 0.5 g DTT and, immediately before use, 50 mg Pefabloc® proteinase inhibitor (VWR).
- SDS sample solubilization buffer [1% (w/v) SDS, 100 mM Tris-HCl (pH 9.5)]
  To prepare 50 ml of SDS sample buffer, dissolve 0.5 g of SDS and 0.6 g of Tris base in about 40 ml of deionized H_2O. Titrate to pH 9.5 with 4 N HCl, filter, and adjust the volume to 50 ml with deionized H_2O.
- 20% Trichloroacetic acid + 0.2% dithiothreitol in ice-cold acetone (–20°C)
- 0.2% dithiothreitol in ice-cold acetone (–20°C)
- Phosphate-buffered saline (PBS) [0.9% (w/v) sodium chloride in phosphate buffer (10 mM), pH 7.4; is also available commercially]

Important notes: (**1**) Lysis buffer should be freshly prepared. Alternatively, make 1-ml aliquots and store at –70°C (for up to several months, at least). Lysis buffer that has been thawed once should not be refrozen! (**2**) Never heat urea solutions above 37°C to avoid protein carbamylation!

Equipment

Aluminum foil; centrifuge (capable of 40,000 *g*), filter paper; hammer; liquid nitrogen; mortar and pestle, agate stone, plastic spatula; scalpel; stainless steel centrifuge tube; stainless steel sieve, 0.2 mm; ultrasonic homogenizer (*e.g.*, Bandelin Sonoplus HD 60).

METHODS

Microorganisms

The growth conditions of ***microbial cell cultures*** such as bacteria or yeast need to be optimized and standardized. Since the growth phase has an enormous impact on the biochemical state of the cells, the time of sample collection also must be predetermined and standardized. Because the cells may excrete proteases and other extracellular enzymes into the growth medium, and because compounds in the medium can interfere with extraction, the cultures first need to be washed with an isotonic buffer such as phosphate-buffered saline (PBS) or sucrose. To avoid stress induction, these wash buffers should be at the same temperature and pH as the culture (Drews *et al.,* 2002). This wash step also ensures that cell conditions are defined before harvesting.

For cells surrounded by a cell wall, such as yeast, osmotic shock is insufficient for protein extraction. Extensive disruption of these cells is required, either by vigorously shaking the cells in the presence of glass beads, sonicating on ice in (urea/thiourea) lysis buffer, or by heating the sample in the presence of SDS. If necessary, protease-free DNAse and RNAse can be added to digest nucleic acids.

Solubilization of bacterial cell proteins

1. Harvest the cells in a predetermined growth phase after growing under defined conditions. Chemically defined media are preferable if available. At high optical densities, it should be kept in mind that more nonviable cells will contribute to the analyzed proteome. Therefore, the early logarithmic growth phase is widely preferred for harvesting.

2. Centrifuge the cells at 10,000g for 3 minutes. These conditions are sufficient for most types of bacteria. Start with a defined volume, *e.g.*, 10 ml of the culture with an optical density of about 0.5. The pellet should be visible after spinning. Resuspend it in an equal volume of pre-warmed PBS and centrifuge again. Several repetitions might be necessary at this step to get rid of interfering substances (*e.g.*, extracellular enzymes, growth media compounds, etc.

   Notes: If the resulting 2-D gel pattern is not satisfying, *e.g.*, if proteolyis of proteins happened due to incomplete removal of extracellular proteolytic enzymes, the high molecular mass proteins are missing on the 2-D gel; horizontal streaks and/or protein precipitates on the 2-D gel are often caused by high salt concentrations.

3. Suspend the pellet in 0.2 ml of SDS sample solubilization buffer and transfer it to a 1.5-ml microfuge tube. The SDS buffer should be ice-cold, but boiling at 95°C might be necessary if highly active proteases are present.

   Note: The 0.2-ml volume is adapted to a 4-mm diameter sonification tip.

4. Sonicate the sample with an ultrasonic homogenizer (2 x 30 pulses, interval: 1 Hz, pulse duration: 0.3 seconds, 20 kHz homogeneous sound, power output: 60 W).

5. Centrifuge the disrupted cells in an Eppendorf microfuge at maximum speed (14,000*g*) for 30 minutes, preferably at 4°C
6. Carefully collect the supernatant and freeze it in aliquots of 50 µl at –70°C. The extract, if not thawed and refrozen, is stable for up to one year.
7. Dilute the aliquot before IEF with 150 µl of thiourea/urea lysis buffer to displace the SDS from the proteins.

Mammalian tissue samples

Collect ***mammalian tissue samples*** (*e.g.,* mouse liver; biopsy samples) as rapid as possible and freeze immediately in liquid nitrogen at –196°C. Disrupt the samples while still deep-frozen. Small tissue specimens (*e.g.*, biopsy samples) can be wrapped in aluminium foil, immediately frozen in liquid nitrogen, and crushed with a hammer, whereas larger tissue pieces should be ground under liquid nitrogen using a mortar and pestle and then solubilized in lysis buffer.

Solubilization of mouse liver proteins

1. Precool a mortar with liquid nitrogen. Grind the deep-frozen mouse liver in the mortar under liquid nitrogen in the presence of traces of Pefabloc® proteinase inhibitor powder.
2. Immediately after grinding, transfer 60 mg of the ground liver to a microfuge tube containing 1.0 ml of urea (or thiourea/urea) lysis buffer.
3. For improved cell lysis, sonicate the sample on ice five times for 2 seconds each with a 10-second pause between sonication intervals to prevent overheating. Incubate the sample for 30 minutes at room temperature and centrifuge at 40,000*g* for 60 minutes at 15°C.
4. Store the supernatant at –70°C. The protein concentration of the extract should be ~5 - 10 mg/ml.

Enrichment of alkaline mouse liver proteins by TCA/acetone precipitation

1. Grind deep-frozen mouse liver with a mortar and pestle in the presence of liquid nitrogen.
2. Suspend the ground mouse liver (typically 100 mg) in a precooled (–20°C) stainless steel 50 ml centrifuge tube containing 25 ml of 20% (w/v) TCA and 0.2% (w/v) dithiothreitol (DTT) in ice-cold (–20°C) acetone.
3. Keep the centrifuge tube at –20°C overnight to ensure complete protein precipitation. Centrifuge at 40,000*g* for 60 minutes at -10°C. Discard the supernatant and resuspend the pellet in 20 ml of ice-cold acetone containing 0.2% DTT.

4. Centrifuge the sample again and dry the pellet under vacuum. Solubilize the sample by repeated vortexing and/or sonication on ice in an appropriate volume (2-3 ml) of lysis buffer. The resultant protein concentration should be approximately 5-10 mg/ml. Store the supernatants in small aliquots at -70°C.

   Notes: (**1**) The protein pellet may also be solubilized directly in lysis buffer without vaccum-drying. (**2**) This procedure can be simplified by using *GE Healthcare Lifesciences*' 2D sample clean up kit.

Plant samples

Dry plant seeds*,* in which proteases are usually not active, are simply smashed with a hammer and ground with a mortar and pestle, with or without cooling by liquid nitrogen. The plant tissue is then solubilized in lysis buffer, centrifuged, and aliquoted.
Note: It is also possible to extract specific protein fractions, such as H_2O-soluble proteins (albumins) or alcohol-soluble proteins (gliadins) or before IEF (for more details *see* Weiss *et al.,* 1992, 1993).

Plant leaves not only contain proteases, but also high concentrations of phenols which can adsorb proteins and cause streaks on 2-D gels. To avoid the deleterious effects of these substances, disrupt the cells with a mortar and pestle in the presence of liquid nitrogen. Then precipitate the proteins with 20% TCA in pre-chilled acetone (–20°C) essentially as described for enrichment of alkaline mouse liver proteins (*see* Section II). Rinse the pellet twice with cold acetone (–20°C) to remove plant phenols, dry the pellet *in vacuo*, and solubilize the proteins in lysis buffer.

Solubilization of wheat (*Triticum aestivum*) grain proteins

1. Remove the husks with a scalpel if necessary. If necessary, wash the grains briefly with deionized H_2O to remove any adhering impurities and then remove any adhering liquid with filter paper.
2. Transfer the grains into a small bag made from glossy paper (*e.g.*, from an HPLC recorder) and smash them with a hammer.
3. Transfer the smashed grains into an agate stone mortar and grind them into a fine powder that passes through a 200 µm wide stainless steel sieve. For uniform extraction, it is important to obtain particles of similar size. Particles that do not pass through the sieve should be transferred back into the mortar and ground again.
4. Transfer 100 mg of the meal into a centrifuge tube, add 150 µl of deionized H_2O and 350 µl of urea lysis buffer. Gently mix with a plastic spatula and sonicate on ice five times for 2 seconds each with a 10-second pause between sonication intervals to prevent overheating. Leave the sample for 1 hour at room temperature, sonify again, and centrifuge at 40,000*g* for 1 hour at 15°C. Remove the supernatant with a pipette and store it at –70°C.

II. Sample prefractionation using Sephadex-IEF

Complex sample mixtures can be prefractionated in flat-bed (granulated) Sephadex gels containing urea, thiourea, zwitterionic detergents, dithiothreitol (DTT), and carrier ampholytes. After IEF, up to ten Sephadex fractions alongside the pH gradient are removed with a spatula and directly applied onto the surface of the corresponding narrow-range IPG strips for the first dimension of 2D-PAGE. Proteins in the Sephadex gel fraction are transported electrophoretically into the IPG gel with high efficiency and without any sample dilution. This technology has been successfully used for the prefractionation of mouse liver proteins (Görg *et al.*, 2002).

Note: If the slope of the carrier ampholyte-generated pH gradient is unknown, there are several ways to determine where to "cut" and remove individual Sephadex fractions to fit within the corresponding narrow pH range IPG strips. For example, after completion of the separation, it is possible to measure the pH of the fractions with a pH electrode or pH indicator paper. Alternatively, as a preliminary test, small portions of the Sephadex IEF gel fractions can be applied onto an IPG 3–10 strip for the first dimension of 2D-PAGE (*cf.* Görg *et al.*, 2002). The simplest method is to use coloured pI markers which do not interefere with subsequent 2D-PAGE (*cf.* Görg *et al.*, 2007).

MATERIALS

Reagents

- Reswelling buffer [8 M urea, 200 mM hydroxyethyldisulfide (HED), 1% (w/v) CHAPS, 2.5% Pharmalyte (pH 3–10)]

  To prepare 50 ml of reswelling buffer, dissolve 25.0 g of urea (Merck) in deionized H_2O and adjust the volume to 50 ml. Add 0.5 g of Serdolit MB-1 mixed bed ion exchange resin (Serva), stir for 10 minutes and filter. Add 0.5 g CHAPS, 1.2 ml HED (Sigma), and 1.25 ml of Pharmalyte pH range 3–10 to 48 ml of the urea solution.

- Colored low molecular mass isoelectric point (pI) markers (gift from Dr. Karel Slais, Institute of Analytical Chemistry, Brno, Czech Republic). A saturated solution of individual *pI* markers was prepared and stored at 4°C.

- DeStreak® reagent; Sephadex G-100SF; silicone oil (GE Healthcare)

Equipment

IPGphor; IPGphor cup-loading strip holder; Multiphor II horizontal electrophoresis apparatus with power supply and thermostatic circulator; IPG DryStrip Kit; Immobiline DryStrips; IEF electrode paper strips, 1 mm thick ; (GE Healthcare); magnetic strirrer; pH electrode or pH indicator paper; spatula; template made of plastic (customized); plastics sheet for freezing Sephadex fractions and IPG gel strips.

Protein Sample

Mouse liver proteins, solubilized in lysis buffer; protein concentration ~5-10 mg/ml

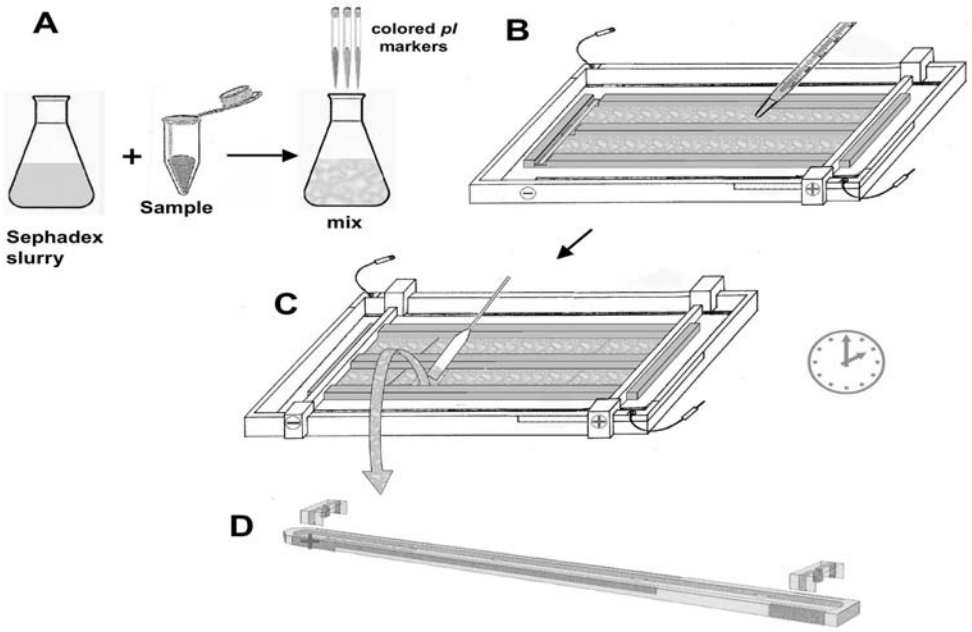


Figure 2 Sample prefractionation by Sephadex IEF (Görg *et al.*, 2002 & 2007)

METHOD

1. Prepare a Sephadex slurry by gently mixing with a spatula: 210 mg of Sephadex G-100 SF with 3 ml of reswelling buffer or DeStreak. [For prefractionation of the more hydrophobic proteins, use a mixture of 6 M urea and 2 M thiourea, 0.4% (w/v) dithiothreitol, 1% (w/v) CHAPS, and 2.5% (v/v) Pharmalyte (pH range 3–10)].
2. Let the Sephadex gel reswell for approximately 24-48 hours at 20°C (**Fig. 2A**). Avoid trapping air bubbles.
3. Insert the customized, tight fitting template made *e.g.* from methacrylate or poly-carbonate into the tray of the IPG DryStrip kit and position on the cooling plate of the Multiphor electrophoresis apparatus (**Fig. 2B and Fig 3**).).
4. Add 1.5 ml of sample solution and 5 µl of each colored *pI* marker solution to 3.0 ml of Sephadex gel slurry by gently mixing with a spatula.
5. Pipette this mixture into the trough from Step 3. Apply 4.5 ml of Sephadex–sample slurry into each individual lane of the template to form an 100 x 20 mm wide and approximately 2 thick gel layer. If the high viscosity of the Sephadex slurry prevents it from spreading evenly, gently tap the tray on the cooling plate until the surface of the slurry is flat and devoid of air bubbles. ***Note:*** Templates of different shapes and sizes (up to 250 x 100 mm) may be used.

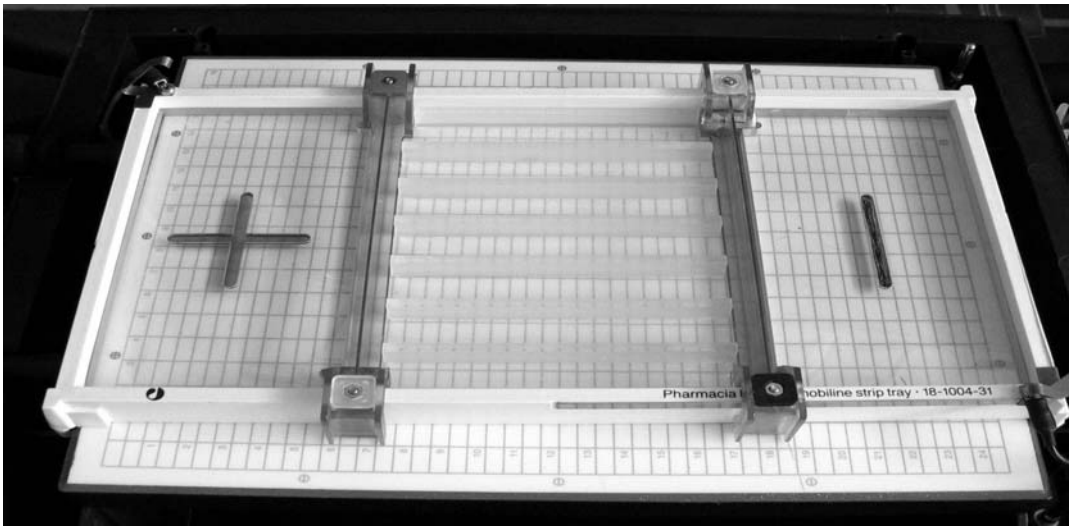


Figure 3 Customized polycarbonate template insetred into the IPG DryStrip kit and placed onto the cooling plate of the Multiphor II unit (Görg *et al.*, 2007)

6. Soak two layers of 1-mm thick IEF electrode paper strips in 10 mM sulfuric acid (anode) and 10 mM sodium hydroxide (cathode) (or simply in deionized H_2O) and insert them in the notch (**Fig. 2B**) at the anodic and cathodic ends of the trough, respectively. Cut off protruding parts so that the strips fit into the template.

7. Apply platinum electrodes on the short side of the trough, so that the proteins are separated along the long axis.

8. Cover the Sephadex gel with a thin layer of silicone oil (1 ml for 20 cm^2 of gel surface) to prevent evaporation or desiccation during the IEF step.

9. Perform IEF at 20°C. Typically, initial voltages of 10 V/cm and terminal voltages of 100 V/cm are applied. For example, for a pH gradient 3–10 and a separation distance of 10 cm, IEF is run with an initial voltage of 100 V for 30 minutes, followed by 300 V for 30 minutes, 600 V for 1 hour, and 1000 V for 2 hours until the proteins have focused.

10. After IEF, up to ten fractions can be collected by slicing the gel with the help of a fractionation grid or by simply scraping off the gel with a spatula (**Fig. 2C**). The Sephadex fractions are either applied immediately onto reswollen narrow pH range IPG strips (*see* Chapter 3), or sealed in plastic sheets and stored at –70°C until further use. Because of problem of instability of carrier ampholyte generated pH gradients, it is important to determine precisely the slope of the pH gradient and to define the exact position where to “cut” and remove the individual Sephadex fractions to fit to the corresponding narrow pH range IPG strips. The most convenient and reliable method is to incorporate synthetic, differentially colored low molecular mass

pI markers (Stastna *et al.,* 2005) (which do not interefere with subsequent 2D-PAGE) in the Sephadex-sample slurry (*see* Step 4).

11. Rehydrate narrow-range IPG strips with 8 M urea (or 6M urea + 2M thiourea), 1% (w/v) CHAPS, 0.4% dithiothreitol (DTT) (or 200 mM HED) and 0.5% (v/v) Pharmalytes (*see* Chapter 3). Alternatively, use DeStreak® as rehydration solution. Place the rehydrated strips in IPGphor strip holders.

12. Transfer the fractionated proteins from Step 9 into the IPG gel by spreading the Sephadex fractions onto the surface (near the anode) of the corresponding narrow-range IPG gel strips (**Fig. 2D**). Add a layer of silicone oil, as in Step 8.
No protein elution from Sephadex fraction prior to IEF is performed and, therefore, no dilution of the fractionated sample happens.

13. Perform IPG–IEF to the steady state using the IPGphor instrument. Running conditions for IEF using the IPGphor are identical to those described in Chapter 3. For better performance, insert moist filter papers (size: 4 x 4 mm^2) between the electrodes and the IPG strip during IEF

14. After termination of IEF, freeze the IPG gel strips at –70°C between two sheets of plastic film. Most of the Sephadex slurry sticks to the plastic film, whereas the other components of the strips are rinsed off during the IPG strip equilibration step. Perform the second dimension (SDS-PAGE) as described in Chapter 3.3. An example of results (comparison of unfractionated and prefractionated samples) is presented in **Fig. 4**.

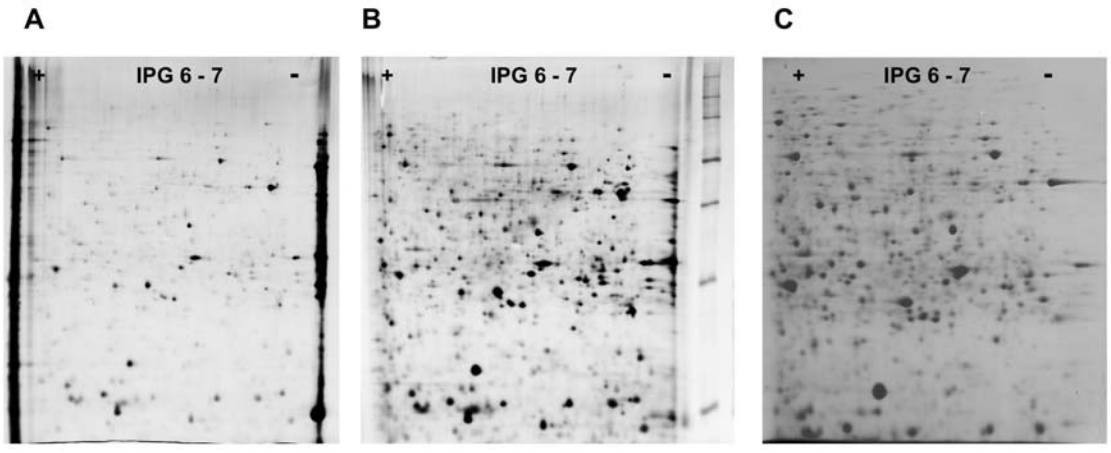


Figure 4 Effect of sample prefractionation for high protein loads applied to 2-DE gels. First dimension: IEF in a narrow pH range IPG 6-7. Second dimension: SDS-PAGE (13%T). Sample: mouse liver proteins. (A) unfractionated sample, protein load 250 µg; silver stain. (B) sample prefractionated with Sephadex-IEF; protein load 250 µg; silver stain. (C) Sample prefractionated with Sephadex-IEF. Protein load 1000 µg. Coomassie Blue stain (Görg *et al.*, 2002).

3 TWO-DIMENSIONAL ELECTROPHORESIS WITH IMMOBILIZED pH GRADIENTS (IPG-DALT)

The challenge for proteome analysis is to separate proteins from complex biological samples with high reproducibly and high resolution. Despite all merits of O'Farrell's (1975) carrier-ampholyte based 2-DE technology, setting a world-wide standard for the separation of complex protein samples by adding urea and detergents for protein solublization and IEF under denaturing conditions -in contrast to Scheele's (1975) native IEF/SDS-PAGE and Kloses's (1975) native IEF/PAGE approach (based on Stegemann's method (Macko & Stegemann, 1969)) for water-soluble proteins- it is, however, often difficult to obtain reproducible results even within a single laboratory, let alone between different laboratories. The problem of limited reproducibility is largely due to the synthetic carrier ampholytes (CA) used to generate the pH gradient required for IEF, for reasons such as pH gradient instability over time, cathodic drift, and batch-to-batch variability of CAs (Righetti & Drysdale 1973; Chrambach *et al.,* 1973). In practice, carrier ampholyte-generated pH gradients rarely extend beyond pH 7.5, with resultant loss of alkaline proteins. For the separation of these alkaline proteins, O'Farrell *et al.* (1977) developed an alternative procedure, known as non-equilibrium pH gradient electrophoresis (NEPHGE), however at the expense of reproducibility, since this procedure is extremely difficult to control and to standardize.

The above mentioned difficulties of 2-DE have been largely overcome by the development of immobilized pH gradients (IPG) (Bjellqvist *et al*., 1982), based on the use of the bifunctional Immobiline® reagents, a series of ten chemically well defined acrylamide derivatives with the general structure CH_2=CH-CO-NH-R, where R contains either a carboxyl or a (tertiary) amino group. These form a series of buffers with different pK values between pK 1 and 13. Since the reactive end is co-polymerized with the acrylamide matrix, extremely stable pH gradients are generated, allowing true steady-state IEF with increased reproducibility, as has been demonstrated in several inter-laboratory comparisons (Corbett *et al.*, 1994; Blomberg *et al*., 1995). Other advantages of IPGs are increased resolution by the ability to generate (ultra)narrow pH gradients (Δ pI = 0.001) (Görg *et al*., 1985, 1988; Wildgruber *et al.*, 2000), reproducible separation of alkaline proteins (Görg 1991; Görg *et al*., 1997-2000; Wildgruber *et al.*, 2002; Drews *et al.*, 2004) and increased loading capacity (Bjellqvist *et al.*, 1993). Consequently, IEF with IPGs is the current method of choice for the first dimension of 2-D PAGE for most proteomic applications.

The original protocol of 2-DE with immobilized pH gradients (IPG-Dalt) was described by Görg *et al*. (1988; updated in 2000 and 2004), summarizing the critical parameters inherent to isoelectric focusing with IPGs and a number of experimental conditions. The first dimension of IPG-Dalt, IEF, is performed in individual, 3-mm wide and up to 24 cm long IPG gel strips cast on GelBond PAGfilm (laboratory-made or commercial Immobiline

Dry-Strips). Samples can be applied either by cup-loading or by in-gel rehydration. IPG-IEF has been simplified by use of an integrated system such as the IPGphor (Islam *et al*., 1998; Görg *et al*., 1999) where rehydration with sample solution and IEF can be performed in a one-step automated procedure. After IEF, the IPG strips are equilibrated with SDS buffer in the presence of urea, glycerol, DTT and iodoacetamide, and applied onto horizontal or vertical SDS gels in the second dimension. After electrophoresis, the separated proteins are visualized by staining with silver staining, organic or fluorescent dyes, or autoradiography (or phosphor-imaging) of radiolabelled samples.

Limitations (not only of 2-DE, but of almost all current proteome analysis technologies) remain in the field of the analysis of very hydrophobic and/or membrane proteins, as well as in the lack of highly sensitive and reliable techniques for detection and quantitation of low abundant proteins. Yet, the recent introduction of more powerful chaotropes and detergents such as thiourea and sulfobetaines, as well as the advent of sensitive fluorescent dyes, in particular of dual label techniques for the visualization of differentially expressed proteins (Ünlü *et al.*, 1997) have improved the situation.

3.1 First dimension: IEF with immobilized pH gradients (IPGs)

3.1.1 IPG gel casting. Procedure and recipes

Linear or non-linear wide pH range (*e.g.*, IPG 3-12), medium (*e.g.*, IPG 4-7), narrow (*e.g.*, IPG 4.5-5.5), and/or ultra-narrow (*e.g.*, IPG 4.9-5.3) IPGs can be cast in different pH ranges between pH 2.5 and pH 12, as well as in different lenghts, usually from 7-24 cm (however, IPG strips up to 54 cm long have been applied (Poland *et al.*, 2003). Besides laboratory-made IPG gels, a variety of commercial IPG dry strips can now be purchased from different suppliers. Ready-made IPG dry strips are increasingly popular due to easier handling, better comparability of results and exchange of data, and have significantly contributed to the widespread application of 2-DE in proteomics.

Whereas narrow (one pH unit) and ultra-narrow (< 1 pH unit) linear immobilized pH gradients can be calculated with the help of the Henderson-Hasselbalch equation with relative ease, or by using nomograms (Righetti 1990), computer assisted programs (Altland, 1990; Righetti & Tonani 1991) are mandatory for wider (> 1 pH unit) and/or more complex (*e.g.*, non-linear) IPGs. IPG slab gels with linear gradients such as pH 4-7, 4-9, 6-10, 6-12, 3-12 and 4-12 are formed by mixing two immobiline starter solutions in a gradient mixer according to the gradient casting technique for ultrathin gels described by Görg *et al.* (1980) with the recipes of Righetti (1990) and Görg *et al.* (1998, 1999). IPG gels are IPG gels are 0.5 mm thick and cast on GelBond PAGfilm. The gel casting mold, which is typically loaded in a vertical position from the top, consists of two glass plates, one covered with the GelBond PAGfilm, whereas the second one bears a 0.5 mm thick 'U-frame' as spacer. Besides Immobiline^{R} chemicals, the gel casting solutions contain an

acrylamide/bisacrylamide mixture (typically 4%T / 3%C). For narrow-range alkaline IPG gels (*e.g.*, IPG 9-12), acrylamide may be substituted by N,N-dimethyl-acrylamide for improved stability of the gel matrix (Görg *et al.*, 1997). After poly-merization (50°C), the IPG gel is washed with deionized water, impregnated in 2% (w/v) glycerol, and dried. The surface of the dry IPG gel is protected with a sheet of plastic film before it is stored in a sealed plastic bag at -20°C. Prior to use, the dried gel is cut into 3 mm wide IPG dry strips with a paper cutter. For detailed information on IPG gel casting *see* Görg & Weiss (2000).

PROTOCOL: IPG gel casting

MATERIALS

Chemicals

Immobiline II chemicals, repel silane, acrylamide, bisacrylamide, ammonium persulfate, tetramethylethylenediamine (TEMED) (*GE Healthcare Lifesciences*), Serdolit MB-1 mixed bed ion exchanger resin (*Serva*)

Reagents

- Acrylamide/Bisacrylamide solution (30% T, 3% C)
  29.1% (w/v) acrylamide and 0.9% (w/v) N, N'-methylenebisacrylamide. To make 100 ml of the solution dissolve 29.1 g of acrylamide and 0.9 g of bisacrylamide in deionized water and fill up to 100 ml. Add 1 g of Serdolit MB-1, stir for 10 min and filter. This solution can be stored for one week at 4°C. However, for optimal results it is better to prepare it fresh the day you use it.
- Ammonium persulfate solution (40% (w/v) in deionized water).
  To prepare 1 ml of the solution, dissolve 100 mg of ammonium persulfate in 250 µl of deionized water. This solution should be prepared just before use.
- Solutions for casting Immobiline gels
  To prepare 15 ml each of acidic and basic solutions mix chemicals and reagent solutions as described in **Table 1**. For improved polymerization, the acidic and basic solutions are titrated to pH 7 (with sodium hydroxide and acetic acid) prior to gel casting. A huge selection of recipes for many types of narrow or broad-range pH gradients has been calculated (Righetti 1990). **Table 1** contains recipes for those IPGs which are preferred for 2-D electrophoresis in our lab. IPG solutions should be prepared fresh just before use.

Table 1. Recipes for casting immobiline gels pH gradients 4-7, 4-9, 6-12 and 4-12, respectively

Linear pH gradient	pH 4-7		pH 4-9		pH 6-12		pH 4-12	
	acidic solution	basic solution	acidic solution	basic solution	acidic solution	basic solution	acidic solution	basic solution
	pH 4	pH 7	pH 4	pH 9	pH 6	pH 12	pH 4	pH 12
Immobiline pk 3.6	578 µl	302 µl	829 µl	147 µl	1367 µl	_	950 µl	_
Immobiline pk 4.6	110 µl	738 µl	235 µl	424 µl	_	_	352 µl	74 µl
Immobiline pk 6.2	450 µl	151 µl	232 µl	360 µl	188 µl	251 µl	319 µl	206 µl
Immobiline pk 7.0	_	269 µl	22 µl	296 µl	323 µl	125 µl	294 µl	103 µl
Immobiline pk 8.5	_	_	250 µl	71 µl	365 µl	84 µl	48 µl	522 µl
Immobiline pk 9.3	_	876 µl	221 µl	663 µl	497 µl	32 µl	52 µl	219 µl
Immobiline pk 10.0	_	_	_	_	_	485 µl	41 µl	325 µl
Immobiline pk >13	_	_	_	_	_	345 µl	_	531 µl
Acrylamide / Bis	2.0 ml	2.0 ml	2.0 ml	2.0 ml	2.25 ml	2.25 ml	2.5 ml	2.5 ml
Deionized water	8.9 ml	10.7 ml	8.3 ml	11.1 ml	7.0 ml	11.4 ml	7.4 ml	10.5 ml
Glycerol (100%)	3.75 g	_	3.75 g	_	3.75 g	_	3.75 g	_
TEMED (100%)	10.0 µl	10.0 µl	10.0 µl	10.0 µl	10.0 µl	10.0 µl	10.0 µl	10.0 µl
Persulfate (40%)	15.0 µl	15.0 µl	15.0 µl	15.0 µl	15.0 µl	15.0 µl	15.0 µl	15.0 µl
Final volume	15.0 ml	15.0 ml	15.0 ml	15.0 ml	15.0 ml	15.0 ml	15.0 ml	15.0 ml

Note: For effective polymerization, acidic and basic solutions are adjusted to pH 7 with 4 N sodium hydroxide and 4 N acetic acid, respectively, before adding the poymerization catalysts (TEMED and ammonium persulfate).

Equipment

Gradient mixer (2 x 15 ml), glass plates with a 0.5 mm thick U-frame (200 x 260 mm^2), plain glass plates (size 200 x 260 mm^2), clamps, GelBond PAGfilm (200 x 260 mm^2), repel silane, roller *(GE Healthcare Lifesciences*), heating cabinet (*Heraeus, Germany*), laboratory shaker (rocking platform) (*GFL, Germany*) and Milli-Q System (*Millipore*)

METHOD

Two immobiline starter solutions (an acidic one and a basic one) are prepared as described in **Table 1**. The acidic solution, which additionally contains 25% glycerol to form a density gradient which stabilizes the pH gradient during the gel casting procedure, is pipetted into the mixing chamber, and then the basic, „light" (*i.e.*, without glycerol) solution into the reservoir of the gradient mixer. If a pH plateau (typically 2 cm wide) for the sample application area is desired, an extra portion of the dense solution is pipetted into the precooled (refrigerator) cassette prior to pouring the gradient. After pouring, the cassette is kept at room temperature for 10-15 min to allow adequate levelling of the density gradient. After polymerization (60 min, 50°C), the IPG gel is removed from the gel cassette, extensively washed with deionized water, impregnated with 2% glycerol, and air-dried. The surface of the dried IPG gel is covered with a sheet of plastic film. The dried IPG gels can be stored frozen at -20°C. for at least one year.

1. Wash the glass plates with a mild detergent, then rinse them with deionized water and let them air-dry in a dust-free cabinet. If brand new glass plates are used, pipette (in an extractor hood!) 1-2 ml of repel silane on the glass plate that bears the U-shaped spacer and distribute it evenly on the surface with a lint-free filter paper (Kimwipe). Let the solvent evaporate (1-2 minutes), clean the glass plate with a mild detergent, rinse with deionized water, water and let it air-dry. Repeat this procedure every 2-3 months in order to prevent the IPG gels from sticking to the glass plate.
   Note: The detergent used for cleaning of the glass plates should not contain any proteins (enzymes)

2. To assemble the polymerisation cassette, wet the plain glass plate (size 260 x 200 mm^2) with a few drops of water. Place the Gelbond PAGfilm, hydrophilic side upwards, on the wetted surface of the plain glass plate. The GelBond PAGfilm should overlap the upper edge of the glass plate for 1-2 mm to facilitate filling of the cassette. Expel excess water with a roller (**Fig. 5**). Place the glass plate which bears the U-frame (0.5 mm thick) on top of the GelBond PAGfilm and fix the cassette with clamps. Put it in the refrigerator for 30 min.

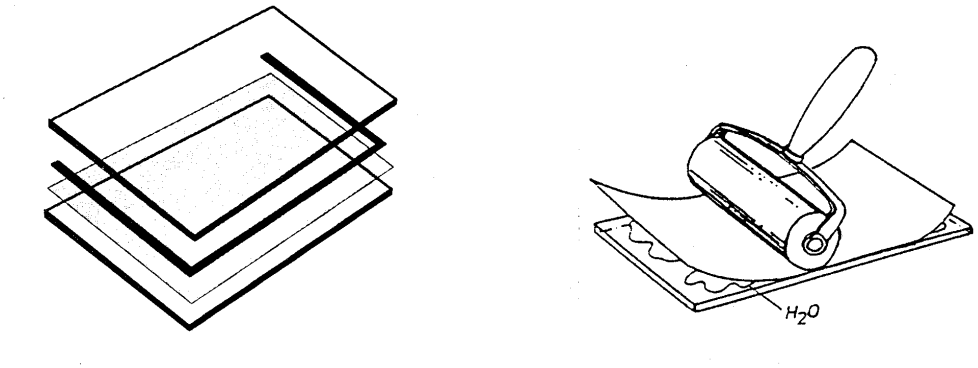


Figure 5 Assembly of the gel casting cassette

(Left): Assembly of the polymerisation cassette for IPG and SDS gel casting on plastic backing (Glass plates; GelBond PAGfilm; Spacer (U-frame), thickness 0.5 mm)

(Right): Application of the GelBondPAGfilm onto the glass plate

3. To cast the IPG gel, pipette 12.5 ml of the acidic, dense solution into the mixing chamber of the gradient mixer (**Fig. 6**). Outlet and valve connecting the mixing chamber and reservoir have to be closed! Add 7.5 µl of TEMED and 12 µl of ammonium persulfate and mix. Open the connecting valve between the chambers for a second to release any air bubbles.

   Note: In order to ensure the reproducibility of the IPG gradient, the volume of the cassette should be not vary. Therefore, we recommend to check the volume of the cassette from time to time since it may diminish on ageing of the U-frame.

4. Pipette 12.5 ml of the basic, light solution into the reservoir of the gradient mixer. Add 7.5 µl of TEMED and 12 µl of ammonium persulfate and mix with a spatula.

5. Switch on the magnetic stirrer at a reproducible and rapid rate (approximately 600 rpm). Avoid excessive vortex! Remove the polymerisation cassette from the refrigerator and put it underneath the outlet of the gradient mixer. Open the valve (1) connecting the chambers and, immediately afterwards, the pinchcock (2) on the outlet tubing so that the gradient mixture is applied slowly, but steadily into the cassette from a height of about 5 cm just by gravity flow. Take care that the level in both chambers drops equally fast. Formation of the gradient is completed in 2-3 min.

   Note: If one of the chambers is emptying faster than the other, the resulting pH gradient will not be linear. Check if there is an air bubble in the connecting line or whether the speed of the magnetic stirrer is unappropriate!

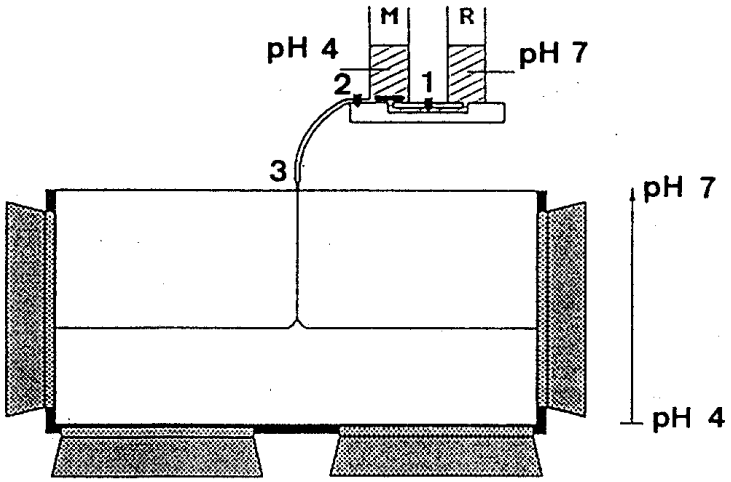


Figure 6 IPG gel casting (1) Gradient mixer and connecting valve; (2) outlet tubing valve; (3) gel casting cassette

6. Keep the mold at room temperature for 15 min to allow adequate levelling of the density gradient. Then polymerize the gel for 1 h at 50°C in a heating-cabinet.
7. After polymerization at 50°C, the cassette is allowed to cool down to room temperature (at least 15 min). Then insert a spatula between the glass plates and the gel, pry apart the glass plates and carefully remove the gel from the cassette. Wash the IPG gel for 1 h with 10-min changes of deionized water (500 ml each) in a glass tray on a rocking platform. Equilibrate the gel in 2% (w/v) glycerol for 30 min and dry it overnight at room temperature, using a fan, in a dust-free cabinet. Afterwards, protect the surface of the dry gel with a sheet of plastic film. The dried IPG gel can be stored in a sealed plastic bag at -20°C for at least several months without loss of quality. Dried IPG gels in several pH ranges are also commercially available (Immobiline DryPlate).

3.1.2 IPG strip rehydration and sample application

Prior to IEF, the IPG dry strips must be rehydrated (usually overnight) to their original thickness of 0.5 mm with a rehydration buffer containing 8M urea (or, alternatively, 2M thiourea and 6M urea), 1- 2% non-ioninc or zwitterionic detergents (*e.g.*, 2% CHAPS), a reductant (typically 0.4% DTT) and 0.5% (v/v) carrier ampholytes (*e.g.*, IPG buffer or Pharmalyte 3-10). Rehydration buffer should be prepared fresh before use, or stored frozen in aliquots at –70°C. It is important to deionize the urea with an ion exchange resin prior to adding the other components, because urea in aqueous solution exists in equilibrium with ammonium cyanate which can react with protein amino groups and introduce charge artifacts, giving rise to additional spots on the IEF gel. Carrier ampholytes are added for improved protein solubility, but also as a cyanate scavenger.

IPG dry strips are either rehydrated with sample already dissolved in rehydration buffer ("sample in-gel rehydration"), or with rehydration buffer without sample, followed by sample application by "cup-loading". Rehydrated IPG strips can be stored at –70°C for instantaneous use (Görg *et al.*, in preparation).

For *sample in-gel rehydration* (Schupbach *et al.*, 1991; Rabilloud *et al.*, 1994; Sanchez *et al.*, 1997), the extract (1-10 mg protein/ml) is directly solubilized in a defined volume of rehydration buffer. For 240 mm long and 3 mm wide IPG dry strips, 450 µl of this solution is pipetted into the grooves of the reswelling tray or into the IPGphor strip holder (*GE Healthcare Lifesciences*). For shorter IPG strips, rehydration volume has to be adjusted accordingly (*e.g.*, 350 µl or 180 mm long IPG strips). The IPG strips are applied, gel side down, into the grooves without trapping air bubbles. The IPG strip, which must still be moveable and not stick to the tray, is then covered with silicone oil or DryStrip cover fluid and rehydrated overnight at approximately 20°C. Higher temperatures (>37°C) hold the risk of protein carbamylation, whereas lower temperatures (< 10°C) should be avoided to prevent urea crystallization on the IPG gel. Improved entry of higher molecular weight proteins (Mr > 100 kDa) into the IPG gel matrix is facilitated by 'active' rehydration, *i.e.*, by applying low voltages (30-50 V) during reswelling (Görg *et al.*, 1999, 2000).

Sample in-gel rehydration is not recommended for samples containing very high molecular weight, very alkaline and/or very hydrophobic proteins, since these are taken up into the gel only with difficulty, *e.g.*, due to hydrophobic interactions between proteins and the wall of the tray, or because of size-exclusion effects of the gel matrix. The latter phenomenon is particularly pronounced if the sample volume significantly exceeds the calculated volume of the IPG strip after reswelling, since higher Mr proteins preferably remain in the excess reswelling solution instead of entering the IEF gel matrix. Cross contamination is another problem; hence the reswelling tray must be thoroughly cleaned between different experiments. In conclusion, sample-in-gel rehydration is less reliable than cup-loading, in particular for quantitative analyses.

For *cup-loading*, IPG drystrips are reswollen in rehydration buffer, either in a reswelling cassette or, more convenient, in a reswelling-tray, however without sample. After IPG strip reydration, samples (20-100 µl) dissolved in lysis buffer are applied into disposable plastic or silicone rubber cups placed onto the surface of the IPG strip. Best results are obtained when the samples are applied at the pH extremes, *i.e.*, either near the anode or cathode. Sample application near the anode proved to be superior to cathodic application in most cases. When using basic pH gradients such as IPGs 6-12 or 9-12, anodic application is mandatory for all kinds of samples investigated (Görg *et al.*, 2000).

PROTOCOL: IPG drystrip rehydration

The dried IPG gels (or ready-made Immobiline DryPlates) are cut into individual 3 mm wide strips with the help of a paper cutter (**Fig. 7**). Alternatively, ready-made IPG strips (Immobiline DryStrip) are used. IPG drystrips are reyhdrated to their original thickness of 5 mm, either in a reswelling cassette (**Fig. 8A**), or in a reswelling tray (**Fig. 8B**), or in a IPGphor strip holder (**Fig. 8C**). The correct IPG gel thickness is achieved by rehydrating the IPG drystrips with the proper amount of rehydration buffer (**B,C**), or by using an appropriate spacer (**A**).

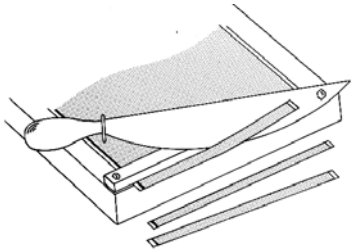


Figure 7 IPG strip cutting

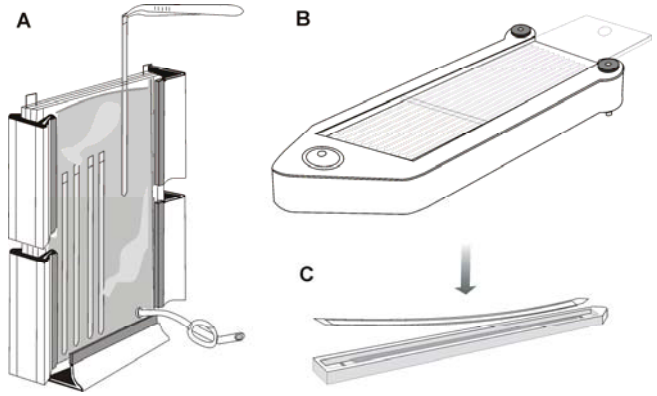


Figure 8 Rehydration of IPG Drystrips
(**A**) Reswelling cassette; (**B**) Reswelling tray;
(**C**) IPGphor strip holder

MATERIALS

Chemicals

Pharmalyte (pH range 3-10), IPG buffers, repel silane, IPG cover fluid, DeStreak® reagent (*GE Healthcare Lifesciences*), Serdolit MB-1 mixed bed ion exchanger resin, hydroxyethyldisulfide (HED) (Sigma); silicone oil (*Serva*), CHAPS (*Roche*), urea (*Merck*), thiourea (Fluka).

Reagents

- IPG drystrip rehydration buffer *(*8 M urea, 1% CHAPS, 0.4% DTT and 0.5% carrier ampholyte). To prepare 50 ml of the solution, dissolve 25.0 g of urea in deionized water and complete to 50 ml. Add 0.5 g of Serdolit MB-1, stir for 10 min and filter. To 48 ml of this solution add 500 mg CHAPS, 200 mg DTT, and 0.25 ml IPG buffers (*alternatively:* Pharmalyte pH 3-10) and complete to 50 ml with deionized water. Rehydration solution should be prepared freshly the day you use it or stored frozen in small portions at –70°C until use.

***Notes*: (1**) For hydrophobic proteins, 6 M urea and 2 M thiourea instead of 8 M urea is recommeded. (**2**) For alkaline IPGs (*e.g.*, IPG 6-12), DTT should be substituted by 100 mM hydroxyethyldisulfide; alternatively, use DeStreak® reagent. (**3**) Do not heat urea solutions > 37°C to avoid protein carbamylation!

Equipment

IPGphor, IPGphor strip holders, reswelling tray, reswelling cassette (125 x 260 mm^2), Immobiline DryPlates, Immobiline DryStrips *(GE Healthcare Lifesciences*), paper cutter (*Dahle, Germany*), Parafilm (roll, 50 cm x 15 m) (*ANC, USA*), Milli-Q System (*Millipore*).

METHODS

PROTOCOL I: Rehydration of IPG DryStrips with sample solution

Particularly for micropreparative purposes, samples are applied by in-gel rehydration. However, one has to be aware that high Mr proteins, very basic proteins, and/or membrane proteins do not always enter the gel matrix properly! For improved entry of high Mr proteins, IPGphor strip holders, which facilitate ‚active" rehydration by applying low voltage 30-50 V during rehydration, are superior to the reswelling tray that allows „passive" rehydration only (Görg *et al.*, 1999, 2000).

Rehydration of IPG strips in the reswelling tray

1. Dilute the extract (approximate protein concentration ≈ 10 mg/ml) with IPG dry strip rehydration buffer. Recommended dilution is 1+1 or 1+2 for micropreparative IEF, and 1+9 or 1+19 for analytical IEF (depending on the pH gadient; the narrower the pH gradient, the higher the protein load) to a final volume of 350 µl for 180 mm long IPG strips (or 450 µl for 240 mm long IPG strips, respectively).
2. Pipette 350 µl (or 450 µl, respectively) of sample-containing rehydration buffer into the grooves of the reswelling tray (**Fig. 8B**). Peel off the protective cover sheets from the IPG strips and insert the IPG strips (gel side-down) into the grooves. Avoid trapping air-bubbles. The IPG strip must still be moveable and not stick to the tray. Cover the IPG strips with 1-2 ml of silicone oil (or IPG DryStrip cover fluid), close the lid and rehydrate the IPG DryStrips overnight at approximately 20°C.
3. After the IPG strips have been rehydrated, briefly rinse them with deionized water and place them, gel side up, on a sheet of water saturated filter paper. Wet a second sheet of filter paper with deionized water, blot it slightly to remove excess water and put it onto the surface of the IPG gel strips. Blot them gently for a few seconds to remove excess rehydration solution to prevent urea crystallization on the surface of the gel during IEF.

Rehydration of IPG strips in the IPGphor strip holder

1. Dilute the extract (approximate protein concentration ≈ 10 mg/ml) with sample IPG dry strip rehydration buffer. Recommended dilution is 1+1 or 1+2 for micropreparative IEF, and 1+9 or 1+19 for analytical IEF (depending on the pH gadient; the narrower the pH gradient, the higher the protein load) to a final volume of 350 µl for 180 mm long IPG strips (or 450 µl for 240 mm long IPG strips, respectively).
2. Apply the required number of strip holders (**Fig. 8C**) onto the cooling plate / electrode contact area of the IPGphor (**Fig. 9**).

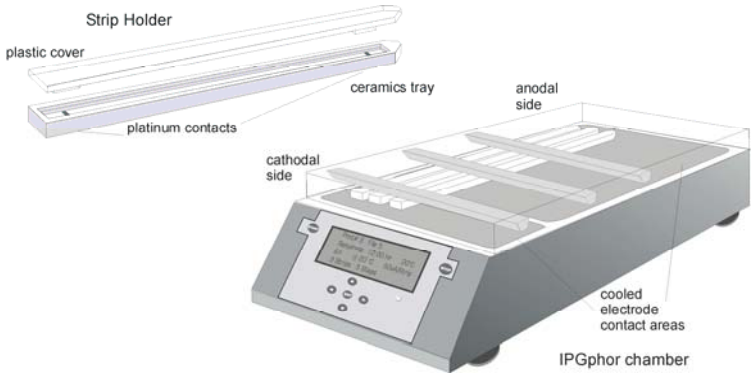


Figure 9 IPGphor for IPG-IEF

3. Pipette 350 µl of sample-containing rehydration buffer (for 180 mm long IPG strips) into the strip holder base. For longer (*e.g.,* 240 mm) IPG strips in longer strip holders use proportionately more liquid. Peel off the protective cover sheets from the IPG strip and slowly lower the IPG strip (gel side-down) onto the rehydration buffer. Avoid trapping air-bubbles. The IPG strip must still be moveable and not stick to the tray. Cover the IPG strips with 1-2 ml of IPG DryStrip cover fluid and apply the plastic cover. Pressure blocks on the underside of the cover assure that the IPG strip keeps in good contact with the electrodes as the gel swells. Apply low voltage (30-50V) during rehydration for improved entry of high Mr proteins (Görg *et al.*, 1999, 2000).

PROTOCOL II: Rehydration of IPG DryStrips for sample cup-loading

Up to 30 IPG drystrips can be rehydrated simultaneously in a reswelling cassette (**Fig. 8A**), whereas in the reswelling tray (**Fig. 8B**) this number is restricted to 12 IPG strips. The correct IPG gel thickness (5 mm) after reswelling is achieved by means of a U-shaped spacer (cassette), or by rehydrating with the proper amount of rehydration buffer (tray).

IPG strip rehydration in a resewelling cassette

1. To rehydrate the IPG gel strips to their original thickness (0.5 mm) in a reswelling cassette (*GE Healthcare Lifesciences*), cut a U-frame from two layers of Parafilm of the same shape and size as the the glass plate's U-frame. Thus, after assembly of the rehydration cassette, a 0.7 mm thick U-frame (0.5 mm original thickness plus two layers of Parafilm, 0.1 mm each) is formed that to compensates for the 0.2 mm wide GelBond PAGfilm on which the IPG gel is polymerized.
   Note: If sample volumes > 100 µl will be applied on the rehydrated IPG strip, it is better to use one layer of parafilm only.

2. Take an appropriate number of IPG dry strips and pull off their protective covers. Level the lower glass plate (*i.e.*, that one that does not carry the U-shaped spacer) horizontally, and pipette approximately 25 µl deionized water as small droplets over the entire length of the glass plate. Lower the first IPG drystrip, gel side up (!), onto the water droplets and slide the it back and forth to remove any air bubbles under the strip. Repeate this procedure with the remaining IPG drystrips. Leave 1-2 mm space between them, and at least 5 mm distance to the edges of the glass plate.

3. Clamp the cassette together, put it in an inclined (or vertical) position and carefully fill in IPG drystrip rehydration buffer (approximately 25 ml) from the bottom with a syringe (**Fig. 6A**). Make sure that the cassette does not leak. Allow the strips to rehydrate overnight (16-18 h) at approximately 20°C. Higher temperatures (> 37°C) hold the risk of protein carbamylation, whereas lower temperatures (< 15°C) should be avoided to prevent urea from crystallization on the IPG gel.

4. After the IPG gel strips have been rehydrated, pour the rehydration solution out of the reswelling cassette, remove the clamps and open the cassette with the help of a spatula. Using clean forceps, briefly rinse the rehydrated IPG gel strips with deionized water and place them, gel side up, on a sheet of water saturated filter paper. Wet a second sheet of filter paper with deionized water, blot it to remove excess water and put it onto the surface of the IPG gel strips. Blot them gently for a few seconds to remove excess rehydration solution in order to prevent urea crystallization on the surface of the gel during focusing.

IPG strip rehydration in the reswelling tray

1. Pipette 350 µl of rehydration solution (for 180 mm long IPG strips) into the grooves of the reswelling tray (GE Healthcare Lifesciences) (**Fig. 6B**). Peel off the protective cover sheets from the IPG strips and insert the IPG strips (gel side-down) into the grooves. To help coat the entire IPG drystrip, gently lift and lower the strip and slide it back and forth along the surface of the solution. The IPG strip must be moveable and not stick to the tray. Avoid trapping air-bubbles under the strip. Then Cover the IPG strips with 1-2 ml of

silicone oil (or IPG DryStrip cover fluid) which prevents evaporation during reswelling, close the lid and let the strips rehydrate overnight at room temperature.

2. After the IPG gel strips have been rehydrated, briefly rinse them with deionized water and place them, gel side up, on a sheet of water saturated filter paper. Wet a second sheet of filter paper with deionized water, blot it slightly to remove excess water and put it onto the surface of the IPG gel strips. Blot them gently for a few seconds to remove excess rehydration solution in order to prevent urea crystallization on the surface of the gel during IEF.

3.1.3. IEF using IPGs from pH 2.5 to 12. General aspects and guidelines

The first dimension of IPG-Dalt, isoelectric focusing in immobilized pH gradients, is performed as originally described by Görg *et al*. (1988) in individual 3-mm wide IPG gel strips cast on GelBond PAGfilm. The original IPG-IEF procedure of running IPG strips on a flat-bed isoelectric focusing unit has been simplified by the introduction of an integrated system, the IPGphor. This instrument features ceramic strip holders that facilitate rehydration of individual IPG strips with or without sample, optional sample cup loading, and subsequent IEF, all without handling the strip after it is placed in the holder. Other important features are precise temperature control and an 8000 V power supply, allowing shorter running times and concomitant better performance of alkaline IPGs.

General guidelines for IEF with IPGs

The ***amount of protein*** which which can be loaded onto a single IPG gel strip for optimum resolution, maximum spot numbers and minimum streaking/background smearing depends on parameters such as pH gradient used (wide or narrow), separation distance, and protein complexity of the sample. For analytical purposes, typically 100 µg of protein can be loaded on an 18 cm long, wide pH range gradient, and 500 µg on narrow range IPGs. For micropreparative purposes, five to ten times more protein can be applied. For micro-preparative 2-DE, in-gel rehydration is often preferred, but up to several mg of protein may also be applied by (repeated) cup-loading. If the protein concentration of the sample is unknown, one can do a rough, but quick estimation by making a dilution series of both the sample and a known standard (*e.g.* BSA) by spotting them on a nitrocellulose membrane and staining it with Amido Black or Coomassie blue. The optimum ***sample volume*** for cup loading is 20 µl – 100 µl, whereas volumes less than 20 µl (IPGphor: 50 µl) are not recommended because of the increased risk of protein aggregation and precipitation at the point of sample application, resulting in loss of proteins and/or horizontal or vertical streaking. For the same reason, the ***protein concentration*** of the sample solution should not exceed 10 mg/ml.

Samples can be applied to the IPG strip either by cup-loading or by in-gel rehydration. In case of cup-loading, samples are typically applied at the pH extremes, *i.e.* either near the

anode or cathode. In our experience, sample application near the anode proved to be superior to cathodic application in most cases, with some exceptions. *E.g.* human cardiac proteins were preferably applied at the cathode, whereas not only most kinds of plant proteins but also mouse liver, yeast cell proteins etc. yielded best results when applied at the anode. For basic pH gradients such as IPGs 6-10, 7-10 or 6-12, anodic application was mandatory for all kinds of samples investigated.

Settings are usually limited to 50 µA per IPG strip and 150 V to avoid Joule heating, because the conductivity is initially high due to salts. As the run proceeds, the salt ions migrate to the electrodes, resulting in decreased conductivity and allowing high voltages to be applied. Samples with high salt concentrations can be desalted directly in the IPG gel by restricting the voltage to 50 -100 V during the first 4-5 hours with several changes of the electrode filter paper strips (Görg *et al.*, 1995, 2000). Likewise, voltage should be limited to 100 V overnight for improved sample entry in case of large sample volumes (micropreparative runs and/or narrow IPGs) prior to continuing IEF at higher voltages (> 3500 V). Final settings up to 8000 V are particularly useful for zoom-in gels and alkaline pH gradients (*see* below). The longer the IPG strip and the narrower the pH gradient, the more volt-hours are required to achieve steady state separation for high reproducibility. If the second dimension cannot be performed directly after IEF, the IPG strips should be immediately frozen and stored at –70 °C between two plastic sheets.

Too short ***focusing times*** will result in horizontal streaking, but severe over-focusing should also be avoided. Although in contrast to the classical O'Farrell (1975) method, over-focusing does not result in migration of proteins towards the cathode (cathodic drift), but will result in excess water exudation at the surface of the IPG gel due to active water transport (reverse electroendosmotic flow). This leads to distorted protein patterns, horizontal streaks at the basic end of the gel, and loss of proteins. The optimum focusing time must be established empirically for each combination of protein sample, protein loading and the particular pH range and length of IPG gel strip used. Detailed protocols including optimum focusing parameters for a number of different wide and narrow pH range IPGs have been published by Görg *et al*. (2000, 2004).

Temperature during IEF has an important effect on the resulting 2-DE patterns, since spot positions vary along the pH axis with different applied temperatures. For enhanced reproducibility it is extremely important to run the separations under precise temperature control, where 20 °C proved to provide the optimal conditions (Görg *et al.*, 1991).

Narrow overlapping IPGs ('zoom-in' gels) and extended separation distances

The ***choice of pH gradient*** primarily depends on the sample's protein complexity. Wide or medium range IPGs, such as IPGs 3–12, 4–9, or 4–7, are typically used to analyze simple proteomes (small genome, organelle, or other subfraction), or to get an overview of a more complex proteome, respectively. Although at first glance the resolution of 2-D

PAGE seems impressive, it is, however, still not sufficient compared to the enormous diversity of proteins from higher eukaryotic proteomes, where extensive co-and post-translational modifications of proteins and differential gene splicing lead to expression of more proteins than the total number of genes in their genomes. With samples such as total lysates of eukaryotic cells or tissues, 2-D PAGE on a single wide-range pH gradient reveals only a small percentage of the whole proteome. The best remedy, preferably in combination with prefractionation procedures, is to use ***multiple narrow overlapping*** IPGs ("zoom-in" gels, *e.g.*, IPG 4–5, IPG 4.5–5.5, or 5.0-6.0) and/or extended separation distances (up to 24 cm, or even longer) to achieve an optimal resolution to avoid multiple proteins in a single spot for unambiguous protein identification and to facilitate the application of higher protein amounts for the detection of minor components Wildgruber *et al.* (2000) have demonstrated the improved separation and visualization of the *S. cerevisiae* proteome by using narrow overlapping IPGs. Combining IPGs 4-5, 4.5-5.5, 5-6, 5.5-6.7 and 6-9 and excluding the overlap, so that every protein was counted only once, it was possible to detect three times more protein spots than in the IPG 3-10. Interestingly, not only did spots become further apart, but several single spots divided into two or more protein spots. This observation is in perfect agreement with the detection by mass spectrometry of different proteins in a single spot in about 10-40% of all spots detected on broader pH range IPG gels. Similarly, Westbrook *et al*. (2001) demonstrated not only improved resolution and higher spot numbers by using narrow-range IPGs, but also the identification by mass spectrometry of additional protein species and isoforms of proteins from apparent single spots, or unresolved spot clusters, on broader-range IPGs.

"Zoom" gels in the acidic and neutral pH range between pH 4 and 7 (*e.g.*, IPG 4-5, IPG 4.5 - 5.5, IPG 5-6) work with in-gel rehydration or cup-loading. These gels are typically used for micropreparative purposes with sample loads up to several milligrams. In order to avoid protein precipitation and horizontal streaking, low voltages (approximately 50-100 V) should be applied during the initial stage of IEF, in particular when samples are applied by cup-loading. Because of the high number of volthours (> 100,000) and concomitant long focusing time (up to 24h) required for focusing to the steady-state, the surface of the IPG strips has to be protected by a layer of silicone oil to prevent them from drying out. The paper strips beneath the electrodes should be removed after several hours and replaced by fresh ones. This is of particular importance when the sample contains high amounts of salt, but also to remove proteins with pIs outside the chosen pH interval (Görg *et al.*, 2000). Much better results, however, are obtained when prefractionated samples are separated on "zoom" gels (Zuo & Speicher 2001, 2002; Görg *et al.*, 2002).

Very ***long separation distances*** (> 30 cm) for maximum resolution of complex protein patterns have been described (Levenson *et al.*, 1990). However, size, stability, and handling of the fragile tube gels used in carrier-ampholyte 2-DE is often a problem. This is in contrast to IPG gel strips, which are cast on plastic backings so that they can neither

stretch nor shrink, which contributes significantly to improved reproducibility. Meanwhile, 24 cm long IPG gel strips (Görg *et al.*, 1999) are routine. Recently, 54 cm long IPG strips were successfully applied (Poland *et al.*, 2003).

IEF of very alkaline proteins

Theoretical 2-D maps calculated from sequenced genomes indicate that approximately 30% of all proteins possess alkaline pIs up to pH 12. Wide range IPGs 3-12 and 4-12 (Görg *et al*., 1998, 1999) are ideally suited to provide an overview of the proteome of a cell or tissue. In particular, the IPG 4-12 which is flattened between pH 9-12, proved to be a most useful gradient for the separation of very alkaline proteins. Prefractionation procedures such as TCA/acetone precipitation of proteins are recommended for enrichment and visualization of basic proteins exceeding pI 10, since these are usually not included in lysis buffer extracts of eukaryotic organisms (Görg *et al*., 2000).

Strongly alkaline proteins such as ribosomal and nuclear proteins with closely related pIs between 10.5 and 11.8 were focused to the steady state by using IPGs 3-12, 6-12 and 9–12 (Görg *et al*., 1997; Wildgruber *et al.*, 2002; Drews *et al*., 2004). For highly resolved 2-D patterns, different optimization steps with respect to pH engineering and gel composition were necessary, such as the substitution of dimethylacrylamide for acrylamide, the addition near the cathode of a paper strip soaked with DTT providing a continuous influx of DTT to compensate for the loss of DTT (which is a weak acid and migrates out of the basic part of the gel) (Görg *et al.*, 1995; Altland *et al.*, 1990), and the addition of isopropanol to the IPG rehydration solution in order to suppress the reverse electroendosmotic flow which causes highly streaky 2-D patterns in narrow pH range IPGs 9-12 and 10-12 (Görg *et al.*, 1997). With the advent of the IPGphor, the procedure was greatly simplified by applying high voltages (8000 V) to shorten run times considerably, which permits ro run these IPGs under “standard“ conditions without isopropanol, at least with analytical sample amounts (Wildgruber *et al.*, 2002; Drews *et al.*, 2004) (*see* **Fig. 10**).

For optimized separation, cup-loading at the anode is mandatory, and the use of high voltages (final settings up to 8000 V) is strongly recommended (Görg *et al.*, 2000; Wildgruber *et al.*, 2002; Drews *et al.*, 2004). With IPGs above pH 7, horizontal streaking due to DTT depletion can occur at the basic end. To avoid streaking, the cysteines should be stabilized as mixed disulfides by using hydroxyethyl-disulfide (HED) reagent (DeStreak™ *GE Healthcare Lifesciences*) in the IPG strip rehydration solution instead of a reductant. Besides the elimination of streaking, the use of HED results in a simplified spot pattern and improved reproducibility (Olsson *et al.*, 2002).

Analysis of very hydrophobic membrane proteins

Analysis of very hydrophobic proteins such as integral membrane proteins remains a challenge for both 2-DE and LC-based proteomic approaches. Besides their low

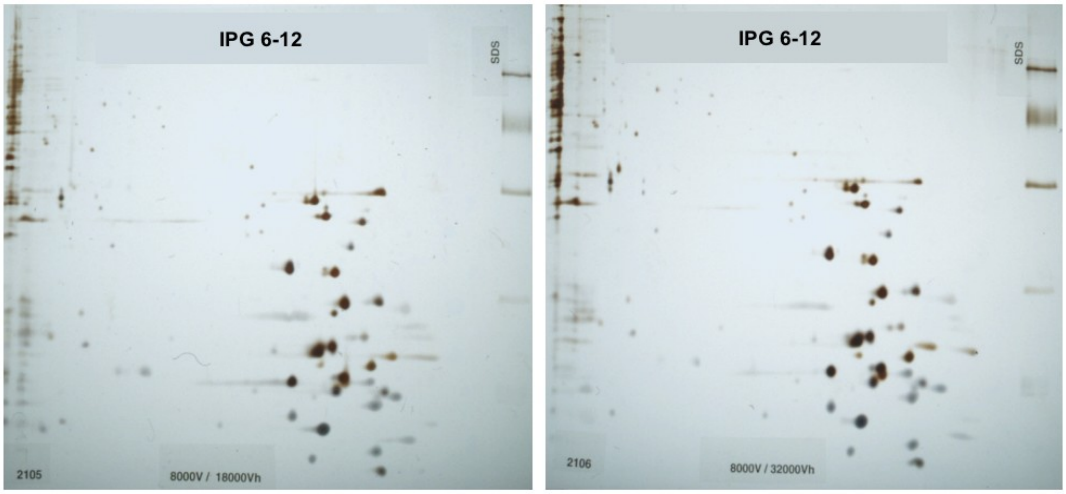


Figure 10A Steady-stade IEF of very alkaline proteins. Running conditions *see* Table 4.

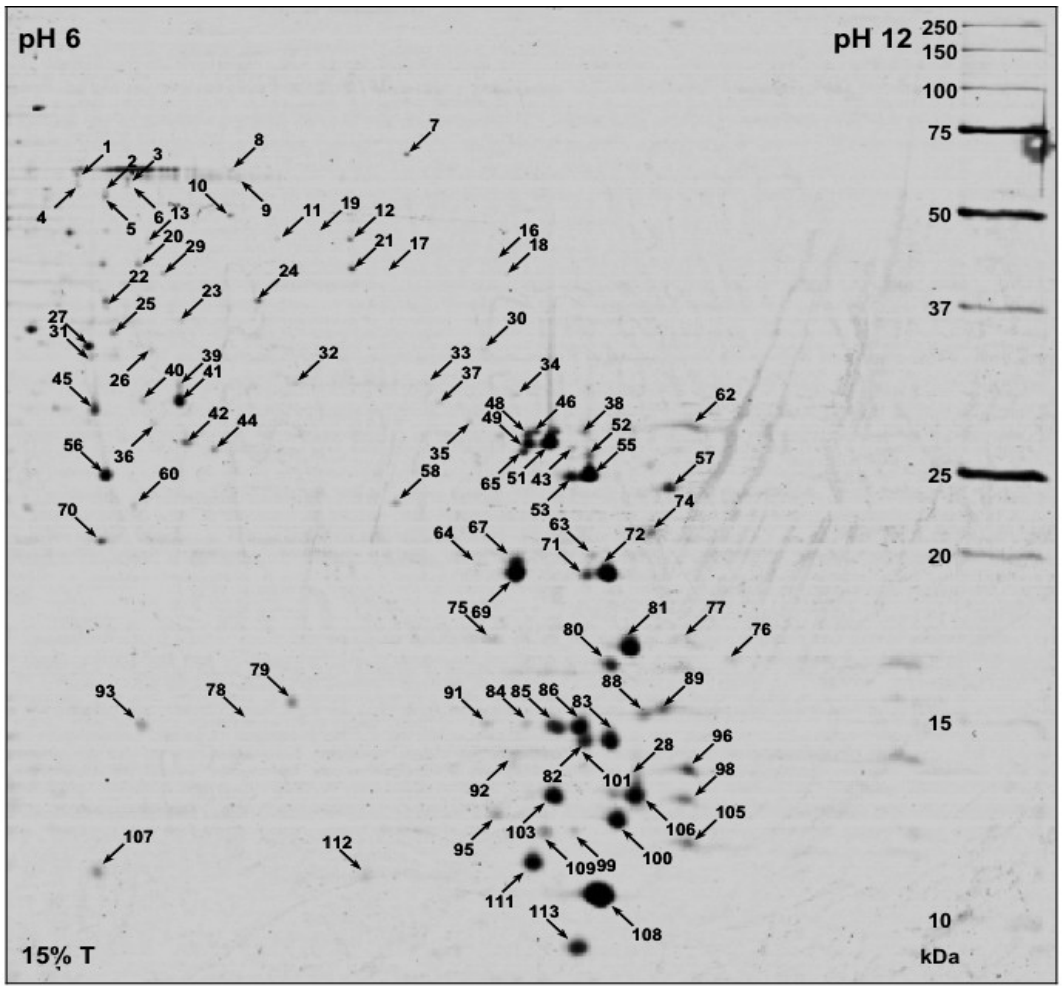


Figure 10B 2D database of very alkaline *Lactococcus lactis* proteins
(http://www.wzw.tum.de/proteomik/lactis)

solubility and their tendeny to aggregate and precipitate in aqueous media, many membrane proteins possess basic pIs and/or expressed in low copy numbers. Certain “loss“ of membrane proteins on 2-D gels may be attributed to the fact that these proteins, once solubilized, may in fact enter the IPG strips and also focus properly, but do not elute during the transfer step from first to second dimension. Nevertheless, some progress has been made towards improving solubilization and separation of membrane proteins, *e.g.*, by the introduction of thiourea and novel zwitterionic detergents which made it possible to display at least some membrane proteins on 2-DE gels.

PROTOCOL: IPG-IEF

Equipment

IPGphor unit; IPGphor strip holders and cup loading strip holders; Multiphor II horizontal electrofocusing unit; power supply (3500 V); Multitemp II thermostatic circulator; IPG DryStrip Kit; sample cups; IEF sample applicator strip *(GE Healthcare Lifesciences*)

I. IEF on the Multiphor unit. Running conditions for different IPGs

Ia. Sample application by cup loading

For analytical purposes, typically 50-100 µg of protein are applied onto a single, 180 mm long IPG gel strip, whereas for micropreparative purposes up to several mg of protein can be applied in case that GE Healthcare Lifesciences‘ Immobiline DryStrip Kit with sample cups is used. When IEF is performed using the DryStrip Kit, the IPG gel strips can be covered by a layer of silicone oil (or Drystrip cover fluid) which is mandatory when running very basic pH gradients (*e.g.* IPG 10-12) (Görg *et al.*, 1997) or narrow pH gradients with long focusing times for micropreparative IPG-Dalt (Bjellqvist *et al*., 1993).

Analytical IEF for multiple runs

1. Cover the flat-bed cooling block with 2-3 ml of kerosene and place the rehydrated (*see* section 3.1.2) IPG gel strips *(up to 40!)* side by side, 1-2 mm apart, on it (**Fig. 11**). The acidic end of the IPG gel strips must face towards the anode!
2. Cut two IEF electrode strips or paper strips prepared from 2 mm thick filter paper (*e.g.* MN 440, *Macherey & Nagel, Germany*) to a length corresponding to the width of all IPG gel strips lying on the cooling plate. Soak the electrode strips with deionized water (however not Milli-Q quality!) and remove excessive moisture by blotting with filter paper.
3. Place the IEF electrode strips on top of the aligned IPG gel strips at the cathodic and anodic ends.

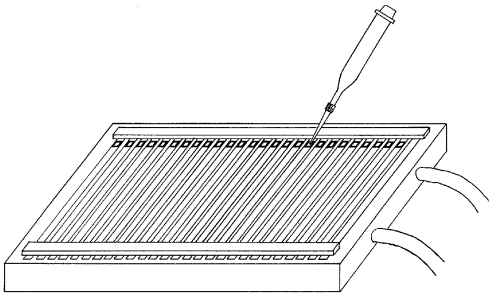


Figure 11 IEF in individual IPG strips

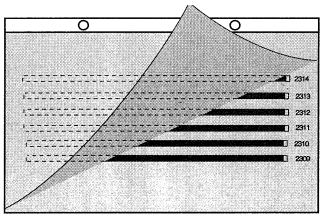


Figure 12 Storage of IPG strips after IEF

4. When running basic IPGs (*e.g.* IPG 6-10 or IPG 10-12), an extra paper strip soaked with 20 mM DTT should be placed onto the IPG gel surface near the cathodic electrode strip. Alternatively, include the DeStreak™ reagent (*GE Healthcare Lifesciences*) instead of DTT in the IPG drystrip rehydration buffer (*cf.* Section 3.1.2).

5. Apply silicone rubber frames (2 x 5 mm^2 internal diameter) onto the IPG gel surface, 5 mm apart from anode or cathode, for sample application.

6. Pipette the samples (not less than 20 µl, preferably dissolved in Lysis buffer) into the silicone frames. Protein concentration should not exceed 5-10 mg/ml. Otherwise, protein precipitation at the sample application area may occur. To avoid this, it is advantageous to dilute the sample with Lysis buffer and apply larger volumes instead (*see below*; Dry strip kit).

7. Position the electrodes and press them gently down on top of the IEF electrode strips.

8. Place the lid on the electrofocusing chamber, connect the cables to the power supply and start IEF. Running conditions depend on the pH gradient and the length of the IPG gel strips used. An appropriate time schedule for orientation is given in **Table 2** (Görg *et al.*, 2000). For improved sample entry, voltage is limited to 150 V (30-60 min), 300 V (30-60 min) and 600 V (30-60 min) at the beginning. Then continue with maximum settings of 3500 V to the steady state. Optimum focusing temperature is 20°C (Görg *et al.*, 1991).

9. After IEF, store those IPG gel strips which are not used immediately for second dimension run (and/or are kept for further reference) between two sheets of plastic film at -78 °C for up to several months (*see* **Fig. 12**).

Table 2 Running conditions using the Multiphor II IEF unit (Görg *et al.*, 2000)

IPG strip length	180 mm
Temperature	20°C
Current max.	0.05 mA per IPG strip
Power max.	0.2 W per IPG strip
Voltage max.	3500 V

I ANALYTICAL IEF

Initial IEF:

Cup loading (20-50 µL)	**In-gel rehydration** (350 µL)
150 V, 1-3 h*	150 V, 1-3h*
300 V, 1-3 h*	300 V, 1-3 h*
600 V, 1 h	

IEF to the steady state at 3500 V:

1-1.5 pH units		**4 pH units**		**7 pH units**	
e.g. IPG 5-6 ..	24 h	IPG 4-8	10 h	IPG 3-10 L	6 h
e.g. IPG 4-5.5	20 h	IPG 6-10	10 h	IPG 3-10 NL	6 h
3 pH units		**5-6 pH units**		**8-9 pH units**	
IPG 4-7	12 h	IPG 4-9	8 h	IPG 3-12	6 h
IPG 6-9	12 h	IPG 6-12	8 h	IPG 4-12	8 h

II EXTENDED SEPARATION DISTANCES (240 mm)

IEF to the steady-state at 3500 V:

IPG 3-12 ..... 8 h

IPG 4-12 ..... 12 h

IPG 5-6 ..... 40 h

III MICROPREPARATIVE IEF

Initial IEF:

Cup loading (100 µL)	**In-gel rehydration** (350 µL)
50 V, 12-16 h	50 V, 12-16 h
300 V, 1 h	300 V, 1h

IEF to the steady state at 3500 V:

Focusing time of analytical IEF plus approximately 50%

* Time required for initial IEF (sample entry) depends on salt concentration of the sample. The more salt, the longer.

Analytical and micropreparative IEF using the DryStrip Kit

The application of higher sample volumes (> 20 µl) is facilitated when the sample cups of the *Immobiline DryStrip Kit* are used (**Fig. 13**). A sample volume of 100 µl can be applied at a time, and it is possible to apply a total of up to 200 µl, portion by portion, onto a single IPG gel strip. Typical sample load is 50-100 µg protein/strip for silver stained 2-D patterns, whereas for preparative purposes up to 10 mg of protein from a cell lysate can be loaded onto a single IPG gel strip.

For IEF in very basic (pH > 10), or for micropreparative runs in narrow pH gradients (pH-range <1 unit), the surface of the IPG strips must be protected by a layer of silicone oil, whereas in case of broad pH gradients not exceeding pH 10 (*e.g.* IPG 4-7, 4-9 or 3-10), the DryStrip Kit can be used without silicone oil overlay.

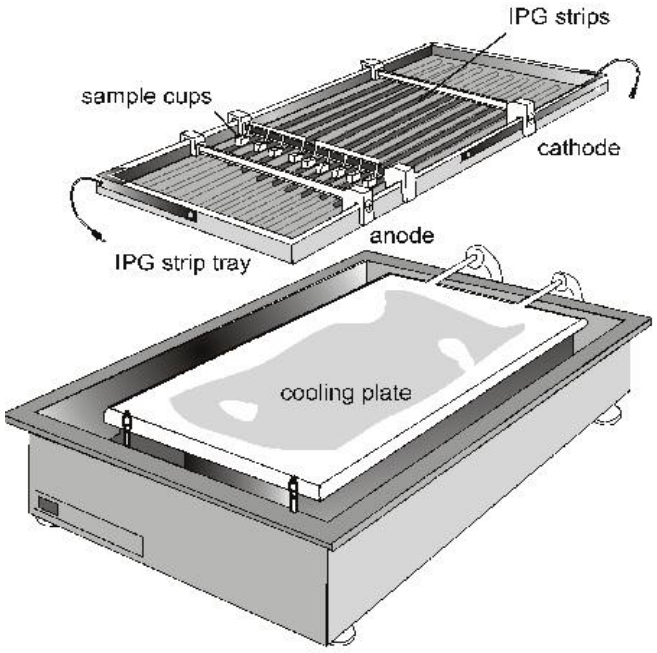


Figure 13 IEF in individual IPG gel strips using the DryStrip kit

1. Place the cooling plate into the Multiphor II electrophoresis unit. Pipette 3-4 ml of kerosene or silicone oil onto the cooling plate and position the Immobiline DryStrip tray on the cooling plate. Avoid trapping large air bubbles between the tray and the cooling plate.
2. Connect the electrode leads on the tray to the Multiphor II unit.
3. Pour about 10 ml of of silicone oil (or IPG Drystrip civer fluid) into the tray.

4. Place the Immobiline strip aligner into the tray on top of the oil.

5. Transfer the rehydrated IPG gel strips (gel-side up and acidic end towards the anode) into adjacent grooves of the aligner in the tray. Align the strips such that the anodic gel edges are lined up (**Fig. 13**).

6. Cut two IEF electrode strips or paper strips prepared from 2 mm thick filter paper (*e.g.* MN 440, *Macherey & Nagel, Germany*) to a length corresponding to the width of all IPG gel strips lying in the tray. Soak the electrode strips with deionized water, remove excessive moisture by blotting with filter paper and place the moistened IEF electrode strips on top of the aligned strips near the cathode and anode.

7. Position the electrodes and press them gently down on top of the IEF electrode strips.

8. Put the sample cups on the sample cup bar. Place the cups high enough on the bar to avoid touching the gel surface. Put the sample cup bar in position so that there is a distance of a few millimeters between the sample cups and the anode (or cathode, in case of cathodic sample application).

9. Move the sample cups into position, one sample cup above each IPG gel strip, and finally press down the sample cups to ensure good contact with each strip.

10. Once the sample cups are properly positioned, pour about 80 ml of silicone oil (or Drystrip cover fluid) into the tray so that the IPG gel strips are completely covered. If the oil leaks into the sample cups, suck the oil out, re-adjust the sample cups and check for leakage again. Fill up each sample cup with a few drows of silicone oil.
    Note: *In case of IEF using wide pH gradients in the range between pH 3 and 10, the oil-step can be omitted!*

11. Pipette the samples into the cups by underlaying. Watch again for leakage!

12. Close the lid of the Multiphor II electrophoresis chamber and start the run according to the parameters given in Table 2. For improved sample entry, voltage is limited to low voltage for the first few hours. Then continue with maximum settings of 3500 V to the steady state. Optimum focusing temperature is 20°C (Görg *et al.*, 1991).

13. If sample volumes exceeding 100 µl are to be applied, pipette in 100 µl, run IEF with limited voltage until the sample has migrated out of the cup. Then apply another 100 µl and repeat the procedure until the whole sample has been applied.

14. When the IEF run is completed, remove the electrodes, sample cup bar and IEF electrode strips from the tray. Use clean forceps and remove the IPG gel strips from the tray. Those IPG gel strips which are not used immediately for second dimension run and/or are kept for further reference are stored between two sheets of plastic film at -70°C up to several months (**Fig. 12**).

Ib. IEF of in-gel rehydrated samples

1. Cover the flat-bed cooling block of the horizontal IEF apparatus with 2-3 ml of kerosene and place the IPG gel strips which had been rehydrated with sample (*cf.* Section 3.1.2) on it. Line them up side by side and 1-2 mm apart. The acidic end of the IPG gel strips must face towards the anode! If IEF is performed under a protective layer of silicone oil, use the DryStrip kit instead, however without sample cups.

2. Cut two IEF electrode strips to a length corresponding to the width of all IPG gel strips (and spaces between them) lying on the cooling plate. Soak the electrode strips with deionized water and remove excessive moisture by blotting with filter paper.

3. Place the IEF electrode strips on top of the aligned IPG gel strips at the cathodic and anodic gel ends. When running basic IPGs (*e.g.,* IPG 6-10 or 8-12), put an extra paper strip soaked with 20 mM DTT on the surface of the IPG strips next to the cathode. Alternatively, include the DeStreak™ reagent (*GE Healthcare Lifesciences*) instead of DTT in the rehydration buffer.

4. Position the electrodes and press them gently down on top of the IEF electrode strips.

5. Place the lid on the electrofocusing chamber, connect the cables to the power supply and start IEF. Running conditions depend on the pH gradient and the length of the IPG gel strips. An appropriate time schedule for orientation is given in **Table 2**.

For improved sample entry, voltage is limited to 150 V, 300 V and 600 V for 1 h each, followed by 3500 V to the steady state. Current is limited to 0.05 mA/IPG strip. Optimum focusing temperature is 20°C (Görg *et al.*, 1991).

6. After IEF, IPG gel strips which are not used immediately for second dimension run are stored between two sheets of plastic film at -70°C up to several months (**Fig. 12**).

II. IEF using the IPGphor unit. Running conditions for different IPGs

IPG-IEF for 2D electrophoresis can be simplified by the use of an integrated instrument, the *IPGphor* (**Fig. 9**) (Isalm *et al.,* 1998). The IPGphor includes a Peltier element for precise temperature control (between 19.5°C and 20.5°C) and a programmable power supply. The central part of this instrument are so-called strip holders made from an aluminium oxide ceramic, in which IPG strip rehydration with sample solution and IEF are performed without further handling after the strip is placed into the strip holder (**Fig. 9**). The IPGphor can handle up to 12 strip holders of different lengths (7, 11, 13, 18 or 24 cm). Alternatively, the IPGphor can also be used with a cup loading procedure using cup-loading strip holders or a multiple cup-loading strip holder ('Manifold'), which allow(s) the application of quantities up to 100 µl. The strip holder platform regulates temperature and

serves as the electrical connector for the strip holders. Besides easier handling, a second advantage of the IPGphor is shorter focusing time, since IEF can be performed at rather high voltage (up to 8000 V).

The IPGphor is programmable and can store nine different programs. A delayed start is also possible, which allows the user to load the strip holders with sample dissolved in rehydration buffer in the afternoon, then automatically start IEF during the night so that IEF is finished the next morning.

Typical running conditions for IEF using the IPGphor are given in **Tables 3 & 4**. As indicated earlier, low voltage (30-50 V) is applied during the rehydration step for improved sample entry of high Mr proteins into the polyacrylamide gel which otherwise can be a problem with sample in-gel rehydration. Then voltage is stepwise increased up to 8000 V (when IPG strips with separation distances < 11 cm are used, voltage should be limited to 5000 V only). For optimum results for samples with high salt concentrations, or when narrow pH intervals are used, it is beneficial to insert moist filter paper pads (size: 4 x 4 mm^2) between the electrodes and the IPG strip prior to raising voltage to 8000 V. After termination of IEF, the IPG strips are stored as described above (**Fig. 12**).

IIa. IEF of in-gel rehydrated samples

1. Apply the required number of strip holders onto the cooling plate / electrode contact area of the IPGphor (**Fig. 9)**. Pipette the appropriate amount of sample-containing rehydration solution into the strip holders, lower the IPG dry strips gel-side down into the rehydration solution and overly with cover fluid (*see* section 3.1.2)
2. Program the instrument (desired rehydration time, volthours, voltage gradient).
3. After the IPG gel strips have been rehydrated (which requires six hours at least, but typically overnight), IEF starts according to the programmed parameters listed in Table 4.
4. After completion of IEF, store those IPG gel strips which are not used immediately for second dimension run between two sheets of plastic film at -70°C (**Fig. 12**).

IIb. IEF after sample cup loading

When protein separation is performed in alkaline pH ranges (> pH 10), much better separations are obtained by applying sample *via* cup loading on separately rehydrated IPG strips than by sample in-gel rehydration. Sampe cup loading is accomplished with special cup-loading („universal“) IPGphor strip holders, or with a multiple cup-loading strip holder (’Manifold’) (**Fig. 14**).

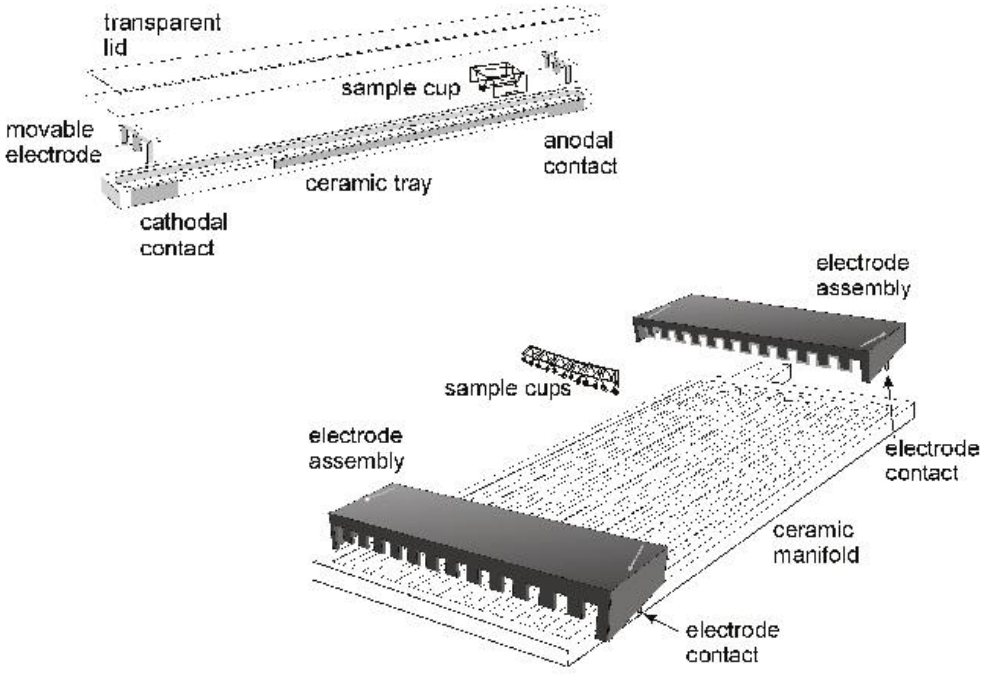


Figure 14 Individual cup loading strip holder (*top*) and multiple cup-loading strip holder („Manifold“) (*bottom*)

1. Rehydrate IPG drystrips with rehydration buffer, however without sample, in a reswelling tray or cassette. After the IPG strips have been rehydrated, use clean foreceps to remove the reswollen IPG strips from the reswelling tray or reswelling cassette. Rinse them with deionized water and blot for a few seconds between two sheets of moist filter paper to remove excess liquid in order to prevent urea crystallization on the surface of the gel during IEF as described in section 3.1.2.

2. Apply the required number of the cup-loading (or „universal“) IPGphor strip holders onto the cooling plate / electrode contact area of the IPGphor instrument, and make sure that the pointed (anodic) ends contact the anodic electrode area.
   Note: Instead of individual strip holders, the Manifold device may be used.

3. Apply the rehydrated IPG gel strips into the cup-loading strip holders, gel side upwards and pointed (acidic) ends facing toward the anode. Make sure that the cathodic end of the IPG strip is approximately 1.5 cm from the end of the channel and in electrical contact with the elecrode rails via the electrode clips.

4. Moisten two filter paper electrode pads (size: 4 x 10 mm^2) with deionzed water, remove excess liquid by blotting with a filter paper, and apply the moistended filter paper pads on the surface IPG gel at the anodic and cathodic ends of the IPG strip between the IPG gel and the electrodes.

5. Position the the movable electrodes above the electrode filter paper pads. Clip the electrodes firmly onto the electrode paper pads.

6. Position the movable sample cup either near the anode or cathode, and gently press the sample cup onto the surface of the IPG gel strip. The sample cup should form a good seal with the IPG strip but not damage its surface.

8. To confirm that the sample cup does not leak, pipette 100 µl of IPG cover fluid into the cup. If a leak is detected, remove the fluid and use a tissue paper to remove the cover fluid and reposition the sample cup. Check again for leakage. Remove the cover fluid before loading the sample.

9. Overlay each IPG strip with 2-4 ml of cover fluid. If the fluid leaks into the sample cup, rearrange the cup and use tissue paper to remove the cover fluid from the cup. Check again for leakage, and pipette (50-100 µl) sample solution (***Note:*** Do NOT apply less than 50 µl in the sample cup; do NOT use silicone oil or kerosene instead of IPG strip cover fluid!)

10. Program the instrument (desired volthours, voltage gradient, temperature etc.) and run IEF according to the recommended settings in **Tables 3 & 4**.
    Note: Omit the low-voltage rehydration step recommended in **Table 3** for sample in-gel rehydration.

11. After IEF is complete, procedded with equilibration (Section 3.2) and second dimension IEF (SDS-PAGE) (Section 3.3), or store the IPG strips, for up to several months, between two plastic sheets at –70°C.

Notes / experimental tips:

(1) When IPG strips with separation distances ≤ 110 mm are used, voltage should be limited to 5000 V.

(2) Theoretically, no further intervention is required after the start of IEF until IEF has been completed. In practice, however, superior results are obtained if the electrode filter pads are replaced by new ones after the sample has entered the IPG strip. This is particularly important for samples that contain high amounts of salts and/or protein, or when very alkaline IPGs (*e.g.*, IPG 6-12 or IPG 9-12) are used. In these cases, the filter paper pads should be replaced every 2 hours.

Table 3 IPGphor running conditions (for sample cup-loading or in-gel rehydration)

Gel length	180 mm		
Temperature	20°C		
Current max.	0.05 mA per IPG strip		
Voltage max.	8000 V		
I ANALYTICAL IEF			
Reswelling*:	• 30 V, 12 - 16 h*		
Initial IEF:	• 200 V, 1 h, 500 V, 1h, 1000 V, 1h • Gradient from 1000 V to 8000 V within 30 min		
IEF to the steady-state:	• 8000 V to the steady-state, depending on the pH used used:		
1-1.5 pH units	**4 pH units**		**7 pH units**
e.g. IPG 5-6 .. 8 h	IPG 4-8 ..... 4 h		IPG 3-10 L ..... 3 h
e.g. IPG 4-5.5 8 h			IPG 3-10 NL .. 3 h
3 pH units	**5-6 pH units**		**8-9 pH units**
IPG 4-7 ..... 4 h	IPG 4-9 ..... 4 h		IPG 3-12 ..... 3 h
			IPG 4-12 ..... 3 h
II MICROPREPARATIVE IEF			
Reswelling*:	• 30 V, 12-16 h*		
IEF to the steady state:	• Focusing time of analytical IEF + additional 50% (approx.)		

* Omit the low-voltage reswelling step in case of sample cup-loading

Table 4 IPGphor running conditions for very alkaline IPGs (sample cup loading)

Gel length	180 mm
Temperature	20°C
Current max.	0.07 mA per IPG strip
Voltage max.	8000 V
IPGs	6-12, 9-12, 10-12
Sample application	anodic
Initial IEF:	• 150 V, 1 h • 300 V, 1h • 600 V, 1h
IEF to the steady-state:	• Gradient from 600 V to 8000 V within 30 min • 8000 V to the steady-state
Total volthours:	• 32,000 Vh

3.2 IPG strip equilibration for proper protein transfer and pattern quality

Prior to the second-dimension separation (SDS-PAGE), it is essential that the IPG strips are equilibrated to allow the separated proteins to fully interact with SDS. Due to the observation that the focused proteins bind more strongly to the fixed charged groups of the IPG gel matrix than to carrier ampholyte gels, relatively long equilibration times (10–15 minutes), as well as urea and glycerol to reduce electroendosmotic effects are required to improve protein transfer from the first to the second dimension (Görg *et al.*, 1988). Thiourea is sometimes recommended for more efficient transfer of hydrophobic proteins (Pasquali *et al.*, 1997), but may cause vertical streaks in the 2-DE pattern. The by far most popular protocol is to incubate the IPG strips for 10–15 minutes in the buffer originally described by Görg *et al.* (1987) [50 mM Tris-HCl (pH 8.8), containing 2% (w/v) SDS, 1% (w/v) dithiothreitol (DTT), 6 M urea and 30% (w/v) glycerol]. This is followed by a further 10–15 minute equilibration in the same solution containing 4% (w/v) iodo-acetamide instead of DTT (**Fig. 15**). The latter step is used to alkylate any free DTT, as otherwise it migrates through the second-dimension SDS-PAGE gel, resulting in an artifact known as point-streaking that can be observed after silver staining. More importantly, iodoacetamide alkylates sulfhydryl groups and prevents their reoxidation. This two-step reduction/alkylation procedure is highly recommended, since it considerably simplifies downstream sample preparation (protein in-gel digestion) for spot identification by mass spectrometry. After equilibration, the IPG strips are applied onto the surface of the second-dimension horizontal or vertical SDS-PAGE gels.

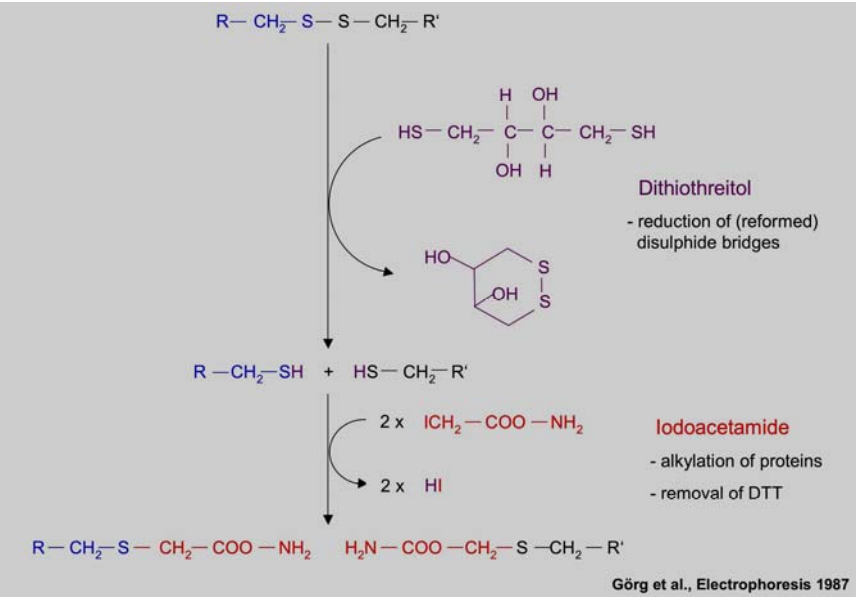


Figure 15 Reduction-Alkylation of proteins (Görg *et al.,* 1987, 1988)

Loss of proteins during the equilibration step and subsequent transfer from the first to the second dimension has been reported and is primarily due to (i) proteins which remain in

the IPG strip because of adsorption to the IPG gel matrix and/or insufficient equilibration times, and (ii) wash-off effects. Experiments with radioalabelled proteins have shown that up to 20% of the proteins get lost during equilibration (Zuo 2000). The majority of these proteins (most probably, those located near the surface of the IPG strip) is lost during the very first minutes of equilibration and is quite reproducible for a given sample, whereas protein losses in the second equilibration step are only marginal.

PROTOCOL: IPG strip equilibration

MATERIALS

Chemicals

Sodium dodecyl sulfate (SDS) (*Serva*), iodoacetamide, D,L-dithiothreitol (DTT) tris(hydroxymethyl)aminomethane (Tris) (*Sigma*), bromophenol blue, glycerol, urea, sodium azide (*Merck*)

Reagents

- Bromophenol blue solution [0.25% (w/v) of bromophenol blue in Tris-HCl buffer]
  To make 20 ml, dissolve 50 mg of bromophenol blue in 20 ml of Tris-HCl buffer. Store at 4°C.
- Equilibration buffer [6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer, (pH 8.8)]. To make 500 ml, combine 180 g of urea, 150 g of glycerol, 10 g of SDS and 16.7 ml of Tris-HCl buffer. Dissolve in deionized H_2O and adjust the volume to 500 ml. This buffer can be stored at room temperature up to 2 weeks.
- Tris-HCl buffer [1.5 M Tris-HCl (pH 8.8), 0.4% (w/v) SDS]. To make 25 ml, dissolve 4.55 g of Tris base (*GE Healthcare Lifesciences*) and 0.1 g of SDS in about 20 ml of deionized H_2O. Adjust the pH of the solution with 4 N HCl and adjust the volume to 25 ml with deionized H_2O. Add 2.5 mg of sodium azide, and filter. The buffer can be stored at 4°C up to 2 weeks

Equipment

Glass test tubes for equilibration (200–250 mm long; 20 mm internal diameter); laboratory shaker; parafilm

METHOD

1. Dissolve 100 mg of DTT in 10 ml of equilibration buffer to make equilibration buffer A. Make 10 ml per IPG strip. Place the focused IPG strips into individual test tubes (**Fig. 16**). Add 10 ml of equilibration buffer A and 50 µl of bromophenol blue solution to each tube. Seal the tubes with Parafilm, rock them for 15 minutes on a shaker, and pour off the equilibration buffer.

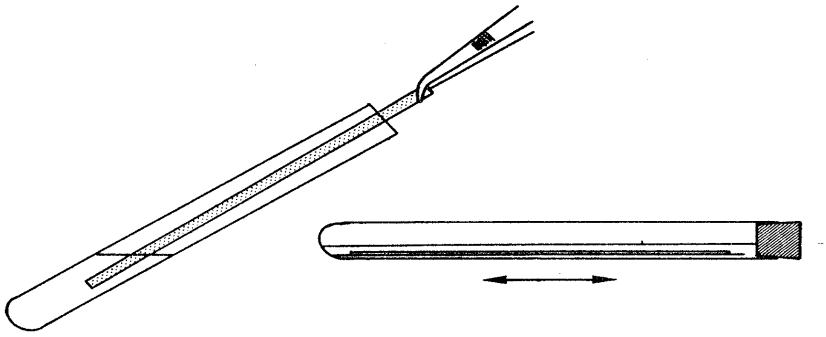


Figure 16 Equilibration of IPG strips prior to SDS-PAGE

2. Dissolve 0.4 g of iodoacetamide in 10 ml of equilibration buffer to make equilibration buffer B. Make 10 ml per sample. Add this buffer and 50 µl of bromophenol blue solution to each tube, and equilibrate for another 15 minutes with gentle agitation.

Note: Shorter equilibration times (10 min) may be applied, however at the risk that some proteins may not migrate out of the IPG gel strip during sample entry into the SDS gel.

3. Pour off equilibration buffer B, and proceed to SDS-PAGE (see section 3.3). If SDS-PAGE is performed on a horizontal electrophoresis unit (*e.g.*, Multiphor II), briefly rinse the IPG gel strip with deionized water and place it on a piece of filter paper at one edge for a few minutes to drain off excess equilibration buffer. If SDS-PAGE is performed on a vertical electrophoresis unit (*e.g.*, Ettan Dalt), briefly rinse the equilibrated IPG strip with electrode buffer.

Table 5 IPG strip equilibration protocol (Görg *et al.*, 1987, 1988)

Reagent	**Purpose**
50 mM Tris-Hcl pH 8.8	
+ 2% SDS	Improved protein transfer from IPG strip into SDS gel
+ 6 M urea	Improved protein transfer from IPG strip into SDS gel
+ 30% glycerol	Improved protein transfer from IPG strip into SDS gel
+ 1% dithiothreitol	Complete reduction of disulfide bonds
+ 4% iodoacetamide	Removal of excell DTT (no point streaking) Alkylation of SH-groups Simplifies sample preparation for mass spectrometry

	Dithiothreitol	**Iodoacetamide**
1st step, 15 min	**+**	**-**
2nd step, 15 min	**-**	**+**

3.3 Second Dimension: SDS-PAGE on horizontal and vertical systems

SDS-PAGE can be performed on horizontal or vertical systems (Görg *et al.*, 1995). Horizontal setups (Görg & Weiss, 1999) are ideally suited for ready-made gels (*e.g.*, ExcelGel SDS; *GE Healthcare Lifesciences*), whereas vertical systems are preferred for multiple runs in parallel, in particular for large-scale proteome analysis which usually requires simultaneous electrophoresis of batches of second-dimension SDS-PAGE gels for higher through-put and maximal reproducibility (Anderson & Anderson 1978). The most commonly used buffers for the second dimension of 2-DE are the discontinuous buffer system of Laemmli (1970) and modifications thereof, although for special purposes other buffer systems are employed, such as borate buffers for the separation of highly glycosylated proteins (Patton *et al.*, 1991). Typically, gel sizes of 20 x 25 cm^2 and a gel thickness of 1.0 mm are employed. In contrast to horizontal SDS-PAGE systems, it is not necessary to use stacking gels with vertical setups, as the protein zones within the IPG strips are already concentrated and the nonrestrictive, low polyacrylamide concentration IEF gel acts as a stacking gel (Dunn, 1993).

The analysis of low Mr (< 15 kDa) and high Mr (> 150 kDa) proteins is somewhat intricate since there is no “standard“ 2-DE system which effectively allows separation of proteins over the entire Mr range between 5 kDa and 500 kDa. A common approach is to combine several gels optimized for the approximate Mr ranges 5-30 kDa, 15-200 kDa, and > 150 kDa instead of using a single “standard“ 2-DE system, or to use polyacrylamide „pore“ gradient gels. Conventional Tris-glycine gels do not allow efficient separation of proteins below 15 kDa. Hence, Schägger & von Jagow (1987) have described a Tris-Tricine buffer systems for the separation of low Mr (3-30 kDa) polypeptides. Fountoulakis *et al*. (1998) have improved this gel system, which permits efficient and reproducible separation of proteins with Mrs between 5 kDa and 20 kDa.

The major problem associated with high Mr proteins is that a significant proportion of these proteins is rather hydrophobic, and, consequently, will not readily dissolve in “standard“ urea lysis and/or rehydration solutions used for sample solubilization and IEF. Even though these proteins were solubilized, they will not always enter the IEF gel matrix, or are not transferred from the first to the second dimension. Several strategies have been proposed to overcome at least some of these obstacles. For example, it has been demonstrated that sample application of high Mr proteins to IPG gels *via* cup-loading is more efficient than “passive“ sample application by sample in-gel rehydration If samples are applied by in-gel rehydration, “active“ reswelling by applying low voltage (30 - 50V) during the rehydration step is superior to “passive“ loading and improves the entry of high Mr proteins into the polyacrylamide matrix (Görg *et al.*, 2000). The transfer of high Mr proteins from the IPG strip onto the SDS gel is enhanced by sufficiently long equilibration steps (2 x 15 min; *see* Section 3.2). The same holds true for application of low voltages

during the transfer step, *i.e.*, 50 V for vertical SDS gels, and 100 V for horizontal SDS-PAGE systems, respectively (Görg *et al.*, 2000).

3.3.1 SDS Gel casting

Due to simplified handling and higher reproducibility, in most cases, SDS gels of a homogeneous polyacrylamide concentration are preferred, but polyacrylamide concentration ("pore") gradients, which extend the range over which proteins of different molecular mass can be effectively separated, are also used. The following protocol describes the procedure for casting horizontal SDS pore gradient gels on plastic backing according to Görg *et al.* (1980), and multiple SDS slab gel casting according to Anderson and Anderson (1978). Our preferred gel thickness is 0.5 mm for horizontal, and 1.0 mm for vertical SDS gels. Typical polyacrylamide concentrations are 10% -15%, which allows effective separation of proteins with Mrs between 10 kDa and 150 kDa.

PROTOCOL: SDS gel casting

MATERIAL

Note: Not all of the materials listed below are required for all types of gels.

Chemicals

Repel silane, ammonium persulfate, acrylamide, methylene bisacrylamide, tetramethyl-ethylenediamine (TEMED) (*GE Healthcare Lifesciences*); Serdolit MB-1 mixed bed ion exchanger resin, sodium dodecyl sulfate (SDS) (*Serva*), tris(hydroxymethyl) aminomethane (Tris) (*Sigma*), glycerol, sodium azide (*Merck*).

Reagents for horizontal SDS gels on plastic backing

- Acrylamide/Bisacrylamide solution (30% T, 3% C): 29.1% (w/v) acrylamide and 0.9% (w/v) methylenebisacrylamide in deionized water.
  To make 100 ml, dissolve 29.1 g of acrylamide and 0.9 g of methylenebisacrylamide in deionized water and fill up to 100 ml. Add 0.5 g of Serdolit MB-1, stir for 10 min and filter. The solution can be stored up to 2 weeks in a refrigerator.
- Stacking gel buffer stock: 0.5 M Tris-HCl, pH 6.8 and 0.4 % (w/v) SDS.
  To make 100 ml, dissolve 6.05 g of Trizma base and 0.4 g of SDS in about 80 ml of deionized water. Adjust to pH 6.8 with 4N HCl and fill up to 100 ml with deionized water. Add 10 mg of sodium azide and filter. The buffer can be stored at 4°C for two weeks.

Note: In most cases, stacking gel buffer can be replaced by resolving gel buffer without any loss of resolution.

- Resolving gel buffer stock: 1.5 M Tris-HCl, pH 8.8 and 0.4% (w/v) SDS
  To make 100 ml , dissolve 18.15 g of Trizma base and 0.4 g of SDS in about 80 ml of deionized water. Adjust to pH 8.8 with 4N HCl and fill up to 100 ml with deionized water. Add 10 mg of sodium azide and filter. The buffer can be stored at 4°C up to two weeks.
- Ammonium persulfate solution: 40% (w/v) of ammonium persulfate in deionized water. To prepare 1 ml of the solution, dissolve 0.4 g of ammonium persulfate in 1 ml of deionized water. This solution should be prepared fresh before use.

Reagents for multiple vertical SDS gels

- Acrylamide/Bisacrylamide solution (30.8% T, 2.6% C): 30% (w/v) acrylamide and 0.8% (w/v) methylenebisacrylamide in deionized water.
  To make 1000 ml, dissolve 300.0 g of acrylamide and 8.0 g of methylene-bisacrylamide in deionized water and fill up to 1000 ml. Add 1 g of Serdolit MB-1, stir for 10 min and filter. The solution can be stored up to 2 weeks in a refrigerator.
- Resolving gel buffer: 1.5 M Tris-HCl, pH 8.6 and 0.4% (w/v) SDS (Laemmli 1970).
  To make 500 ml, dissolve 90.85 g of Trizma base and 2 g of SDS in about 400 ml of deionized water. Adjust to pH 8.6 with 4 N HCl and fill up to 500 ml with deionized water. Add 50 mg of sodium azide and filter. The buffer can be stored at 4°C up to 2 weeks.
- Ammonium persulfate solution: 10% (w/v) of ammonium persulfate in deionized water. To prepare 10 ml of the solution, dissolve 1.0 g of ammonium persulfate in 10 ml of deionized water. This solution should be prepared just before use.
- Overlay buffer: Buffer-saturated 2-butanol. To make 30 ml, mix 20 ml of resolving gel buffer (*see* above) with 30 ml of 2-butanol, wait for a few minutes and pipette off the butanol layer.
- Displacing-solution: 50% (v/v) glycerol in deionized water and 0.01% (w/v) bromo-phenol blue. To make 500 ml, mix 250 ml of glycerol (100%) with 250 ml of deionized water, add 50 mg of bromophenol blue and stir for a few minutes.

Equipment

Gradient mixer (2 x 15 ml), glass plates with a 0.5 mm thick U-framed spacer (200 x 260 mm^2), plain glass plates (size 200 x 260 mm^2), spacers (1 mm thick), clamps, GelBond PAGfilm (200 x 260 mm^2), roller *(GE Healthcare Lifesciences*), heating cabinet (Heraeus, Germany), Milli-Q System (*Millipore*)

METHOD

I. Casting of horizontal SDS gels on plastic backing

1. Assemble the polymerisation cassette consisting of two glass plates, one covered with GelBond PAGfilm, the other bearing the U-frame (0.5 mm thick) as described in Section 3.1.1 for casting IPG gels (*see* **Fig. 13**). GelBond PAGfilms should be washed 6 x 10 min with deionized water prior to use to avoid spot streaking upon silverstaining.

2. Immediately before casting add 5 µl of TEMED and 10 µl of ammonium persulfate to the SDS gel solutions (**Table 6**). For casting an SDS gel (size 250 x 190 x 0.5 mm^3) with a stacking gel length of 50 mm, pipette 6.0 ml of stacking gel solution into the precooled (4°C) cassette. Then cast the pore gradient on top of the stacking gel by mixing 9.0 ml of the dense (9% or 12% T, 25% glycerol) and 9.0 ml of the light (15% or 18% T, no glycerol) solution with the help of a gradient mixer in the same way as described in section 3.1.1 for casting IPG gels. The high glycerol concentration of the stacking gel solution allows overlaying of the pore gradient mixture without an intermediate polymerization step (**Fig. 17 A**).

   Note: Pore gradient gels as well as SDS gels with an identical polyacrylamide concentration in the resolving gel („homogeneous gels“) may be used for the second dimension. For casting homogeneous SDS gels, the stacking gel solution is simply overlayed with resolving gel solution with the help of a pipette (**Fig. 17 B**).

3. After pouring, leave the cassette for 15 min at room temperature to allow adequate levelling of the density gradient. Then place it in a heating cabinet at 50°C for 30 min for polymerization. The polymerized gel can be stored in a refrigerator overnight.

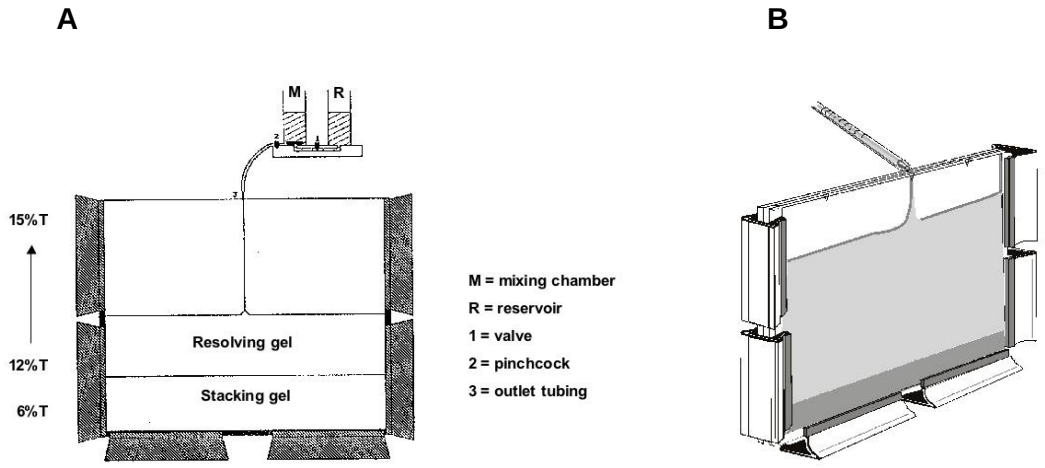


Figure 17 (**A**) Casting of horizontal SDS pore gradient gels and (**B**) homogeneous SDS gels on plastic backing

Gel composition:	***stacking gel*** (50 mm long): 6% T, 3% C, 0.1% SDS, 125 mM Tris/HCl pH 6.8 (alternatively: 375 mM Tris/HCl pH 8.8; 0.1 % SDS ***resolving gel*** (140 mm long): 9-18 % T or 12-15% T, 3% C, 0.1% SDS, 375 mM Tris/HCl pH 8.8
Gel size:	190 x 250 x 0.5 mm^3

Table 6 Recipes for casting SDS pore gradient gels (9-18%T or 12-15%T, 3%C)

	Stacking gel	**Resolving gel**		**Resolving gel**	
		dense solution	light solution	dense solution	light solution
	6% T	9% T	18% T	12% T	15% T
Glycerol (100%)	3.75 g	2.5 g	_	2.5 g	_
Stacking gel buffer*	2.5 ml	_	_	_	_
Resolving gel buffer	_	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Acrylamide/Bisacryl-amide	2.0 ml	3.0 ml	6.0 ml	4.0 ml	5.0 ml
Deionized water	2.5 ml	2.5 ml	1.5 ml	1.5 ml	2.5 ml
TEMED (100%)	5 µl	5 µl	5 µl	5 µl	5 µl
Ammonium persulfate (40%)	10µl	10µl	10µl	10µl	10µl
Final volume	10 ml	10 ml	10 ml	10 ml	10 ml

Notes: (1) Stacking gel buffer can be substituted by resolving gel buffer; (2) TEMED and ammonium persulfate are added to the solutions immediately before gel casting

II. Casting of vertical SDS gels for multiple runs

Vertical SDS-PAGE is usually performed in the DALT apparatus originally described by Anderson & Anderson (1978), or in the Ettan Dalt II vertical electrophoresis unit. These systems allow 12 (Ettan Dalt II) or even 20 (Dalt apparatus) SDS slab gels to be run simultaneously under identical conditions. A stacking gel is usually not necessary with these instruments. Ready-made gels on plastic backing (*GE Healthcare Lifesciences*) for the Ettan Dalt II electrophoresis unit are also available.

1. The gel casting cassettes (200 x 250 mm^2) are made in the shape of books consisting of two glass plates connected by a hinge strip, and two 1.0 mm thick spacers in between them. Stack 22 cassettes (Dalt apparatus) or 14 cassettes (Ettan Dalt II) vertically into the gel casting box with the hinge strips to the right, interspersed with plastic sheets (*e.g.,* 0.05 mm thick polyester sheets). The stack is firmly held in position with a 10 mm thick polyurethane sheet on both ends.

2. Put the front plate of the casting box in place and screw on the nuts (hand tight).

3. Connect a polyethylene tube (*i.d.* 5 mm) to a funnel held in a ring-stand at a level of about 30 cm above the top of the casting box. The other end of the tube is placed in the grommet in the casting box side chamber (**Fig. 18**).

4. Fill the side chamber with heavy displacing solution.

5. Immediately before gel casting, add TEMED and ammonium persulfate solutions to the gel solution (Table 8). To cast the gels, the gel solution (1400 ml in case of the Dalt chamber, and 950 ml in case of the Ettan II unit) is poured into the funnel. Avoid introduction of any air bubbles into the tube!

   Notes: (**1**) Do not fill the cassettes with acrylamide solution completely, as some space at the top (~10 mm) is needed to fix the IPG strip to the SDS gel with hot agarose. (**2**) It is also possible to cast polyacrylamide contentration gradient SDS gels –with the help of a gradient maker- as described for horizontal SDS pore gradient gels.

6. When pouring is complete, the tube is removed from the side chamber grommet so that the level of the displacing solution in the side chamber falls.
7. Very carefully pipette about 1 ml of overlay buffer onto the top of each gel in order to obtain a smooth, flat gel top surface.

8. Allow the gels to polymerize for at least three hours at room temperature, but preferably over night for higher reproducibility.

9. After gel polymerization, remove the front of the casting box and carefully unload the gel cassettes from the box, using a blade to separate the cassettes. Remove the polyester sheets which had been placed between the individual cassettes.

10. Wash each cassette with water to remove any acrylamide adhered to the outer surface and drain excess liquid off the top surface. Discard unsatisfactory gels, in particular gels with uneven thickness, *i.e.*, usually those at the outer edges of the gel casting cassette.

11. Gels that are not needed immediately can be wrapped in plastic wrap and stored in a refrigerator (4 °C) for up to two days.

Gel composition:	*no stacking gel* *resolving gel:* 10%T, 12.5%T or 15%T homogeneous, 2.6%C, 375 mM Tris/HCl pH 8.8, 0.1% SDS
Gel size:	200 x 250 x 1 mm^3

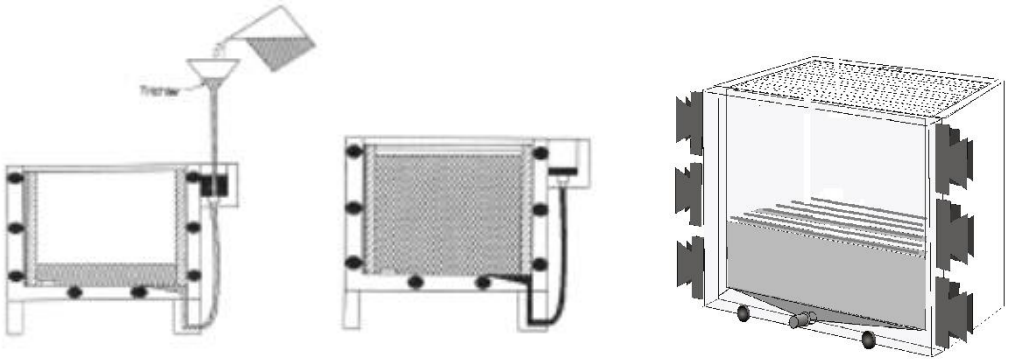


Figure 18 Multiple vertical SDS gel casting

Table 7 Recipes for casting homogeneous vertical SDS gels (10%, 12.5%, or 15% T)

Resolving gel	10% T, 2.6% C	12.5% T, 2.6% C	15% T, 2.6% C
Acrylamide/Bisacrylamide (30.8%T, 2.6%C)	455 ml	568 ml	682 ml
Gel Buffer	350 ml	350 ml	350 ml
Glycerol (100 %)	70.0 g	70.0 g	70.0 g
Deionized water	532 ml	419 ml	305 ml
TEMED (100%)	66 µl	66 µl	66 µl
Ammonium persulfate (10%)	7.0 ml	7.0 ml	7.0 ml
Final volume	1400 ml	1400 ml	1400 ml

3.3.2 Running SDS gels

The second dimension can be performed on horizontal or vertical electrophoresis systems (Görg *et al.* 1988, 1995). Horizontal setups are ideally suited for ready-made gels on film supports (*e.g.*, ExcelGel SDS), whereas vertical systems (*e.g.,* the Ettan-DALT multiple slab gel unit) are preferred for multiple runs in parallel. However, ready-made SDS gels are also available for vertical systems.

For horizontal setups, laboratory-made or ready-made SDS polyacrylamide gels (0.5 mm thick on GelBond PAGfilm) are placed on the cooling plate of the horizontal electrophoresis unit. Electrode wicks or buffer strips made from polyacrylamide are then applied. The equilibrated IPG strip(s) is (are) simply placed gel side down onto the surface alongside the cathodic electrode wick or polyacrylamide buffer strip without any embedding procedure. In the vertical setup, the equilibrated IPG gel strips are placed on top of the vertical SDS gels and embedded in hot agarose.

PROTOCOLS

MATERIAL

Chemicals

Sodium dodecyl sulfate (SDS) (*Serva*), agarose (low-melting, low electro-endosmosis), tris(hydroxymethyl)aminomethane (Tris) (*Sigma*), Bromophenol blue, glycerol, glycine, sodium azide (*Merck*), Mr marker proteins (*BioRad*)

Ready-made polyacrylamide gels

ExcelGel SDS 12-14 and buffer strips; ready-made DALT gel 12.5 (optional) (*GE Healthcare Lifesciences*).

Reagents for horizontal SDS gels

- Electrode buffer
  To make 1000 ml of a 10x solution add: 30.3 g of Trizma base, 144 g of glycine, 10.0 g of SDS and 100 mg of sodium azide. Dissolve in deionized water, fill up to 1000 ml and filter. Electrode buffer stock solution can be kept at room temperature for up to one week. Before use, mix 100 ml of the buffer with 900 ml of deionized water.

Reagents for vertical SDS gels

- Electrode buffer stock solution (96 mM Tris, 800 mM Glycine, 0.4% SDS)
  To make 4x electrode buffer concentrate, dissolve 58.0 g of Trizma base, 299.6 g of glycine and 20.0 g of SDS in deionized water Adjust the volume to 5.0 liters.
- Agarose solution (0.5%)

Suspend 1.0g of agarose in 90 ml of electrode buffer stock solution + 100 ml of deionized water and melt it in a boiling water bath or in a microwave oven. Then add 10 ml of bromophenol blue solution (50 mg of bromophenol blue dissolved in 10 ml of electrode buffer stock solution).

Equipment

Multiphor II horizontal electrophoresis apparatus, power supply, Multitemp II thermostatic circulator, DALT or Ettan Dalt II vertical electrophoresis system, cassette rack (*GE Healthcare Lifesciences),* electrode wicks (200 x 250 mm^2, Ultra pure), Milli-Q System (*Millipore*)

Note: A cassette rack is recommended for storing Dalt cassettes in an upright position to facilitate the application of the IPG strips.

METHOD

I. SDS electrophoresis of horizontal gels on plastic backing

Ia. SDS-PAGE with laboratory-made gels

1. Fill the buffer tanks of the electrophoresis unit with electrode buffer (Laemmli (1970) buffer system). Soak two sheets of filter paper (size 250 x 200 mm^2) in electrode buffer and put them on the cooling block (15°C). Soak the electrode wicks (size 250 x 100 mm^2) in electrode buffer. Place them at the edges of the buffer-soaked filter papers and perform a pre-run (600 V, 30 mA) for 3 h to remove impurities from the electrode wicks. Then remove the filter papers and discard them whereas the purified electrode wicks remain in the electrode buffer tanks and are used repeatedly.

2. During the equilibration step of the IPG gel strips (*see* Section 3.2), open the gel polymerization cassette, pipette a few ml of kerosene on the cooling block (20°C) of the electrophoresis unit and place the SDS gel (gel side up!) onto it. Apply the electrode wicks on the surface of the SDS gel so that they overlap the cathodic and anodic edges of the gel by about 10 mm.

3. Place the blotted IPG gel strip(s) gel side down (!) onto the SDS gel surface adjacent to the cathodic wick, 5 mm apart (**Fig. 19A**). No embedding of the IPG gel strip is necessary. If it is desired to co-electrophorese molecular weight (Mr) marker proteins, put a silicone rubber frame onto the SDS gel surface alongside the IPG gel strip and pipette 5 µl of Mr marker proteins dissolved in SDS-buffer into the frame.

4. Place the lid on the electrophoresis unit and start SDS-PAGE with 100 V for about 75 min with a limit of 20 mA. When the Bromophenol Blue tracking dye has completely moved out of the IPG gel strip, interrupt the run, remove the IPG gel strip and move the cathodic electrode wick forward for 4-5 mm so that it now overlaps the former sample application area (**Fig. 20**). Then continue the run at 600 V with a limit of 30 mA

until the tracking dye has migrated into the anodic electrode wick. Total running time is approximately 6 h (separation distance: 180-200 mm) (**Table 8**). The gel is then fixed in 40% alcohol and 10% acetic acid for at least one hour and stained with either silver nitrate or Coomassie Blue. Alternatively, it can be removed from its plastic backing with the help of a film remover (*GE Healthcare Lifesciences*) and used for blotting.

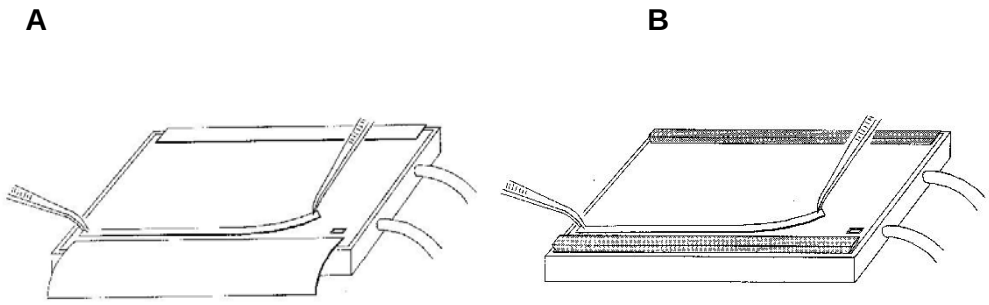


Figure 19 Horizontal SDS-PAGE on (**A**) laboratory-made and (**B**) ready-made gels
Transfer of the equilibrated IPG gel strip onto the surface of horizontal SDS gesl alongside the cathodic paper wick (A) or polyacrylamide buffer strip (B)

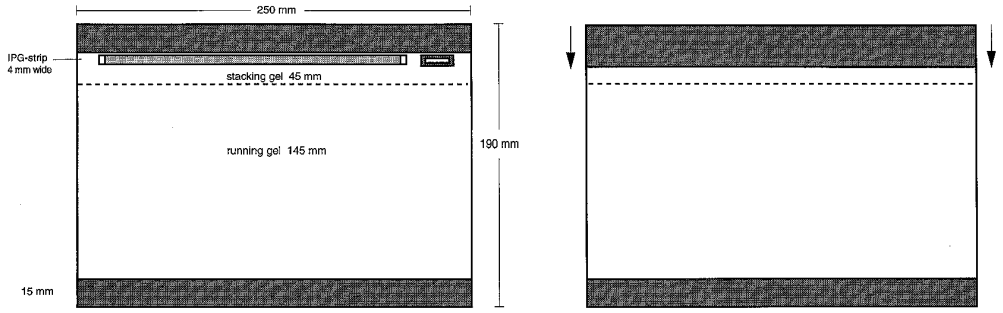


Figure 20 Removal of IPG strips after protein transfer from first to second dimension

Table 8 Running conditions for laboratory-made SDS-pore gradient gels *(12-15% T, 3% C, size 250 x 190 x 0.5 mm^3)*

time	voltage	current	power	temperature
75 min	100 V	20 mA	30 W	20°C
remove the IPG gel strip and move forward the cathodic electrode wick so that it overlaps the former IPG strip application area				
5 h	600 V	30 mA	30 W	20°C

Ib. SDS-PAGE with ready-made Excel gels

1. Equilibrate the IPG strips as described above (*see* Section 3.2).
2. During the equilibration is in progress, assemble the SDS ExcelGel for the second dimension: Remove the ExcelGel from its foil package. Pipette 2-3 ml of kerosene on the cooling plate of the horizontal electrophoresis unit (20°C). Remove the protective cover from the top of the ExcelGel and place the gel on the cooling plate, cut-off edge towards the anode. Avoid trapping air bubbles between the gel and the cooling plate.
3. Peel back the plastic foil of the cathodic SDS buffer strip. Wet your gloves with a few drops of deionized water and place the buffer strip on the cathodic end of the gel. Avoid trapping air bubbles between gel surface and buffer strip.
   Note: Use nitrile or vinyl gloves. Latex gloves are not recommended, as the polyacrylamide gel and buffer strips tend to stick to the latex.
4. Repeat this procedure with the anodic buffer stril.
5. Place the equilibrated and slightly blotted IPG strips (*see* Section 3.2) gel side down on the surface of the ExcelGel, 5 mm apart from the cathodic buffer strip (**Fig. 19 B**).
6. Press gently on top of the IPG gel strips with forceps to remove any trapped air bubbles.
7. Align the electrodes with the buffer strips and lower the electrode holder carefully onto the buffer strips.
8. Start SDS-PAGE at 150 V for about 45 min with a limit of 20 mA. When the Bromophenol Blue tracking dye has moved 4-5 mm from the IPG gel strip, interrupt the run, remove the IPG gel strip and move the cathodic buffer strip forward so that it just covers the former contact area of the IPG gel strip (*cf.* **Fig. 20**). Re-adjust the electrodes and continue electrophoresis at 800 V and 40 mA for about 160 min until the Bromophenol Blue dye front has reached the anodic buffer strip. (**Table 9**) and proceed with protein staining or with blotting.

Table 9 Running conditions of ExcelGel SDS Gradient 12-14 *(12-14% T, 3% C, size 250 x 180 x 0.5 mm^3)*

time	voltage	current	power	temperature
45 min	100V	20 mA	50 W	20°C
remove the IPG gel strip and move forward the cathodic buffer strip so that it overlaps the former IPG strip application area				
160 min	800 V	40 mA	50 W	20°C

II. Multiple vertical SDS-PAGE

IIa. SDS-PAGE using the Ettan Dalt II vertical electrophoresis unit

1. Add 1875 ml of electrode buffer stock solution and 5625 ml of deionized H_2O to the lower electrophoresis buffer tank of the Ettan DALT II unit. Mix, and turn on cooling (25°C).

2. Support the DALT gel cassettes (containing the SDS gels) in a vertical position on the cassette rack to facilitate the application of the IPG gel strips.

3. Briefly rinse the equlibrated IPG strip with electrode buffer (diluted 1:1 with H_2O) and place the IPG strip on top of the DALT gel cassette. Use a thin spatula or a ruler to push against the plastic backing of the IPG strip and slide it into the gap between the two glass plates (**Fig. 21**). Add 2 ml of hot (75°C) agarose solution, and continue to slide the strip down onto the surface of the SDS gel until good contact is achieved. Avoid trapping air bubbles between the IPG strip and the SDS gel surface.
***Note*:** For co-electrophoresis of molecular weight marker proteins, soak a filter paper pad (2x4 mm^2) with 5 µl of SDS marker proteins dissolved in electrophoresis buffer, let it dry, and apply it to the left or right of the IPG strip. Dried filter paper pads soaked with molecular weight marker proteins can be stored in microfuge tubes at –70°C.

4. Allow the agarose to solidify for at least 5 minutes before placing the slab gel into the electrophoresis apparatus (*see* Step 5). Repeat this procedure for the remaining IPG strips.
Note: Embedding in agarose is not absolutely necessary, but it ensures much better contact between the IPG gel strip and the top of the SDS gel.

5. Wet the outside of the gel cassettes by dipping them into electrode buffer to make them fit more easily into the electrophoresis unit. Insert them in the electrophoresis apparatus. If necessary, push blank cassette inserts into any unoccupied slots. Seat the upper buffer chamber over the gels and fill it with 2.5 liters of electrode buffer (1250 ml of buffer stock solution + 1250 ml of deionized H_2O).

6. Place the safety lid on the electrophoresis unit and start SDS-PAGE with 5 mA per SDS gel (100 V maximum setting) for ~2 hours. Continue with 15 mA per SDS gel (200 V maximum setting) for ~16 hours overnight or higher current for faster runs (*see* Table 11)

7. Terminate the run when the bromophenol blue tracking dye has migrated off the lower end of the gel.

8. After electrophoresis, carefully open the cassettes with a plastic spatula. Use the spatula to separate the agarose overlay from the polyacrylamide gel. Carefully peel the gel from the glass plate, lifting the gel by its lower edge and place it in a box of fixing or staining solution (*see* Chapter 4 on Protein Visualization).

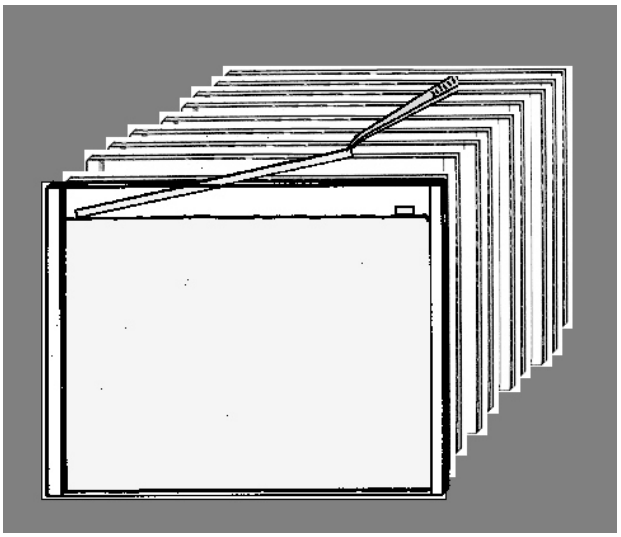


Figure 21 Loading the equilibrated IPG gel strips onto vertical SDS gels

Table 10 Running conditions for 12 vertical SDS gels *(12.5% T, 2.6% C)* using the Ettan-DALT II vertical electrophoresis unit

Fast run

time	current	temp.
2 h	50 mA	25°C
8 h	340 mA	25°C

Slow run (overnight)

time	current	temp.
2 h	50 mA	25°C
16 h	180 mA	25°C

IIb. SDS-PAGE using the Dalt electrophoresis unit

In contrast to most vertical electrophoresis systems, the Dalt electrophoresis unit developed by Anderson & Anderson (1978) consits of a single buffer tank for simplified handling and improved reproducibility.

1. Fill the electrophoresis tank with 15.0 l deionized water and 5.0 l of electrode buffer stock solution, and and turn on cooling (25°C).
2. Support the SDS gel cassettes in a vertical position on the cassette rack to facilitate the application of the first dimension IPG strips.
3. Equilibrate the IPG gel strips as described above (*see* Section 3.2) and immerse them briefly in electrode buffer.
4. Place the IPG gel strip on top of an SDS gel and overlay it with 2 ml of hot agarose solution (75°C). Carefully press the IPG strip with a spatula onto the surface of the

SDS gel to achieve complete contact (**Fig. 21**). Allow the agarose to solidify for at least 5 min. Repeat this procedure for the remaining IPG strips.

5. Insert the gel cassettes in the electrophoresis apparatus and start electrophoresis. In contrast to the procedure of horizontal SDS-PAGE it is not necessary to remove the IPG gel strips from the surface of the vertical SDS gel once the proteins have migrated out of the IPG gel strip.

6. Run the SDS-PAGE gels according to the settings in **Table 11**.

7. Terminate the run when the Bromophenol Blue tracking dye has migrated off the lower end of the gel.

8. Open the cassettes carefully with a plastic spatula. Use a spatula to remove the agarose overlay from the polyacrylamide gel.

9. Peel the gel off the glass plate carefully, lifting it by the lower edge and place it in a tray containing fixing solution or transfer buffer, respectively. Then continue with fixing, protein staining or blotting.

Table 11 Running conditions for 10 vertical SDS gels *(13% T homogeneous, 2.6% C, size 250 x 200 x 1 mm^3) using the Hoefer DALT tank*

time	voltage	current	power	temperature
18 h	150 V	150 mA	50 W	25°C

Protein fixation

After termination of the second dimension run (SDS-PAGE), fixing is necessary to immobilize the separated proteins in the gel and to remove any non-protein components which might interfere with subsequent staining. Depending on gel thickness, the gel is submersed in the fixative for one hour at least, but usually overnight, with gentle shaking.

Widely used fixatives are either 20% (w/v) trichloroacetic acid (TCA), or methanolic (or ethanolic) solutions of acetic acid (*e.g.* methanol / distilled water / acetic acid 45/45/10). A disadvantage of the latter procedure is that low molecular weight polypeptides may not be adequately fixed. When using fluorography, fixing may be carried out in 30% isopropyl alkohol / 10% acetic acid, as methanol can interfere with detection. Several authors also recommend aqueous solutions of glutardialdehyde for covalently cross-linking proteins to the gel matrix (*e.g.* for diamin silver staining). However, in this case subsequent protein identification, *e.g.*, by mass spectrometry, is impractical.

4 PROTEIN DETECTION AND QUANTITATION

After 2-D PAGE, the separated proteins have to be visualized, either by “universal” or by "specific" staining methods. Since the concentrations of individual proteins in a single cell differ between six or seven orders of magnitude, ranging from several millions of copies/cell for some highly abundant proteins (such as glycolytic enzymes) to a few copies/cell for very low abundant proteins, these enormous variations in protein concentrations are a major challenge for almost all currently available protein detection methods. The most important properties of protein visualization methods are high sensitivity (low detection limit), high linear dynamic range (for quantitative accuracy), reproducibility, and compatibility with post-electrophoretic protein identification procedures, such as mass spectrometry. Unfortunately, currently no staining method for 2-D gels meets all requirements for proteome analysis.

Universal detection methods of proteins on 2-D gels include staining with anionic dyes (*e.g.*, Coomassie Blue), negative staining with metal cations (*e.g.*, zinc imidazole), silver staining, fluorescence staining or labelling, and radioactive isotopes, using autoradiography, fluorography, or Phosphor-imaging (*see* **Fig. 22**). For most of these staining procedures, the resolved polypeptides have to be fixed in solutions such as in ethanol/acetic acid/ H_2O for at least several hours (but usually overnight) before staining to remove any compounds (*e.g.*, carrier ampholytes, detergents) that might interfere with detection. ***Specific*** staining methods for detection of post-translational modifications (glycosylation, phosphorylation *etc.*) are employed either directly in the 2DE gel, or after transfer (blotting) onto an immobilizing membrane (Towbin *et al.,* 1979; Kyhse-Andersen, 1984). The blotted proteins can be probed with specific antibodies (*e.g.*, against phosphotyrosine residues) or with lectins (agains carbohydrate moieties). The *pros* and *cons* of most protein detection and quantitation methods applied in proteome analysis have recently been reviewed by Patton (2002) and Rabilloud (2002).

Gels must be dried before autoradiography or for permanent storage. To dry Coomassie- or silver stained gels, they are first soaked for 30-60 min in glycerol, sorbitol, or other polyols. Horizontal, 0.5 mm thin SDS gels on plastic backing are usually impregnated in 30% glycerol for 30 min, air-dried for 2-3 hours and sealed in a plastic bag. Alternatively, they are soaked in 2-3% glycerol for 30 min, covered with a cellophane sheet and air-dried overnight. Problems with gel swelling or shrinking during fixing, staining and drying are usually encountered with gels not polymerized onto plastic backing. Hence, vertical slab gels without plastic backing must first be „shrunk“ to their original size in 10% glycerol + 30% ethanol for 20 min. Then they are air-dired between two sheets of cellophane (soaked with the glycerol/ethanol mixture) in a special frame (*GE Healthcare Lifesciences*). Gels that are exposed to X-ray films (for autoradiography) or phoshor storage screens are placed on filter paper, covered with a sheet of cellophane and dried using a vacuum gel dryer for several hours at 50-60°C.

4.1 Universal protein detection and quantitation methods

Coomassie Brilliant Blue (CBB) staining methods have found widespread use for the detection of proteins on 2-D gels, last not least because of their low price, ease of use and compatibility with most subsequent protein analysis and characterization methods such as MS. However, in terms of the requirements for proteome analysis, the principal limitation of CBB stains lies in their insufficient sensitivity, which does not permit the detection of low abundance proteins (the detection limit of CBB stains is in the range of 200-500 ng protein per spot). Hence, typically no more than a few hundred protein spots can be visualized on a 2-D gel, even if milligram amounts of protein had been loaded onto the gel. CBB in colloidal dispersions according to Neuhoff *et al.* (1988) have been reported to be more sensitive than the "classical" CBB stain, but are still less sensitive than the majority of chemical stains employed in 2-D PAGE for proteomics.

Reverse staining exploits the fact that protein-bound metal cations (*e.g.*, potassium, copper or zinc) are usually less reactive than the free salt in the gel. Thus, the speed of precipitation of free or only weakly bound ions to form an insoluble salt is slower on the sites occupied by proteins than in the protein-free background. This generates transparent protein zones or spots, while the gel background becomes opaque due to the precipitated, unsoluble salt. Compared to the other reverse staining methods, but also CBB, the zinc stain offers some distinct advantages as it can be completed in 15 min for most applications, and is more sensitive than CBB, KCl or copper stains. Hence, zinc or imidazole-zinc stains (Castellanos-Serra *et al.,* 1999) are currently the most sensitive reverse (or „negative") staining methods applied in 2-DE. Zinc-imidazole staining is rapid, simple, and sensitive, and has a detection limit of roughly 20-50 ng of protein per spot. Moreover, it is compatible with subsequent protein identification by mass spectrometry, making the stain quite popular for detection of proteins separated on micropreparative 2-DE gels. The major disadvantage of zinc-staining is its rather restricted linear dynamic range, which makes this staining procedure unsuitable for detecting quantitative differences on 2-DE gels.

Silver staining methods (Merril *et al*., 1981; Oakley *et al*., 1980) are far more sensitive than CBB or imidazole-zinc stains (detection limit is as low as 0.1 ng protein/spot). They provide a linear response with over a 10-40 fold range in protein concentration, which is slightly worse than with CBB staining. However, silver staining methods are far from stoichiometric, and are much less reproducible than CBB stains due to the subjective end-point of the staining procedure which makes them less suitable for quantitative analysis. Silver staining methods are quite laborious and complex, although some progress with respect to automation has been made (Sinha *et al.*, 2001). Silver staining methods using aldehyde-based fixatives/sensitizers are the most sensitive ones, but prevent subsequent protein analysis (*e.g.*, by MS) due to protein cross-linkage. If aldehydes are omitted in the fixative and in the subsequent gel impregnating buffers

(except in the developer), microchemical characterization by peptide mass fingerprinting (PMF) is possible, (Shevshenko *et al.*, 1996; Mortz *et al.*, 2001), however at the expense of sensitivity.

Better and more confident results in terms of sensitivity and linear dynamic range of detection are obtained by protein detection methods relying on ***fluorescent*** compounds, or by ***radiolabeling*** of proteins combined with highly sensitive electronic detection methods.

Prior to the advent of highly sensitive silver staining methods, detection of proteins ***labelled with radioisotopes*** was the only way of sensitive detection for proteins separated on 2-DE gels. Radiolabelling can be accomplished by incorporating radioactive isotopes (*e.g.*, ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, ^{125}I, or ^{131}I into proteins. *In vivo* metabolic radiolabelling of samples by the incorporation of radioactive amino acids (such as ^{35}S-methionine, ^{14}C-leucine, and/or ^{32}P-phosphotyrosine) has been extensively used for proteome analysis of microorganisms and cell culture systems, *e.g.*, for studying the stress response of organisms by „pulse labelling". *In vitro* radiolabelling, *e.g.*, of human tissue proteins, is also possible by using iodination with ^{131}I or ^{125}I, however at the risk of formation of artifacts. The radiolabelled 2-D separated proteins can be detected by autoradiography or fluorography using X-ray films which are exposed to the dried gels and which can be quantified by densitometry (Link, 1999). However, these film-based techniques require long exposure times (up to several weeks) if high sensitivity is desired. Moreover, due to the limited dynamic range of the X-ray film, multiple film exposures combined with computer-aided image processing are required to quantitate high as well as low abundant proteins present in a sample. Nonetheless, even with multiple film detection only a limited dynamic range ($<10^3$) is achievable.

To overcome the limitations of X-ray film-based autoradiography, several electronic methods for the detection of radiolabelled proteins in 2-D gels have been developed. The most popular is ***phosphor-imaging***, where X-ray films have been replaced by so-called storage-phosphor screens that contain a thin layer of special crystals doped with a europium salt. Radioactive radiation excites electrons in the crystals and a latent image is formed on the plate. Scanning the plate with a He-Ne laser results in the emission of a blue luminescence proportional to the original amount of radiation which is then quantified with a photomultiplier. The advantages compared to autoradiography using X-ray films are the possibility to detect very low levels of radioactivity in a considerably shorter time, and the high linear dynamic range (up to five orders of magnitude) (Patterson & Latter, 1993). The major disadvantage (besides the well-known general shortcomings associated with radiolabelling, such as use of hazardous and expensive radiochemicals, waste disposal, safety considerations) lies in the rather high costs for equipment (phosphor-imager, imaging screens).

Another method for the detection of radiolabelled proteins, initially described for detection of radiolabelled DNA (Broude *et al*., 1999) is ***multi photon detection (MPD)***. Although MPD technology has several advantages over conventional autoradiography such as a very high linear dynamic range (up to 7-8 orders of magnitude), high sensitivity, and the possibility of dual isotope detection for multiplexed differential display (provided that the two radiation energies are sufficiently different, which is the case for ^{125}I and ^{131}I labelled proteins). However, the technology suffers from several shortcomings, such as low resolution, low throughput (image capture of a single high resolution 2-DE gel takes up to several days), and high costs of equipment, which have to be solved before MPD technology can be routinely applied in proteome analysis.

Due to the shortcomings of organic dyes, silver staining or radiolabelling for visualization and quantitation of proteins, ***fluorescent detection of proteins*** has increasingly gained popularity for proteome analysis. Two major approaches for the fluorescent detection of proteins on 2-DE gels are currently practiced. These are: (i) covalent derivatization of proteins with fluorophores prior to IEF, and (ii) post-electrophoretic protein staining by intercalation of fluorophores into the SDS micelles coating the proteins, or by direct electrostatic interaction with the proteins (Patton, 2000). „Mid-labelling", *i.e.*, fluorescently labelling the focused proteins while still present in the IEF gel, prior to transfer to the second-dimensional SDS gel (Jackson *et al.*, 1988), is also possible, but this method seems to be applied only rarely. The best known examples for *pre-electrophoretic fluorescent labels* are monobromobimane (Urwin & Jackson, 1993) and the cyanine-based dyes (Ünlü *et al.*, 1997) that react with cysteinyl residues and lysyl residues, respectively. The latter dyes are commercially available as CyDyes (*GE Healthcare Lifesciences*), and their properties will be discussed in more detail in Section 4.3 on fluorescent difference gel electrophoresis (DIGE). The major problem of pre-electrophoretic labelling is the occurence of protein size and/or protein charge modifications which may result in altered protein mobilities alongside the Mr and/or pI axis. Alternatively, proteins can be stained with a fluorescent dye molecule *after* the electrophoretic separation has been completed. The most prominent example is the ruthenium-based dye SYPRO Ruby (Berggren *et al.*, 2002). Staining is accomplished within a few hours in a single step procedure which may be easily adapted for use with automated instrumentation. The detection limit is approximately 1-2 ng protein/spot, and the linear dynamic range of quantitation is about three orders of magnitude. A cost efficient alternative to SYPRO Ruby staining, which is based on ruthenium II tris (bathophenanthroline disulfonate), has been developed by Rabilloud *et al.* (2001). Recently, the staining protocol has been considerably improved with respect to sensitivity by optimizing reagent concentration, pH and solvent composition (Lamanda *et al.*, 2004).

In conclusion, protein detection and quantitation methods based on fluorescent staining and/or labelling are rather promising. They have a comparatively wide linear dynamic

range (>10^3) and are relatively easy to use. Furthermore, most fluorescent staining procedures are compatible with subsequent protein identification methods such as mass spectrometry. The major limitation of most fluorescent staining methods is their lower sensitivity compared to electronic detection methods of radiolabelled proteins. Typically, only proteins expressed at greater than 10^3 copies/cell can be detected on „standard" 2-DE gels by using fluorescent dye technologies, whereas –at least in theory- less than a dozen copies of a protein/cell can be visualized with the most sensitive electronic detection methods for radiolabelled proteins.

4.2 Analysis of protein post-translational modifications (PTMs)

Through genome sequencing no information can be gained on post-translational modifications (PTMs) of proteins. Protein phosphorylation is a key post-translational modification, crucial in the control of numerous regulatory pathways, enzyme activities, and degradation of proteins, whereas glycosylation is associated with biochemical alterations, developmental changes and pathogenesis, *e.g.*, tumorgenesis. Hence, detection and characterization of post-translational modifications are a major task in proteomics. One of the strenghts of 2-DE is its capability to readily locate post translationally modified proteins, as they frequently appear as distinct rows of spots in the horizontal and /or vertical axis of the 2-DE gel. Up to now, several hundred of PTMs, including phosphorylation, glycosylation, acetylation, lipidation, sulfation, ubiquination, or limited proteolysis have been reported. Various methods for the analysis of PTMs have been reviewed recently (Mann & Jensen, 2003; Yan *et al.*, 1998). The analysis of the most important PTMs -phosphorylation and glycosylation- is briefly summarized below.

Phosphoproteins can be detected on 2-DE gels by autoradiography or phosphor-imaging after *in-vivo* incorporation of ^{32}P or ^{33}P orthophosphate into proteins. However, this method is restricted to cell cultures, and cannot be applied for clinical samples obtained from patients. There are other shortcomings of this method: *E.g.*, radioactive phosphate is incorporated not only in proteins, but also in DNA and RNA, and can result in severe background staining. Another drawback is that rapid events, such as protein phosphorylation after application of external stress, cannot always be visualized due to the fact that phosporylation happens in the first minutes after the onset of stress, whereas the time-frame of incorporation of ^{32}P or ^{33}P orthophosphate is much longer in order to achieve sufficient uptake of radioactive phosphate in the cells (Bendt *et al.*, 2003). To avoid dephosphorylation during sample preparation, phosphatase inhibitors should be added.

An alternative method for phosphoprotein detection is immunostaining with phosphoamino acid-specific poly- or monoclonal antibodies after transfer (blotting) of the 2-DE separated proteins onto an immobilizing membrane. Anti phospho-tyrosine specific

antibodies, which are also commercially available, are quite specific, whereas antibodies directed against phosphoseryl and phosphothreonyl residues are more problematic, and often sensitive to the context of a larger epitope (Kaufmann *et al.*, 2001).

A technique that is particularly useful for the characterization of phosphorylation sites is mass spectrometry of 2-D separated proteins, preferably in combination with alkaline or enzymatic hydrolysis of the phosphate esters, after phosphoprotein detection by autoradiography of radiolabelled phosphoproteins, or after immunodetection (reviewed by Mann & Jensen, 2003).

Recently, a fluorescent detection method for gel-separated phosphoproteins using Pro-Q Diamond phosphoprotein dye (available from Molecular Probes) has been introduced. (Steinberg *et al.*, 2003). The method is simple and rapid. However, the specificity of the stain, in particular with complex protein samples such as in 2-DE applications, has sometimes been questioned, since highly abundant, non-phosporylated proteins may also be stained, albeit less intense than the phosphorylated ones.

Glycoprotein*s* separated by 2-DE are usually detected after blotting onto an immobilizing membrane, although several (though less sensitive) methods can also be applied directly in the electrophoresis gel (reviewed by Packer, 1999). Besides detection with autoradiography after incorporation of ^{14}C labelled sugar -a method that is applied only rarely- two major principles prevail: One is detection of glycoproteins after periodate oxidation of vicinal hydroxyls, by coupling a *carbonyl reactive group* (usually a subsituted hydrazine) to the aldehyde groups generated in the carbohydrate part of the glycoproteins. Visualization of the glycoproteins depends on the kind of reporter group attached to the hydrazine, and is achieved by UV illumination in case of fluorescent molecules (*e.g.*, dansyl hydrazine), or through the reaction product (insoluble colour, chemiluminescence *etc.*) in case of hydrazine-conjugated enzymes. Recently, the glycoprotein stain Pro-Q Emerald has been introduced that reacts with periodic acid oxidized carbohydrate groups, and which generates a green fluorescent signal on glycoproteins. The stain permits detection of approximately 1-20 ng of glycoprotein per band/spot, depending upon the nature and the degree of protein glycosylation (Hart *et al.,* 2003).

The second principle for glycoprotein detection is based on sugar binding proteins, so-called *lectins*. A wide range of lectins with different carbohydrate specificities is commercially available, either unlabelled, or labelled with a appropriate reporter groups such as enzymes (peroxidase, phosphatase *etc.*), fluorescent dye molecules (*e.g.*, FITC), or colloidal gold. Whereas it is not possible to discriminate between different carbohyrate moieties of glycoproteins when detection methods based on periodate oxidation have been employed, lectins permit a certain degree of differentiation, depending on the specificity of the lectin. However, for the detailed analysis of the saccharide composition of glycoproteins, HPLC- or MS-based methods are usually preferred.

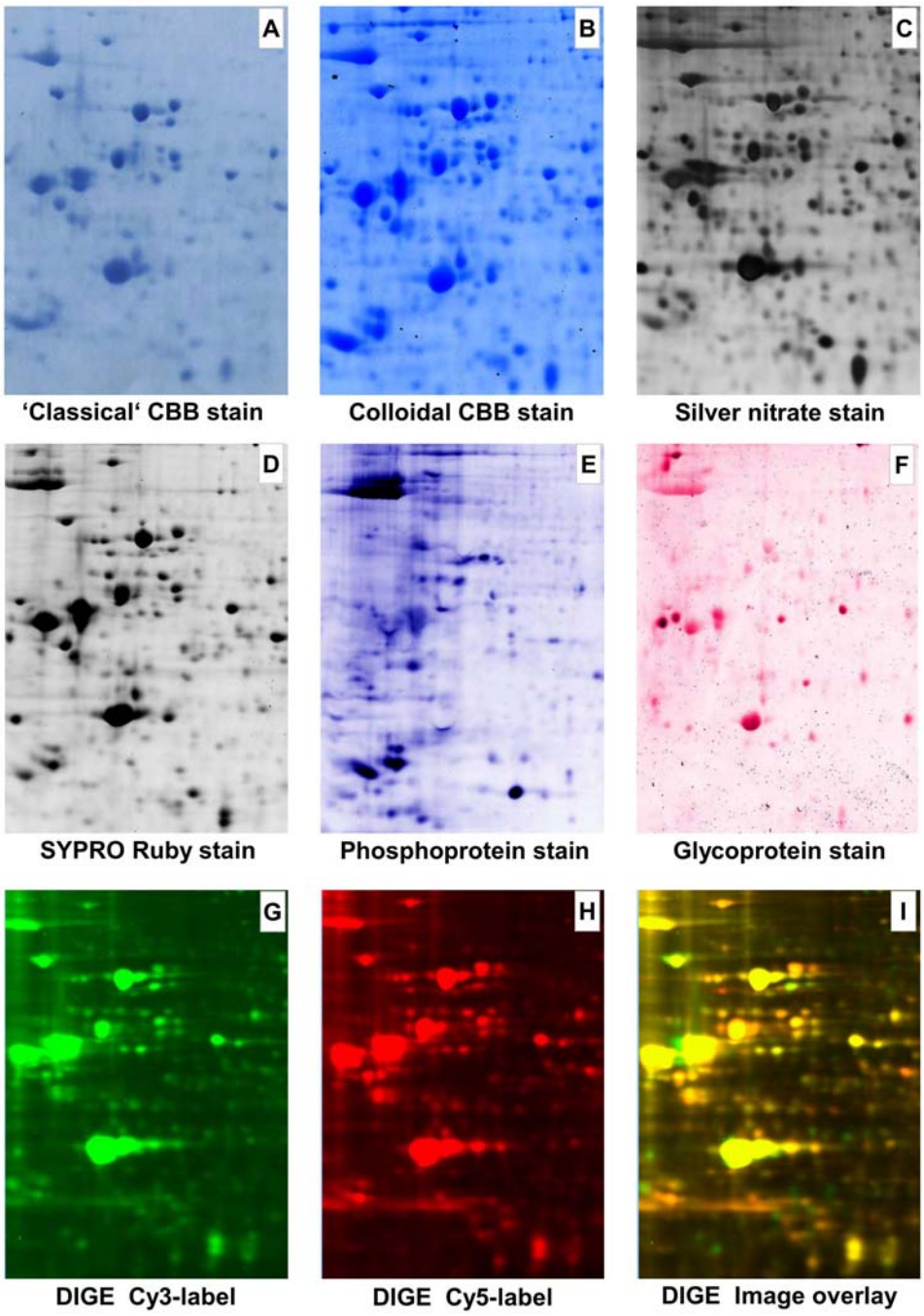


Figure 22: Visualization of total proteins, phosphoproteins, glycoproteins in 2-DE gels, and differentially expressed proteins in DIGE gels, respectively. Gels were stained with either (**A**) Coomassie Brilliant Blue (CBB, 'classical stain'), (**B**) Colloidal Coomassie Brilliant Blue, (**C**) silver nitrate, or (**D**) SYPRO Ruby for total protein, or with (**E**) Pro-Q Diamond phosphoprotein stain or (**F**) Pro-Q Emerald 488 glycoprotein stain. Proteins from two different cell lines were labelled *in vitro* with two Cyanine dyes (Cy3 and Cy5, respectively) for DIGE analysis. The amount of protein loaded onto the 2-DE gels (size 200x250x1 mm^3) was 500 µg for Coomassie Blue staining, 150 µg for glyco- and phosphoprotein, and 100 µg for all other protein detection methods (Weiss, W. Weiland, F., Görg, A., *Meth. Mol. Biol.,* in press).

4.3 Difference gel electrophoresis (DIGE) for differential display and quantitation

A bottleneck for high throughput proteomic studies is image analysis. In conventional 2-DE methodology, protein samples are separated on individual gels, stained, and quantified, followed by image comparison with computer-aided image analysis programs. Because multi-step 2-DE technology often prohibits different images from being perfectly superimposable, image analysis is frequently very time consuming. To shorten this laborious procedure, Ünlü *et al.* (1997) have developed a method called fluorescent difference gel electrophoresis (DIGE), in which two samples are labeled *in vitro* using two different fluorescent cyanine minimal dyes (CyDyes, *GE Healthcare Lifesciences*) differing in their excitation and emission wave-lengths, then mixed before IEF and separated on a single 2-D gel. After consecutive excitation with both wavelengths, the images are overlaid and 'subtracted' (normalized), whereby only differences (*e.g.*, up- or downregulated, and/or post-translationally modified proteins) between the two samples are visualized (**Fig. 23**). Due to the comigration of both samples, methodological variations in spot positions and protein abundance are excluded, and, consequently, image analysis is facilitated considerably.

A third cyanine dye is now available, which makes it possible to include an internal standard, which is run on all gels within a series of experiments. This internal standard, typically a pooled mixture of all the samples in the experiment labelled with this dye, is used for normalization of data between gels thereby minimizing experimental variation and increasing the confidence in matching and quantitation of different gels in complex experimental designs (Alban *et al.*, 2003). Nevertheless, even this approach is still dependent on accurate matching and comparison of large sets of 2-DE gels in order to generate meaningful data on differential protein expression between sets of samples (Dowsey *et al.*, 2003). Applications that profit from the DIGE system include the investigation of differential protein expression of samples generated under various prespecified conditions, the comparison of extracts, and the analysis of biological variance. In short, all analyses in which 2-D gels need to be compared are simplified and accelerated by this method.

The pIs of the cyanine dye labelled proteins remain unaffected, because the cyanine dyes compensate for the loss of the positive charge of the lysyl residues. However, the Mr increases by 434-464 Da (depending on the dye molecule) per labelled lysyl residue. Consequently, labelling of more than one lysine residue per protein molecule must be avoided; otherwise labelling with CyDyes would result in multiple spots in the vertical axis of the 2-DE gel. In practice, approximately only 3-5% of protein is labelled (hence, this procedure is also referred to as "minimal labelling"). Since the bulk of the protein remains unlabelled, the slight increase in Mr, sometimes presents a problem for spot excision for MS analysis, particularly with lower Mr proteins (Shaw *et al*., 2003). This off-set has to be

taken into accout when automatic spot pickers are used. One alternative is to stain the separated proteins additionally with SYPRO Ruby or CBB prior to spot picking from micropreparative gels. Another is so-called “saturation labelling” with similar cyanine dyes which label, cysteine (instead of lysine) residues to saturation. An additional advantage of saturation labelling is the greater sensitivity of the stain (detection limit is approximately 0.1 ng of protein, compared to 1 ng with minimal labelling. However, saturation labelling has also several drawbacks: (i) the labelling reaction must be carried out at a defined protein/dye ratio to obtain an optimal 2-DE spot pattern with a minimal number of spot trains in the vertical (*i.e.*, Mr) dimension; (ii) the dye ratio must be assessed for different types of samples, depending on the percentage of cysteines, which is a time-consuming and laborious procedure; (iii) care must be taken to avoid side-rections, such as lysine labelling; (iv) moreover, currently only two different dyes are available, which excludes the use of an internal standard; (v) up to 25% of the protein material may precipitate during the labelling reaction due to the introduction of the hydrophobic dye molecule, and (iv) the 2-DE spot pattern is significantly altered compared to that of unlabelled or minimal-labelled proteins (Shaw *et al.,* 2003).

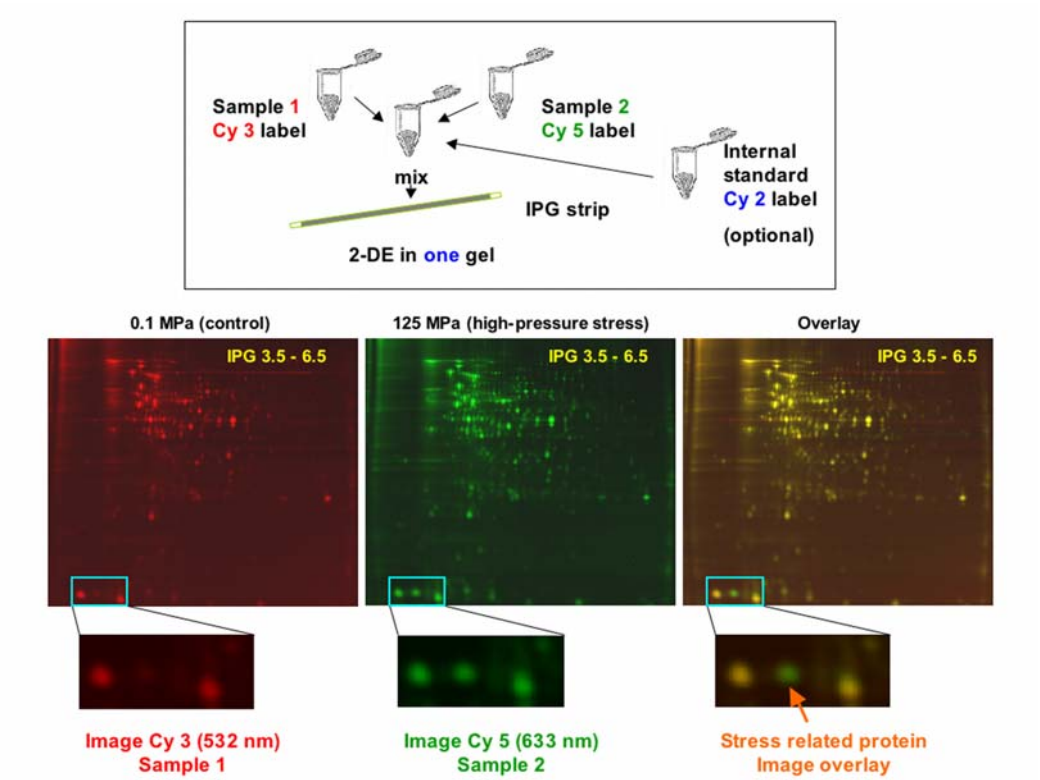


Figure 23 Detection of high-pressure stress induced proteins with fluorescent difference gel electrophoresis (DIGE) (Drews *et al.,* 2004)

Protocols

EQUIPMENT

Staining trays: Trays made of glass, plastic or stainless steel may be used, depending on the type of application. Plastics must be used for fluorescent stains as glass interferes with the staining procedure, whereas glass trays are best suited for Coomassie Brilliant Blue (CBB) and silver staining. For glass, we prefer Pyrex® (http://www.arc-international-cookware.com/en_Home.html) glass ovenware roasters, whereas for plastics we recommend polypropylene food boxes. The trays must be thoroughly clean and it is best to rinse with ethanol prior to use. Staining must be performed with trays that have never been in contact with BSA, nonfat dry milk, or any other protein blocking agent to prevent carryover contamination. The bottom of all trays must be flat and at least 20% larger than the area of the gels to be stained, *i.e.,* 25x30 cm^2 for 'standard' (*i.e.,* 20 x 25 cm^2) 2-DE gels.

Laboratory shaker: Either a slow speed (30-60 rpm, adjustable) orbital shaker (*i.e.,* the platform operates on a horizontal plane in a circular motion), or a slow speed (30-100 strokes per minute, adjustable) reciprokal shaker (*i.e.,* the shaker platform operates on a horizontal plane in a forwards and backwards motion) may be used. The shaker (*e.g.,* GFL 3016) (GFL, Burgwedel, Germany) should be robust and sufficiently large (45x45 cm^2) to accomodate at least two piles of staining trays, and ensure a smooth working motion.

Scanner/Densitometer: For digitizing Coomassie Brilliant Blue and/or silver stained gels, we recommend a high resolution flatbed scanner (*e.g.,* Epson Express 1680 Pro, Epson Europe, Amsterdam). For digitizing gels stained/labelled with fluorescent compounds, a fluorescent imager equipped with either a cooled CCD camera, or a multicolor confocal laser scanner (*e.g.,* Typhoon 9400 from GE Healthcare Life Sciences, Freiburg, Germany; Fuji-FLA-5100, Fujifilm Europe, Düsseldorf, Germany; or Bio-Rad Molecular Imager FX, Bio-Rad, Richmond, CA, USA), as well as appropriate software for image analysis are required. For DIGE analysis, low-fluorescence glass plates (3 mm thick), as well as image analysis software such as DeCyder Differential Analysis Software (GE Healthcare Life Sciences), which is specifically designed for analysis of multiplexed fluorescent images is required.

Always use powder-free non-latex (*e.g.,* nitril or vinyl) gloves when handling gels, as keratin and latex proteins are potential sources of contamination.

Reagent solutions: All solutions should be prepared in water having a resisitivity of 18 $M\Omega\ cm^{-1}$. This standard is referred to as „deionized water" or „Milli Q water" in this text. All chemicals for buffers or reagent solutions should be of analytical or biochemical grade.

METHODS

I. Coomassie Brilliant Blue (CBB) staining

'Classical' Coomassie Brilliant Blue R-250 stain (Fazekas de St. Groth *et al.*, 1963)

Reagent solutions

1. ***CBB R-250 destaining solution***: 40% (v/v) ethanol and 10% (v/v) acetic acid. To prepare 1000 mL, mix 500 mL of deionized water, 400 mL ethanol (Merck, Darmstadt, Germany) and 100 mL glacial acetic acid (Merck).
2. ***CBB R-250 staining solution***: 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250 dye in 40% ethanol and 10% acetic acid. To prepare 500 mL (which is sufficient for staining one 20x25x0.1 cm^3 2-DE gel) dissolve 500 mg CBB R-250 (*e.g.,* GE Healthcare, Bio-Rad, or Serva, Heidelberg, Germany) in 200 mL ethanol (96%; Merck) and stir for approximately 60 min. Then add 250 mL deionized water, and 50 mL glacial acetic acid (Merck) under stirring, and filter.

Method

1. Place the gel into a staining dish on a laboratory shaker and fix the proteins for >30 min in 250 mL trichloroacetic acid (TCA, 20% (w/v)) with gentle shaking.
2. Decant the fixing solution and rinse the gel briefly (1-2 min) with CBB R-250 destaining solution (40% ethanol and 10% acetic acid) to remove excess TCA.
3. Immerse the gel in 500 mL CBB R-250 staining solution and incubate at room temperature on the laboratory shaker for at least three hours. Staining can continue overnight if more convenient.
4. Discard the CBB stain and briefly rinse the gel with deionized water.
5. Destain the gel (however not completely) 2 x 30 min with CBB R-250 destaining solution with shaking until the background is no longer stained dark blue.
6. For higher sensitivity and decreased background staining, immerse the gel in 500 mL of 1% (v/v) acetic acid for up to 24 hours with gentle shaking.

Colloidal Coomassie Brilliant Blue G-250 stain (Candiano *et al.,* 2004)

Reagent solutions

1. ***Fixing solution:*** 40% (v/v) ethanol and 10% (v/v) acetic acid. To prepare 500 mL, mix 250 mL of deionized water, 200 mL ethanol (Merck, Darmstadt, Germany) and 50 mL glacial acetic acid (Merck).
2. ***CBB G-250 staining solution***: 0.12% (w/v) Coomassie Brilliant Blue (CBB) G-250 dye, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol. To prepare 500 mL of the solution, which is sufficient for staining one 2-DE gel (20x25x0.1

cm^3), dilute 50 mL phosphoric acid (85%) (Merck) in 100 mL of deionized water. Add 50 g of ammonium sulfate (Merck) and stir until the ammonium sulfate has dissolved. Add 600 mg CBB G-250 (*e.g.,* GE Healthcare, Bio-Rad, or Serva) and stir for approximately 30 min. Then complete to 400 mL with deionized water, and add 100 mL methanol (Merck) under stirring. This dye suspension is stable at room temperature for up to six months. Stir before use, but do ***not*** filter the dye suspension.

3. ***CBB-G-250 destaining solution***: 5% (w/v) ammonium sulfate and 10% (v/v) methanol. To prepare 1000 mL of the solution, dissolve 50 g of ammonium sulfate (Merck) in 800 mL deinized water and stir until the ammonium sulfate has dissolved. Then add 100 mL methanol and complete to 1000 mL with deionized water.

Method

1. Place the gel into a staining dish on a laboratory shaker and incubate for > 3 hours in 500 mL fixing solution (40% (w/v) ethanol and 10% (w/v) acetic acid).
2. Wash the gel briefly (2 x 30 sec.) with deionized water.
3. Immerse the gel in 500 mL colloidal CBB G-250 staining solution and incubate at room temperature with gentle shaking for at least 3-4 hours. Staining can continue overnight if more convenient.
4. Decant the staining solution and rinse the gel briefly (2x30 sec) with deionized water. Then incubate the gel on the laboratory shaker with 500 mL of CBB-G-250 destaining solution (5% ammonium sulfate and 10% (v/v) methanol) for 20 min, followd by a further 20 min incubation in 250 mL CBB G-250 destaining solution diluted with 250 mL deionized water. ***Note:*** Colloidal Coomassie Brilliant Blue staining kits based on the protocol of Neuhoff *et al.* (1988) are available from different manufacturers, *e.g.,* from Carl Roth (Karlsruhe, Germany). Roti®-Blue staining is quick (2-3 h), and destaining is usually not required.

II. Silver staining (Blum *et al.,* 1987; Chevallet *et al.,* 2006)

Reagent solutions

1. ***Fixing solution:*** 40% (w/v) ethanol and 10% (w/v) acetic acid. To prepare 1000 mL, mix 500 mL of deionized water, 400 mL ethanol (Merck) and 100 mL glacial acetic acid (Merck).
2. ***Sensitizer:*** 0.02% sodium thiosulfate-pentahydrate. To prepare 500 mL, dissolve 100 mg of sodium thiosulfate-pentahydrate in 500 mL deionized water. The solution must be prepared the day of use.

3. ***Silver nitrate solution*****:** 0.2% silver nitrate and 0.02% formaldehyde (37%). To prepare 500 mL, dissolve 1.0 g of silver nitrate (Merck) in 500 mL deionized water and add 0.1 mL formaldehyde solution (37%) (Merck). The solution should be prepared just before use. ***Caution:*** Formaldehyde is toxic and should be handled with care. ***Note:*** Because formaldehyde polymerizes in the cold, the formaldehyde solution (37%) should be stored at room temperature and not used longer than six months as the amount of active formaldehyde will gradually decrease.
4. ***Developer:*** 3% sodium carbonate, 0.05% formaldehyde (37%) and 0.0005% sodium thiosulfate pentahydrate. To prepare 1000 mL, dissolve 30 g of sodium carbonate (Merck) and 5 mg of sodium thiosulfate pentahydrate in 1000 mL deionized water, and add 0.5 mL of formaldehyde solution (37%) (Merck). Developer should be prepared immediately before use.
4. ***Stop solution:*** 0.5% glycine. To prepare 500 mL, dissolve 2.5 g of glycine (Merck) in 500 mL deionized water.

Note: Silver staining kits are available from different suppliers.

Method

1. Place the gel(s) into a glass tray on a laboratory shaker and fix the proteins in 500 mL 40% ethanol, and 10% acetic acid at least for 3 hours. Up to four gels per tray may be fixed simultaneously. In this case, 1000 mL of solution are required. Replacement of the fixing solution after 1 h is recommended. ***Note:*** To remove carrier ampholytes, SDS and other detergents, which otherwise produce a strong background („clouds“) in the lower M_r region, the gel should be fixed for three hours at least, but preferably over night. Fixation can be prolonged for up to 72h.
2. Wash the gel in 500 mL 30% ethanol for 20 min with gentle shaking, followed by two consecutive washes (20 min each) with 15% ethanol and Milli Q water, respectively.
3. Sensitize with 500 mL sodium thiosulfate solution for 1 min with gentle shaking.
4. Discard the sensitizer solution and rinse the gel in Milli Q water for 3 x 20 s while manually shaking. ***Note:*** To ensure even staining, proper and constant agitation by means of a laboratory shaker is required for all incubations lasting longer than one minute. For shorter steps, manual agitation is more convenient.
5. Add 500 mL of silver nitrate solution and gently agitate for approximately 30 min. Protect the tray from bright light.
6. Decant the silver nitrate solution and rinse the gel in Milli Q water for 3 x 20 s while manually shaking.
7. Quickly add 500 mL of developing solution (3% sodium carbonate, 250 µL 37% formaldehyde). Sometimes a grey or brown precipitate may form which normally dissolves within a few seconds by vigorous shaking. The developer may be replaced

after one minute for less background staining. ***Note:*** Optimal temperature of the silver nitrate staining reagent solutions is 20-25°C.

8. Develop until protein spots are clearly visible (5-15 min). The most intense spots will appear within 2-3 minutes.
9. Stop development as soon as an adequate degree of staining has been achieved to avoid excessive background formation. Discard the develope, rinse the gel briefly in deionized water, and immerse it in 500 mL stop solution (0.5% glyine, or, alternatively, 4% Tris and 2% acetic acid) for 20 min with gentle shaking. Then wash the gel (3 x 10 min) with Milli Q water before further processing.

III. SYPRO® Ruby fluorescent stain (Berggren *et al.* 2002)

Reagent solutions

1. ***Fixing solution:*** 50% (v/v) methanol and 7% (v/v) acetic acid. To prepare 1000 mL, mix 430 mL of deionized water, 500 mL methanol (Merck) and 70 mL glacial acetic acid (Merck).
2. ***SYPRO Ruby staining solution:*** Ready-to-use solution (Molecular Probes, Eugene, OR, USA).
3. ***Destaining solution:*** 10% (v/v) ethanol and 7% (v/v) acetic acid. To prepare 1000 mL, mix 830 mL of deionized water, 100 mL ethanol (Merck) and 70 mL glacial acetic acid (Merck).

Method

1. After completion of electrophoresis, place the gel into a high density polypropylene tray (*e.g.,* a food box). Do **not** use a glass vessel! Fix the proteins in 500 mL 40% ethanol, and 10% acetic acid for at least 3 hours at room temperature on a reciprocal or orbital shaker.
2. Discard the fixing solution and add 500 mL SYPRO Ruby staining solution for a 'standard' 2-DE gel with the dimensions 25x20x0.1 cm^3. Up to three gels may be simultaneously stained in one polypropylene box.
3. Cover the box with a tight-fitting lid and with aluminium foil to protect the reagent from bright light. (SYPRO Ruby dye is light-sensitive and thus gel staining must be carried out in an opaque container or in the dark).
4. Shake the box gently, preferably with a circular action on an orbital shaker, at least 3 h at room temperature, or overnight.
5. Pour off the excess stain solution and discard. Wash the gel (3 x 10 min) in deionized water or, preferably, with 10% ethanol and 7% acetic acid to reduce speckling which is sometimes a problem. ***Note:*** When using gels that have been cast on a plastic

backing, the plastic sheet should be removed prior to scanning (*e.g.,* with the help of a film remover available from GE Healthcare). Alternatively, use gels cast on low-fluorescence plastic backings.

6. Capture and save the image using an appropriate fluorescence imager. Use the filter sets that match the excitation (maximum: 450 nm) and emission (maximum: 610 nm) wavelength for SYPRO Ruby. Images may also be obtained by using a simple UV transilluminator, but high sensitivity will only be obtained with dedicated fluorescence imagers, *e.g.,* Molecular Dynamics Fluorimager (Sunnyvale, CA, USA), Molecular Imager FX from Bio-Rad, FLA-5100 image analyzer (Fuji), or similar devices.

IV. Pro-Q Diamond® phosphoprotein stain (Steinberg *et al.,* 2003)

Reagent solutions

1. ***Fixing solution:*** 40% (v/v) ethanol and 10% (v/v) acetic acid. To prepare 1000 mL, mix 500 mL of deionized water, 400 mL ethanol (Merck) and 100 mL glacial acetic acid (Merck).
2. ***Pro-Q Diamond phosphoprotein stain:*** Ready-to-use solution (Molecular Probes, Eugene, OR, USA).
4. ***Pro-Q Diamond phosphoprotein destaining solution****:* 25% acetonitrile and 50 mM sodium acetate/acetic acid, pH 4.0. To prepare 1000 mL, dissolve 4.1 g sodium acetate (Merck) in approximately 700 mL deionized water and titrate to pH 4.0 with glacial acetic acid (Merck). Add 250 mL of acetonitrile (Merck) and complete to 1000 mL with deionized water. *Caution:* Acetonitrile is toxic and should be handled with care.

Method

1. After completion of electrophoresis, place the gel into a high density polypropylene tray (*e.g.,* food box). Do neither use a glass vessel, nor a staining container that has previously been used for SYPRO® Ruby protein gel stain, as residual SYPRO® Ruby stain may interfere with Pro-Q® Diamond phosphoprotein gel staining.
2. Fix the proteins in 500 mL 40% ethanol and 10% acetic acid (or, alternatively, 50% methanol and 10% acetic acid) overnight at room temperature while rotating the tray on an orbital shaker. Replacement of the fixing solution after one hour is highly recommended. Fixation is critical to achieving specific staining as incomplete removal of SDS would otherwise result in very poor or no staining of phosphoproteins.
3. Discard the solution and wash the gel with gentle agitation with Milli Q water (500 mL, three changes, 20 min per wash)

4. Incubate the gel with 500 mL Pro-Q Diamond phosphoprotein stain (supplied as a ready-to-use solution) for 2-3 h (however, not overnight). Cover the box with tight-fitting lid and with aluminium foil to protect the reagent from light.
5. Discard the staining solution and destain in the dark with 500 mL destaining solution (25% acetonitrile and 50 mM sodium acetate/acetic acid, pH 4.0; three changes, 30 min per wash).
6. Wash the gel 2 x 5 min with Milli Q water
7. Continue with image acquisition. Pro-Q® Diamond stain has excitation/emission maxima of ~555/580 nm and can be detected with an appropriate imager. ***Note:*** Subsequent staining with SYPRO® Ruby is recommended, as determining the ratio of Pro-Q® Diamond dye to SYPRO® Ruby dye signal intensities for each spot provides a measure of the phosphorylation level normalized to the total amount of protein.

V. Pro-Q Emerald® 488 glycoprotein stain *(Hart et al., 2003)*

Reagent solutions

1. ***Fixing solution:*** 40% (w/v) ethanol and 5% (w/v) acetic acid. To prepare 1000 mL, mix 550 mL of deionized water, 400 mL ethanol (Merck) and 50 mL glacial acetic acid (Merck).
2. ***Wash solution:*** 3% (v/v) acetic acid. To prepare 1000 mL, mix 970 mL of deionized water and 30 mL glacial acetic acid (Merck).
3. ***Oxidizer:*** Periodic acid in wash solution. To prepare 500 mL, add 250 ml wash solution to the bottle containing the periodic acid (‚Component C'; Molecular Probes) and mix until completely dissolved. Add another 250 mL wash solution before use.
5. ***Pro-Q Emerald glyoprotein staining solution:*** Add 0.5 mL of dimethylsulfoxide (DMSO, Merck) per vial of Pro-Q Emerald 488 reagent (Molecular Probes), mix thoroughly and add to 25 mL of staining buffer (Molecular Probes) just before use. 250 mL of the staining solution is sufficient for staining one 2-DE gel (20x25x0.1 cm^3).

Method

1. After completion of electrophoresis, place the gel into a high density polypropylene tray (*e.g.,* food box). Do not use a glass vessel! Fix the proteins in 1000 mL 40% ethanol, and 10% acetic acid (or, alternatively, 50% methanol and 10% acetic acid) at room temperature while rotating the tray on an orbital rotator. Replace the fixing solution after one hour and continue with fixing over night.

2. Discard the solution and wash the gel with gentle agitation with Milli Q water (1000 mL, two changes, 20 min per wash).
3. Incubate the gel in 500 mL oxidizing solution in the dark for 1 h with gentle agitation.
4. Wash the gel with Milli Q water (1000 mL, six changes, 15-20 min per wash).
5. Incubate the gel with gentle agitation in 250 mL Pro-Q Emerald 488 staining solution for ~3 h (or overnight). Cover the box with tight-fitting lid and with aluminium foil to protect the reagent from light.
6. Incubate the gel with 1000 mL of wash solution (three changes, 30-45 min per wash).
7. Visualize the glycoproteins by *e.g.,* utilizing 488 nm laser excitation and 530 nm bandpass emission filter (the Pro-Q Emerald 488 stain has an excitation maximum at ~510 nm and an emission maximum at ~520 nm).

VI. Fluorescent Difference Gel Electrophoresis (DIGE) (Ünlü *et al.,* 1997)

Whatever the intention of the fluorescent difference gel electrophoresis (DIGE) experiment, the work flow is the same:

- Protein extraction and solubilization
- Protein labeling
- Mixing of the labeled samples
- 2-D electrophoresis of the samples in a single gel
- Image acquisition and image comparison

In the following protocol, the proteins are labelled only to a low extent ("minimal labelling"). This ensures that the dyes do not label more than ~3% of the available protein molecules, and that only a single lysine per protein molecule is labelled. In this way, unlabelled protein can subsequently be analyzed by mass spectrometry. The pI of the proteins is not altered, but the covalently linked, fluorescent dye causes a size increase of ~500 Daltons. Because the CyDye label may have altered the spot position of the proteins, particularly of low molecular weight proteins, additional staining of all proteins (*e.g.,* with Sypro Ruby) may be required before spot picking. After labelling, identical protein amounts of each extract are mixed and loaded on a 2-DE gel. Approximately the same amount of fluorescence-labeled proteins should be loaded as would be needed for silver staining. The common urea lysis buffer is not compatible with the labeling reaction because the reactive group of the CyDyes is an N-hydroxy succinimidyl (NHS) ester, which covalently attaches to the epsilon amino group of lysyl residues. Primary amines such as carrier ampholytes, as well as reducing agents such as dithiothreitol diminish the labeling efficiency and should be added *after* labeling. For a comprehensive list of partially or completely incompatible compounds (*e.g.*, mercapto-ethanol, EDTA, Pefabloc proteinase inhibitor), please see the CyDye manual (*GE Healthcare Lifesciences*).

For sample solublization, SDS buffer can be used, but it must be diluted to 0.2% SDS before the labeling reaction with thiourea/urea/CHAPS buffer (see PROTOCOL for extraction of bacterial proteins in Section 2 of this manual). Direct extraction with urea/CHAPS buffer is simpler, because most proteins are usually unaffected by omitting dithiothreitol (DTT) and carrier ampholytes. Proteins with a high cystine content, however, might be less soluble in urea/CHAPS buffer and tend to precipitate or aggregate if not reduced. For these proteins, the SDS solubilization procedure should be applied. In any case, it is mandatory to ensure that the pH of the sample is pH 8.5 (± 0.1), as this is the optimum pH for the labeling reaction. The final buffer concentration before isoelectric focusing should not exceed 30 mM Tris.

MATERIALS

Reagents

- 2DQuant kit (*GE Healthcare Lifesciences*)
- CyDye DIGE Fluor, Cy2, Cy3 and/or Cy5 minimal dye (*GE Healthcare Lifesciences*)
- Dimethylformamide, 99.8% anhydrous (Sigma-Aldrich)
  Note: The DMF should be fresh (less than three months old after first opening), because it degrades to amine compounds, which interfere with the labeling reaction.
- Dithiothreitol solution, 50% (w/v). To prepare 100 µl, dissolve 50 mg of dithiothreitol in 70 µl of deionized H_2O. Prepare fresh before use!
- pH indicator strips (pH 4.5–10.0)
- Pharmalyte (pH 3-10
- SDS sample buffer [1% w/v SDS, 100 mM Tris-Hcl (pH 8.5)]
  To prepare 50 ml of SDS sample buffer, dissolve 0.5 g of SDS and 0.6 g of Tris base (Sigma) in ~40 ml of deionized H_2O. Titrate to pH 8.5 with 4 N HCl, filter and adjust the final volume to 50 ml with deionized H_2O.
- Stop solution (10 mM lysine)
  To prepare 10 ml of stop solution, dissolve 14.6 mg of lysine in 10 ml deionized H_2O.
- Thiourea/urea/CHAPS buffer **A** [2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 30 mM Tris HCl pH 8.5]
  To prepare 50 ml of buffer, dissolve 22.0 g of urea and 8.0 g of thiourea (Fluka) in ~25 ml of deionized H_2O. Add 0.5 g of Serdolit MB-1 ion exchange resin (Serva), stir for 10 minutes, and filter. Add 2.0 g CHAPS to the filtrate, and 180 mg Tris base to the filtrate. Titrate to pH 8.5 with 4 N HCl, and adjust the final volume to 50 ml with deionized H_2O.
- Thiourea/urea/CHAPS buffer **B** [2 M thiourea, 7 M urea, 4% (w/v) CHAPS]

To prepare 50 ml of buffer, dissolve 22.0 g of urea and 8.0 g of thiourea (Fluka) in ~25 ml of deionized H_2O. Add 0.5 g of Serdolit MB-1 ion exchange resin (Serva), stir for 10 minutes, and filter. Add 2.0 g CHAPS and adjust the final volume to 50 ml with deionized H_2O.

Equipment

- Image analysis software such as DeCyder Differential Analysis Software, which is specially designed for analysis of multiplexed fluorescent images (*GE Healthcare Lifesciences*)
- Low-fluorescence glass plate, 3mm thick
- Multicolor confocal laser scanner, *e.g.*, Typhoon 9400
- Software for image overlay & editing, *e.g.*, ImageQuant Solution

Additional Materials

This protocol requires additional materials (*e.g.*, acrylamide solution, gel buffers etc) used in Protocols described in Chapters 2-4.

METHOD

Labeling the protein samples

1. Extract proteins with SDS-buffer, as described in Chapter 2. Determine the protein concentration of the sample with the 2DQuant kit. Dilute the SDS extract with a fourfold excess of thiourea/urea/CHAPS buffer **B** (final SDS concentration ≤ 0.2%). The protein concentration should be ≥ 1 mg/ml and ≤ 20 mg/ml. Best results are obtained with final protein concentrations between 5 and 10 mg/ml.

   Note: Instead of using SDS buffer, proteins may be extracted directly in thiourea/urea/CHAPS buffer **A.**

2. Reconstitute the CyDyes in DMF to a stock solution of 1 mM (see the CyDyes manual for a detailed protocol). The reconstituted dyes are stable at –20°C for ~6 months.

3. Directly before labeling, dilute the CyDye stock solution to a final concentration of 0.4 mM (400 pmol/µl), *e.g.*, mix 3 µl of DMF with 2 µl of reconstituted CyDye.

4. Prepare an aliquot of 50 µg of the protein sample, and add 400 pmol (1 µl) of the CyDye. Cool on ice.

   Important: (**1**) Make sure the pH is 8.5 by checking with a pH indicator strip. Adjust the pH if necessary. (**2**) Keep the dyes and the samples in the dark and on ice!

5. Vortex and centrifuge the mixture in a microfuge at 10,000*g* for 5 seconds.

6. Incubate the sample in the dark for 30 minutes on ice.

7. Add 1 µl of 10 mM lysine to stop the labeling reaction.

8. Mix, centrifuge briefly, and incubate in the dark for 10 minutes on ice.
9. Add 1 µl of DTT solution and 1 µl of Pharmalyte per 50 µl of sample solution.
10. Pool the protein samples that are going to be separated on the same first- and second-dimension gel, and immediately apply the mixture to the IPG strip for IEF. Alternatively, labeled sample can be stored for at least three months at –70°C.
11. Run IEF and SDS-PAGE according to the Protocols described in Chapter 3. Protect the IPGphor during the IEF and the buffer tank during SDS-PAGE from light to minimize photobleaching of the fluorescence dyes.

Image Acquisition and Analysis

For best results during image acquisition, use low-fluorescence glass plates. Directly scan the gels in the glass cassettes after the second dimension to ensure that all gels have the same dimensions; doing so simplifies spot matching of different gels. The exterior of the glass plates must be carefully cleaned with deionized H_2O and dried with a lint-free laboratory wipe before the gel cassette is positioned on the scanner. Each fluorescent dye should be consecutively excited to avoid fluorescence crosstalk and scanned at a resolution of at least 200 µm with a proper filter (**Table 16**).

After the scan, software such as ImageQuant Solution easily provides an initial image overlay of the scanned channels of one gel, thus giving a quick overview of differences between the labeled extracts is given. It should be kept in mind that the protein patterns of one gel might differ slightly in protein concentration and background. Although the DIGE system is highly reliable in terms of quantitation, especially when an internal standard is included in the experimental design, biological variances or variances during the protein extraction might affect samples. Therefore, at least three gels of three individual protein extracts per experimental condition (*e.g.,* control, stress etc.) should be run, normalized, and compared before qualitative and quantitative changes can be considered significant.

Table 16 Selection guide for proper excitation and filter set during the scan of CyDyes

Dye	Absorption maximum	Fluorescence maximum	Laserlight Wavelength	Emission Filter
Cy2	491 nm	509 nm	488 nm	520 nm, band pass 40
Cy3	553 nm	569 nm	532 nm	580 nm, band pass 40
Cy5	645 nm	664 nm	633 nm	670 nm, band pass 40

VII. Blotting

Electrophoretically separated polypeptides can be visualized by "universal" stains like Coomassie Blue or silver staining, or by "specific" stains such as glycoprotein staining or by immunochemical detection methods (**Fig. 24**). Whereas Coomassie or silver staining are performed in the electrophoresis gel directly, immunochemical detection methods are usually carried out after electrophoretic transfer of the separated polypeptides from the electrophoresis gel onto an immobilizing membrane. This transfer technique is called *"blotting"* (Towbin *et al*., 1979; Kyhse-Andersen 1984). The immobilized proteins cannot diffuse or be washed out, and they are readily accessible to reagents or high-molecular weight ligands such as antibodies or lectins (several examples are shown in **Figs. 25 & 26**). In this manual, the electrophoretic transfer and protein visualization with Coomassie Blue and the more sensitive Indian Ink staining method are described. It is also possible to gain N-terminal or internal amino acid sequence information of proteins blotted onto PVDF membranes. Coomassie Blue stained spots are excised from the immobilizing PVDF membrane and directly applied into an automated protein sequencer.

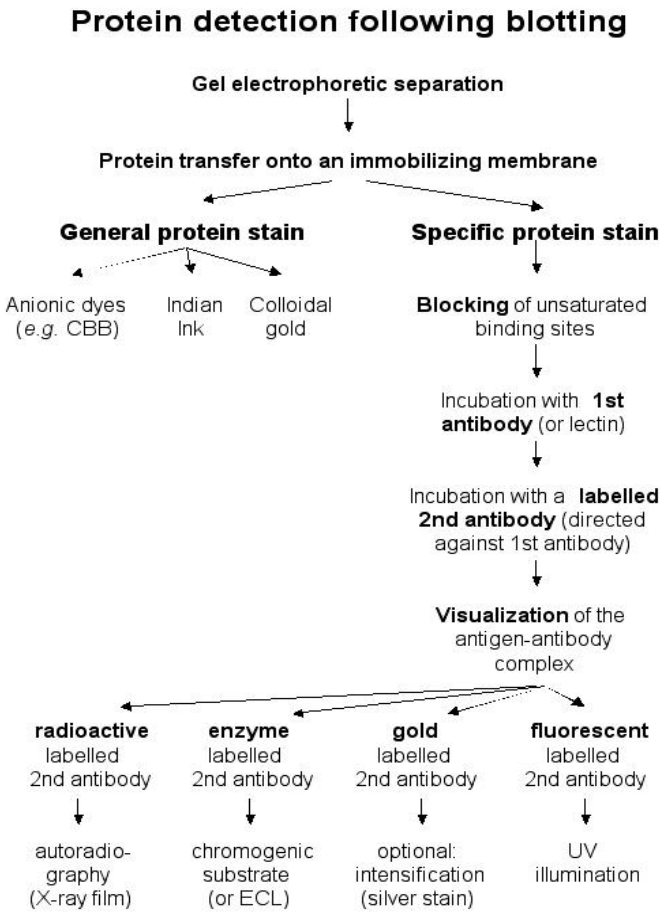


Figure 24 Protein detection methods after blotting

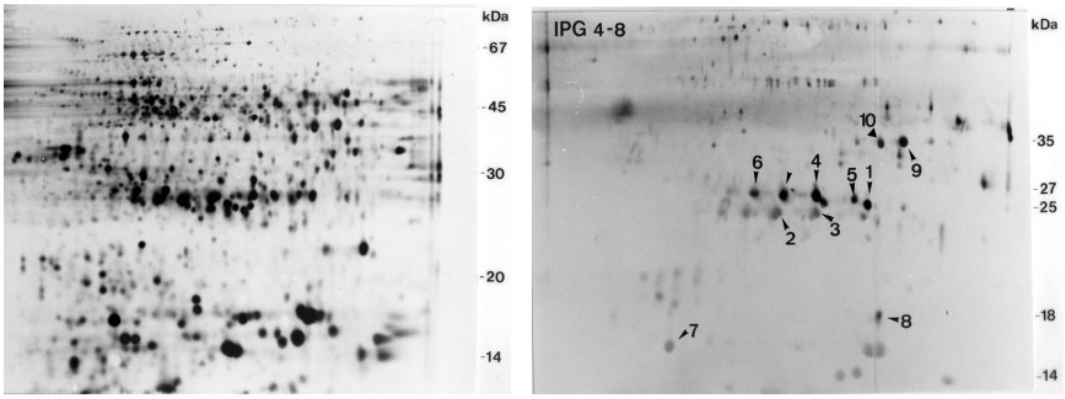


Figure 25 Wheat seed proteins separated by 2DE and electroblotted onto an immobilizing PVDF membrane. *Left hand side*: Total protein staining with Indian Ink. *Right hand side:* Immunostaining with a pooled serum of patients suffering from baker's asthma (first antibody), and alkaline phosphatase labelled anti-human IgE as secondary antibody. Major IgE-binding proteins are labelled with arrows (Posch *et al.,* 1995)

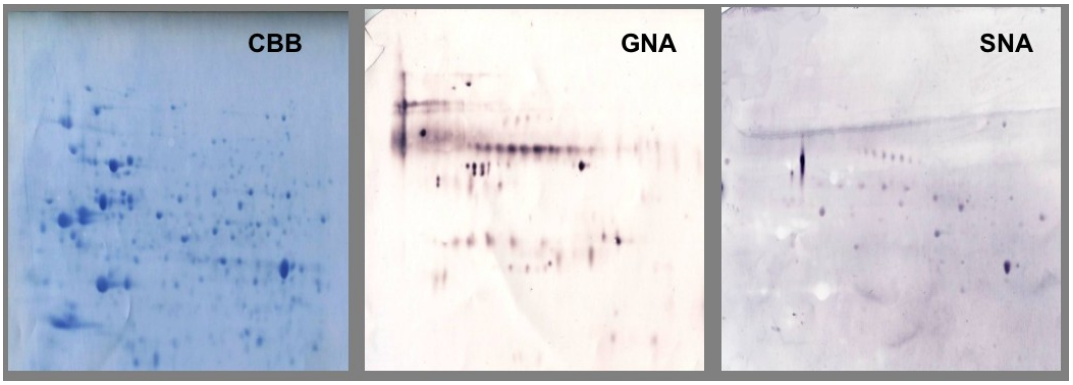


Figure 26 Detection of glycoproteins by lectin-affinity binding after tranfer onto PVDF membrane. *Left hand side:* Total protein stain with Amido Black 10B dye. *Center and right-hand side:* Glycoprotein detection with the the lectins GNA and SNA, respectively. (Weiland, F. & Görg, A., unpublished results)

PROTOCOL

Reagents

- Tris-glycine transfer buffer: 25 mM Tris, 190 mM glycine, 0.1% SDS, 20% methanol, pH 8.5. To make 1000 ml, dissolve 14.4 g of glycine, 3.0 g of Trizma base, and 1.0 g of SDS in 800 ml of deionized water. Add 200 ml of methanol, stir and degas for 20 min. This buffer should be prepared fresh.
- Tris-borate transfer buffer: 50 mM "Tris", 50 mM boric acid, 10% methanol, pH 8.5
  To make 1000 ml, dissolve 6.0 g of Trizma base and 3.1 g of boric acid in 900 ml of deionized water, adjust to pH 8.5, add 100 ml of methanol, stir and degas for 20 min. This buffer should be prepared fresh.
- Sodium chloride solution: 0.9% sodium chloride in deionized water. To prepare 1000 ml, dissolve 9.0 g of sodium chloride in 1000 ml of deionized water.

METHOD

Electrophoretic transfer („Blotting“)

For easier handling, horizontal and/or vertical SDS gels are polymerized onto a plastic backing (GelBond PAGfilm). Before blotting, this plastic film has to be removed from the gel in order to facilitate the electrophoretic transfer of the proteins out of the gel onto an immobilizing membrane. This step needs not to be performed for those vertical SDS gels which are not cast on GelBond PAGfilm.

Tris-glycine buffers are most commonly employed. Borate buffers are recommeded if the blotted proteins are sequenced by Edman degradation, since this buffer does not contain amino acids such as glycine which may interfere with Edman microsequencing.

1. Briefly wet the PVDF membrane in 100% methanol. Then wash the blotting membrane two times (five min each) in deionized water and soak it in transfer buffer. Nitrocellulose membranes are directly soaked with transfer buffer without pre-wetting in methanol.
2. The removal of the plastic backing from the horizontal SDS gel is facilitated by means of a so-called film remover (*GE Healthcare Lifesciences*). Place the gel onto the cylindrical surface of the film-remover, gel side up, and tighten it with the help of a clamp. Then pull the stainless-steel wire, beginning at one end of the gel, between the gel and GelBond PAGfilm towards your body so that the gel is released from its plastic backing (**Fig. 27**).

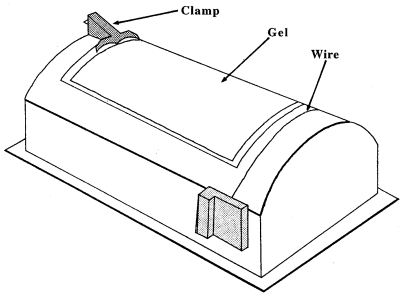


Figure 27 Film remover

3. Transfer the gel into the semi-dry blotting apparatus which has already been prepared for blotting: The lower, anodic graphite electrode is soaked with water and covered with six layers of filter paper soaked in transfer buffer and a nitrocellulose or polyvinylidene difluoride (PVDF) sheet also soaked in transfer buffer.
4. Carefully lower the gel onto the immunoblotting membrane and cover it with another six layers of filter paper soaked in transfer buffer. Squeeze out caught-in air bubbles with the help of a roller or a wetted glass pipette, and cover the blotting "sandwich" with the water-saturated cathodic graphite electrode.

5. Electrophoretic transfer is performed for about one hour at 0.8 mA per cm^2 gel surface (typical settings for a 200 x 180 mm^2 SDS gel: 20 V, 300 mA).
6. When the electrophoretic transfer has been terminated, dismantle the blotting apparatus.Wash the membrane with 0.9 % sodium chloride (3 x 5 min) and let it dry at room temperature. The dried blotting membrane can be stored in a sealed plastic bag at 4°C for at least several weeks. Proteins bound to the blotting membrane are either stained with unspecific protein stains such as Coomassie Brilliant Blue or India Ink, or specifically with antibodies or lectins.

Visualization of the blotted proteins by Coomassie Blue staining

Reagents

• Staining solution: 0.1% Coomassie Blue R-250 in water/methanol/acetic acid (45/45/10). To prepare 500 ml, dissolve 500 mg Coomassie Brilliant Blue R-250 (or Serva Blue R) in 225 ml of ethanol, stir for 30-60 min, add 225 ml of deionized water and 50 ml acetic acid, stir again and filter.

• Destaining solution: 45% ethanol, 45% deionized water and 10% acetic acid. To prepare 1000 ml, mix 450 ml water, 450 ml ethanol and 100 ml of glacial acetic acid.

METHOD

1. Wet the PVDF membrane with methanol (10 sec) and wash with water (2x2 min).
2. Incubate the PVDF membrane in staining solution for 2 minutes on a rocking platform.
3. Destain the PVDF membrane with destaining solution (2 x 5 min)
4. Rinse the membrane in deionized water (3 x 5 min) and air-dry for longer storage.

Protein visualization with India Ink (Hancock & Tsang, 1983)

• Staining solution: Dilute 0.5 ml Indian Ink solution *(Pelikan, Germany)* in 500 ml TBS, add 0.2 ml of Tween 20 and 5 ml of acetic acid, stir for 60 min, and filter.

METHOD

1. Wet the PVDF membrane with methanol and wash with water (2 x 2 min)
2. Place the membrane into an glas or plastic vessel add Indian Ink staining solution
3. Shake the box gently for 2-3 h at room temperature
4. Pour off the excess stain solution and destain several minutes with PBS.

5 COMPUTERIZED 2-D IMAGE ANALYSIS AND DATABASE CONSTRUCTION

One of the key objectives of proteomics is to identify the differential expression between control and experimental samples run on a series of 2-D gels. That is, the protein spots that have been inhibited (disappeared), induced (appeared) or have changed abundance (increased or decreased in size and intensity). Once these gel features have been found, the proteins of interest can be identified using MS. This goal is usually accomplished with the help of computerized image analysis systems (Dowsey *et al*., 2003; Dunn 1992 & 1993; Garrels 1989).

The first step in computerized image analysis of 2-DE protein patterns is capture of the gel images in a digital format. A range of devices, including modified document scanners, laser densitometers, charge-coupled device (CCD) cameras, and fluorescent and phosphor imagers, are available for the acquisition of 2-D gel images. The saved images are then subjected to computer assisted image analysis. The traditional workflow for a 2-DE software package is:

- pre-processing of the gel images, *i.e.*, image normalization, cropping and background subtraction
- spot segmentation, detection and expression quantification
- landmarking, *i.e.*, an initial user guided pairing of a few spots between the reference and sample gels. The sample gel is then warped to align the landmarks
- matching, *i.e.*, automatic pairing of the rest of the spots
- identification of differentially expressed spots
- data presentation and interpretation
- creation of 2-D gel databases

Currently, several 2-D image analysis software packages are commercially available. These programs have been continuously improved and enhanced over the years in terms of faster matching algorithms with lesser manual intervention, and with focus on automation and better integration of data from various sources. New 2-D software packages have also emerged which offer completely new approaches to image analysis and novel algorithms for more reliable spot detection, quantitation and matching. Several programs include options such as control of a spot cutting robot, automated import of protein identification results from mass spectrometry, superior annotation flexibility (*e.g.*, protein identity, mass spectrum, intensity/ quantity, links to the Internet), and/or multichannel image merging of different images to independent color channels for fast image comparison. However, despite these improvements, it is still a long way towards fully automated image analysis systems that do not require user intervention (Dunn, 1993; Fievet *et al.*, 2004).

5.1 Computer assisted 2-D image analysis

Briefly, the digitized image is first subjected to several “clean-up” steps to reduce background smear and to remove horizontal and/or vertical streaks. This procedure is usually quite fast and does not require much user interaction. The individual spots on the 2-D pattern are then detected and quantified. This step is also performed automatically. Regrettably, most image analysis programs do not identify all spots correctly, particularly when the overall quality of the electrophoretic separation is low (*e.g.*, when crowded areas and overlapping spots due to improper sample preparation or insufficient spatial resolution are present on the gel). Hence, manual spot editing with reference to the original stained 2-DE gel (or image) is usually necessary. Depending upon the number of spots, the quality of the 2-DE separation and the algorithms used for spot detection, this process may be quite laborious and time-consuming. After spot editing, each spot on one 2-DE gel must be matched to its counterpart on the other gels, usually by means of a reference (“master”) gel. For this, in most computer aided 2-D image analysis programs, several “landmark” spots (which should be evenly distributed over the entire gel area) must be manually identified on each gel by the operator. Starting from these landmark spots, the program proceeds to match the other spots automatically. Again, mismatches must be carefully checked and edited manually. Typically, at least two 2-D gel patterns are matched (*e.g.*, a diseased *versus* a control) and then compared to each other with respect to qualitative and/or quantitative differences between the 2-D patterns (*e.g.*, induced or repressed proteins). In most cases, however, many gels from different experiments have to be compared, usually by establishing a hierarchical 2-D pattern database (Dunn, 1992).

5.2 Two-dimensional electrophoresis databases

Once the 2-D gel database has been established, information stored in it can be exploited by addressing questions such as “Can particular proteins be identified that are associated with a certain disease or disease state (*e.g.*, disease markers)?”, or “What is the function of a particular protein?” Currently, enormous efforts are being undertaken to display and analyze with 2-DE the proteomes from a large number of organisms, ranging from ***organelles*** such as mitochondriae, nuclei or ribosomes to ***simple prokaroytes*** including *Escherichia coli*, *Bacillus subtilis*, *Haemophilus influenzae*, *Mycobacterium tuberculosis* and *Helicobacter pylori*, to ***single-celled eukaryotes*** such as the yeast *Saccharomyces cerevisiae*, to ***multicellular organisms,*** e.g., *Caenorhabditis elegans, **plants*** such as rice (*Oryza sativa*) or *Arabidopsis thaliana*, and ***mammalian cells and tissues*** including rat and human heart, mouse and human liver, mouse and human brain, different cancer cell lines, HeLa cells, human fibroplasts, human keratinocytes, rat and human serum etc. Most of these and many other studies in progress are summarized at www.expasy.org/ch2D/2d-index.html (“WORLD-2DPAGE Index to 2D PAGE databases“). The Proteomics Standards Initiative (PSI) aims to define community standards for data

representation in proteomics to facilitate data comparison, exchange and verification (http://psidev.sourceforge.net).

6 PROTEIN IDENTIFICATION FROM 2-D GEL SPOTS

Mass spectrometry has become the technique of choice for identification of proteins from excised 2-D gel spots as these methods are very sensitive, require small amounts of sample (femtomols to attomols) and have the capacity for high sample throughput. Recent advances in mass spectrometry also allow the investigation of post translational modifications including phosphorylation and glycosylation. ***Peptide mass fingerprinting (PMF)*** is typically the primary tool for protein identification. This technique, which is user-friendly and quite fast, is based on the finding that a set of peptide masses obtained by MS analysis of a protein digest (usually trypsin) provides a characteristic mass fingerprint of that protein. The protein is then identified by comparison of the experimental mass fingerprint with theoretical peptide masses generated *in silico* using protein and nucleotide sequence databases. This approach proves very effective when trying to identify proteins from species whose genomes are relatively small, completely sequenced, and well annotated, but is not so reliable for organisms whose genomes have not been completed. A second problem is to identify proteins that are extensively postranslationally modified, since the peptides generated from these proteins may not match with the unmodified protein in the database. A third problem is that PMF does not work very well if several *different* proteins are present in the *same* spot. Although search engines such as *ProFound* (Zhang & Chait, 2000) have been developed that enable identification of the correct protein(s) even when the data quality is relatively low or when the sample consists of a simple mixture of proteins, it may prove impossible to identify a protein based on PMF alone. In these cases it is then essential to obtain amino acid sequence information. This is most readily accomplished using either MALDI-MS with post-source decay (PSD) or chemical assisted fragmentation (CAF), or by ***tandem mass spectrometry (MS/MS)***. MS/MS takes advantage of two-stage MS instruments, MALDI-TOF-TOF-MS/MS or ESI-MS/MS triple-quadropole, ion-trap, or Q-TOF machines to induce fragmentation of peptide bonds. One approach is to generate a short partial sequence or ‘tag’ which is used in combination with the mass of the intact parent peptide ion to provide significant additional information for the homology search. A second approach uses a database searching algorithm SEQUEST to match uninterpreted experimental MS/MS spectra with predicted fragment patterns generated *in silico* from sequences in protein and nucleotide databases. The major drawback of MS/MS based protein identification methods is that the process cannot be readily automated, and that considerable time and expertise are required for interpreting the MS/MS spectra.

PROTOCOL: Protein identification by MALDI-MS

Spot picking, excision of gel spots and storage of gels and gel spots

Intact gels are stored in 1% HAc at 4ºC for up to several weeks until spots are excised. Spot detection and spot excision are either carried automatically using a spot picking robot and appropriate software. The protein spots are placed in 96 well plates for digestion. Alternatively are protein spots excised manually using a clean scalpel and tweezers. Excised gel spots are removed with an Eppendorf pipette, put into a 0.5 ml Eppendorf tube, and stored in the freezer until digestion.

Protein digestion

Coomassie and silver stained proteins are digested in 96-well plates. Briefly, the gel spots are destained with 50 mM NH_4HCO_3/ acetonitrile (1:1) followed by dehydration with acetonitrile. The proteins are reduced in 10 mM dithiotreitol (DTT)/ 50 mM NH_4HCO_3 for 1 hour at 56^{o}C, and alkylated in 55 mM iodoacetamide/50 mM NH_4HCO_3 for 1 hour at room temp. The gel pieces are washed several times in 50 mM NH_4HCO_3 followed by dehydration with acetonitrile. The proteins are digested overnight with trypsin at 37^{o}C and the resulting peptide mixtures are analyzed by mass spectrometry.

MALDI MS

MALDI MS data are acquired on a MALDI instrument. An appropriate matrix surface is α-cyano-4-hydroxy-cinnamic acid dissolved in acetonitrile/H_2O (1:1) + 1% trifluoroacetic acid (TFA) to a concentration of 10 g/l. Aliquots of sample are mixed with the matrix, dried and washed twice with 0.1% TFA, crystallized with matrix by the seed layer method and then applied to the MALDI instrument.

METHOD

In gel digestion

1. Excise protein spots from a silver- or Coomassie blue stained 2D gel. Cut a "control" piece of gel from a blank region of the gel and process in parallel with the sample. Put the gel pieces in 0.5 ml Eppendorf tubes.
2. (A) Destain the excised, Coomassie stained gel pieces with 50 mM NH_4HCO_3 / acetonitrile (1:1);
   (B) Destain the excised silver stained gel pieces with 1% H_2O_2 and briefly wash with water after 30 min.
3. Add a volume of 10 mM dithiotreitol (DTT) sufficient to cover the gel pieces, and reduce the proteins for 1 h at 56°C.

4. After cooling to room temperature, replace the DTT solution with the same volume of 55 mM iodoacetamide.
5. After 45 min incubation at room temperature in the dark with occasional vortexing, wash the gel pieces with 50-100 µl of 50 mM NH_4HCO_3 for 10 min.

   Note: If the reduction/alkylation step during IPG strip equilibration (*see* Section 3.2) according to Görg *et al*. (1988) has been performed, steps 3-5 can be omitted!
6. Dehydrate by addition of acetonitrile twice and bring to complete dryness.
7. Remove the liquid phase.
8. Reswell the gel pieces in 10 µl of a digestion buffer containing 50 mM NH_4HCO_3, and 12.5 mg/l of trypsin (Promega, sequencing grade) in an ice-cold bath.
9. After 45 min, remove the supernatant and replace with 5-10 µl of the same buffer, but without trypsin, to keep the gel pieces wet during enzymic cleavage (37°C, overnight).
10. Extract the peptides by one change of 20 mM NH_4HCO_3 and three changes of 5% formic acid in 50% acetonitrile (20 min for each change) at room temperature and dry down.

    Note: The extraction of the peptides is not necessary for routine analysis.

Sample preparation for MALDI-MS analysis

1. Dissolve the matrix material (α-cyano-4-hydroxy-cinnamic acid) in acetonitrile to a concentration of 1.0 mg/ml.
2. Deposit approximately 0.3 µl of the matrix solution on a stainless steel sample stage. And let it dry to form initial crystals of matrix on the target.
3. Dissolve the matrix material (α-cyano-4-hydroxy-cinnamic acid) in acetonitrile/50 mM NH_4HCO_3 + 2% TFA (1:1) to a concentration of 10 mg/ml.
4. Deposit 0.3 µl of the matrix solution on the prepared seed layer of crystals.
5. Depostit aliquots of 0.5 µl of analyte solution into this matrix solution; allow the solvent to evaporate at room temperature.
6. Acquire the MALDI-MS spectra.

7 Pitfalls

1 Blot the rehydrated IPG gel strips to remove excess rehydration solution (not necessary for the IPGphor after sample-in gel rehydration); otherwise urea crystallization on the surface of the IPG gel strips might occur which will disturb IEF patterns.

2 Make sure that the orientation of the IPG gel strips on the cooling block of the IEF chamber is correct (acidic end towards anode). Check the temperature of the cooling block (usually 20°C).

3 Sample solution should not be too concentrated (max. 10 mg protein/ml) to avoid protein precipitation at the sample application point. If you are in doubt, better dilute the sample with Lysis buffer and apply a larger volume instead.

4 When sample is applied in cups, do not apply less than 20 µl (Multiphor unit) or 50 µl (IPGphor unit, respectively) of sample solution.

5 For better sample entry, start IEF at low voltage (150 V for 30 min, followed by 300 V for 60 min). For micropreparative runs (cup loading) with high sample volume (100 µl) start IEF at low voltage for several hours (200 V for 5-6 hours), followed by 1500 V overnight before voltage is raised to 3500 V or 8000 V (IPGphor), respectively.

6 When using the reswelling tray for in-gel rehydration of samples, the sample volume has to be limited to the size of the IPG strip so that no superfluos sample solution is left in the tray. For a 180 mm long and 3 mm wide IPG strip, the correct sample volume is about 350 µl. When the reswelling tray is used for sample application, it should also be checked, whether the high molecular weight proteins have entered the IPG gel matrix properly.

7 Remove the IPG gel strip from the surface of the horizontal SDS PAGE gel as soon as the Bromophenol Blue dye front has migrated 4-5 mm off the IPG gel strip. Then move the cathodic electrode wick (or buffer strip) forward so that it overlaps the area the IPG gel strip once covered.

8 Summarized Protocol of IPG-Dalt

(Görg *et al.*, Electrophoresis *9*, 531-546, 1988)

FIRST DIMENSION	- *IPG gel strips*		- on GelBond PAGFilm - 0.5 x 3 mm - length 4, 7, 11, 18 cm etc.
	- *Rehydration*		- to the original thickness (gel: 0.5 mm -> U-frame 0.7 mm) - over night - 8 M urea, 0.5% CHAPS, 0.2% CA, 10 mM DTT - avoid urea crystals on the surface - prolonges focusing time - creates „empty“ lanes in the 2D map remedy: remove excess reswelling solution with wet filter paper (immediately)
	- *Sample*	- *Solution*	- Lysis buffer with 0.8% CA
		- *Volume*	- 20 µl analytical, 100 µl micropreparative
		- *Entry*	- low field strength for 1-2h (10-20 V/cm) - no prefocusing
	- *IEF*		- to the steady state - at 20°C
	- *Storage*		- at -80°C between plastic sheets
EQUILIBRATION	- *Prolonged* -50 mM Tris-HCl pH 8.8, 2% SDS, 1% DTT + 6 M urea + 30% glycerol 60 mM iodoacetamide		- 2 x 15 min - reduces electroendosmotic effects - in the second equilibration solution eliminates point streaking
SECOND DIMENSION	- *vertical*		- embedding with agarose
	- *horizontal*		- no embedding - low field strength for 1 h (< 10V/cm) - removal of the IPG gel strip after protein transfer from first to second dimension

Appendix I: Trouble shooting

1 General aspects

- Quality of chemicals should be at least of analytical grade (p.a.)
- Double-distilled or deionized (Millipore) water (conductivity < 2 µS) should be used
- Urea and acrylamide/bisacrylamide solutions should be prepared freshly
- Deionize urea prior to use
- Do not heat urea-containing buffers > 37°C; otherwise protein carbamylation may occur
- Filter all solutions. Use clean and dust-free vessels

2 Sample preparation

- Sample extraction buffer (Lysis buffer) has to be prepared freshly. Alternatively, make small portions (1 ml) and store frozen in Eppendorf vials at -70°C. Lysis buffer thawn once should not be refrozen again!
- Add protease inhibitors during cell lysis if necessary. *Note:* several protease inhibitors are inactivated by DTT and/or mercaptoethanol!
- To remove insoluble material, the protein extract should be spun for 1 h at 40,000 g

3 Gel casting

- Ammonium persulfate solution should be prepared freshly. A 40% solution of ammonium persulfate may be used for 2-3 days if stored in a refrigerator, whereas less concentrated solutions should be prepared the day you use them
- TEMED should be stored under nitrogen and replaced every six months
- The glass plate which bears the U-shaped frame should be treated with RepelSilane to avoid sticking of the gel to the glass plate after polymerization
- Glycerol (37.5%) is incorporated into the stacking gel of horizontal SDS gels in order to diminish electroendosmotic effects
- If SDS gels are cast onto GelBond PAGfilm, GelBondPAGfilm should be washed 6 x 10 min prior to use to avoid "*spot streaking"* upon silver-staining
- For proper polymerization, acidic as well as basic Immobiline starter solutions should be titrated to pH 7 with NaOH and HCl, respectively, prior to IPG gel casting
- After polymerization, IPG gels have to be washed thoroughly (6 x 10 min) with deionized water to remove buffer ions and any unpolymerized material
- Washed IPG gels are impregnated with glycerol (2%) for 30 minutes and dried overnight at room temperature in a dust-free cabinet with the help of a fan
- The surface of the dried IPG gels has to be covered with a sheet of plastic film prior to storage at -20°C

Observed problems:

Gel did not polymerize properly

Probable reasons	***Remedies***
• TEMED or ammonium persulfate too old	Replace TEMED and persulfate
• IPG gels: Immobiline starter solutions have not been titrated to pH 7	Titrate to pH 7 with NaOH and HCl, respectively.
• SDS gel: Tris-buffer has not been titrated with HCl	Titrate stacking or resolving gel buffer with HCl (pH 6.8 or 8.8)

Gel is released from the plastic support when the gel casting cassette is opened

Probable reasons	***Remedies***
• Gel has been polymerized onto the hydrophobic side of the GelBond PAGfilm	Gel has to be polymerized onto the hydrophilic side of the gel support
• Wrong support matrix (*e.g.* for agarose gels)	Use support matrices designed for polyacrylamide gels exclusively
• GelBondPAGfilm too old	Do not use GelBondPAGfilms which are older than 12 months

When the gel casting cassette is opened, the gel sticks to the glass plate

Probable reasons	***Remedies***
• Glass plate is not hydrophobic	Treat glass plate with RepelSilane prior to the assembly of the gel casting cassette

4 Reswelling of IPG strips

- Prior to IEF, IPG dry gels have to be cut into individual IPG strips with the help of a paper cutter. During cutting, the surface of the IPG strips has to be protected by a sheet of plastic film to avoid damage of the gel surface
- IPG strips have to be rehydrated to their original thickness of 0.5 mm
- IPG gel reswelling time depends on the composition of the rehydration buffer. If the rehydration solution contains high concentrations of urea (> 8 M) and detergents (>1%), rehydration should be performed for 6 hr at least or, better, overnight
- Rinse and blot the rehydrated IPG gel strips to remove excess rehydration solution (not to be done with the IPGphor); otherwise urea crystallization on the surface of the IPG gel strips might occur and disturb IEF patterns
- If IPG strips are rehydrated in the IPG reswelling tray or in IPGphor strip holders, avoid trapping air bubbles between the IPG strip and the bottom of the tray or the strip holder Distribute the sample solution evenly beneath the IPG gel strip. Cover the IPG strips with a layer of silicone oil during reswelling to prevent evaporation of the reswelling buffer

5 First dimension (IEF-IPG)

5.1 Application of rehydrated IPG strips onto the cooling plate of the electrophoresis chamber

• Use kerosene exclusively to facilitate contact between IPG strips and cooling block

• Use distilled water as electrode solution exclusively

• Make sure that the orientation of the IPG gel strips on the cooling block of the IEF chamber is correct (acidic end facing towards anode)

5.2 Sample application

• Sample may be applied by in-gel rehydration or by cup-loading

5.2.1 Sample application by cup-loading

• When sample is applied into cups, do not apply less than 20 µl (50µl) of sample solution

• Samples may be applied near anode or cathode. In the case of unknown samples it should be checked which sample application area provides better results

• Sample solution should not be too concentrated (max. 10 mg protein/ml) to avoid protein precipitation at the sample application point. If you are in doubt, better dilute the sample with Lysis buffer and apply a larger volume instead

• Sample solution shoud not contain too high concentrations of salt. Either desalt, or dilute with lysis buffer and apply a larger volume instead. Apply low voltage for slow sample entry.

5.2.2 Sample application by in-gel rehydration

• When using the reswelling tray for in-gel rehydration, the sample volume has to be limited to the size of the IPG strip so that no superfluous sample solution is left in the tray. For a 180 mm long and 3 mm wide IPG strip, the correct sample volume is about 350 µl. When the reswelling tray is used for sample application, one should be aware that high molecular weight, alkaline and/or membrane proteins may not enter the IPG gel matrix properly

5.3 Isoelectric focusing

• Never pre-focus IPG strips; otherwise poor sample entry occurs due to the very low conductivity of the gels

• Electrode strips should be humid, but not too wet. Remove superfluous liquid by blotting with filter paper

• Keep temperature of the cooling block at 20°C

- For better sample entry, start IEF with a low voltage gradient (150 V for 30 min, followed by 300 V for 60 min). For micropreparative runs (cup loading) with a high sample volume (100 µl) start IEF at low voltage for several hours (200 V for 5-6 hours), followed by 1500 V overnight, before voltage is raised to 3500 V

• IPGphor: For improved sampe entry apply low voltage (30 V) during rehydration. Then raise voltage gradually (200 V, 500 V, 1000 V for 1 h each) and continue with max. 8000 V up to the steady state

- Focusing time depends on gel length, pH-gradient and gel additives (carrier ampholytes etc.). Focusing time is shorter when separation distance is shorter, or when wide-range pH-gradients are used, or when carrier ampholytes are added to the reswelling solution
- When running very basic and/or narrow-range IPGs, cover the IPG strips with a layer of degassed silicone oil flushed with argon
- After completion of IEF, IPG strips should be stored frozen at -78°C (unless immediately used for the second dimension)

Observed problems:

IPG gel strips "burn" near the electrode (strips)

Probable reasons	***Remedies***
• Gel strips have dried out at the anodic or cathodic ends due to electroendosmotic flow buffer	Add 10% glycerol to the reswelling
• " Wrong" electrode solutions	Use deionized water only

Zero or low voltage; voltage readings rapidly change

Probable reasons	***Remedies***
• No or bad contact between electrodes (or electrode strips) and the IPG gel	Check contact
• Lid not properly connected with power supply	Check connection or electrodes
• Malfunction of electrodes, lid or power supply	Check accordingly

Current does not drop during initial stage of IEF

Probable reasons	***Remedies***
• Wrong orientation of IPG strips (acidic end facing towards cathode)	Check orientation
• Wrong electrode solutions	Use deionized water
• High salt concentration in the sample	Desalt or dilute sample; replace electrode strips after 1-2 h of IEF

Water condensating on the gel surface or on the lower side the glass plate which carries the electrodes

Probable reasons	***Remedies***
• High salt concentration in the sample	Desalt or dilute sample; add carrier ampholytes; reduce initial current
• Humidity too high	Cover IPG strips with a layer of silicone oil. If IEF is performed in the Multiphor apparatus, seal the holes in the lid with adhesive tape
• Power or current too high	Max. 0.05 mA / IPG strip; max. 5 W

Water exudation near the sample application area

Probable reason	*Remedies*
• High salt concentration in the sample	Desalt or dilute sample; add carrier ampholytes; limit voltage (100V) during sample entry; prolong sample entry time

Formation of urea crystals on the IPG gel surface

Probable reasons	*Remedies*
• Temperature of cooling plate too low	Temperature optimum 20°C
• Very low humidity resulting in evaporation of water from the gel	Add glycerol or sorbitol to the reswelling buffer; seal the Multiphor apparatus; put wet filter paper into the IEF chamber
• Excess reswelling buffer sticking to the IPG strips	Rinse IPG strips with deionized water for a second after rehydration and blot them with wet filter paper

Protein precipitation near the sample application zone

Probable reasons	*Remedies*
• Sample too concentrated	Dilute sample with Lysis buffer
• Initial field strength too high	Start with low field strength (10V/cm)
• Proteins poorly soluble	Add high amounts of urea (> 8M) and/or proper detergent (> 1%)
• Very low initial conductivity	Never pre-focus IPG gels

6 IPG strip equilibration and second dimension (SDS-PAGE)

- Equilibration time should be sufficiently long (2 x 10 min at least)
- Equilibration buffer contains Tris-HCl buffer (pH 8.8), SDS (1%), high amounts of urea (6 M) and glycerol (30%) for improved protein solubility and to suppress electroend-osmotic effects. In the first equilibration step, DTT (1%) is added to the equilibration buffer for proper unfolding of proteins, and iodoacetamide (4%) during the second step to remove excess DTT held responsible for "point streaking" during silver staining
- For very hydrophobic and/or S-S containing proteins, tributylphosphine may be advantageous compared to DTT and iodoacetamide
- Horizontal SDS-PAGE: High amounts of glycerol (37%) are incorporated into the stacking gel to suppress electroendosmotic effects
- Horizontal SDS-PAGE: Stacking gel length should at least exceed 25 mm
- Protein transfer from the first dimension (IPG-Strip) to the second (SDS-gel) should be performed rather slowly (field strength: < 10 V/cm) in order to avoid streaking and to minimize loss of high Mr proteins
- Horizontal SDS-PAGE: Remove IPG gel strips from the surface of the SDS gel as soon as the Bromophenol Blue dye front has migrated 4-5 mm off the IPG gel strip. Then move

the cathodic electrode wick (or buffer strip) forward so that it overlaps the area the IPG gel strip once covered

Observed problems:

Horizontal streaks on SDS gel

Probable reasons	***Remedies***
• Focusing time too short (especially for high Mr proteins) or too long (proteins are not stable for an unlimited period of time)	Perform time-course to find out optimum focusing time
• Concentration of detergent too low; or inappropriate detergent used	Check concentration of detergent; test different detergents
• Urea concentration too low	Urea concentration in IPG reswelling solution: > 8 M
• IPG strip has not been reswollen to its original thickness	Reswell IPG strip to a thickness of 0.5 mm
• Insufficient amount of DTT in the sample solubilization buffer or in the IPG strip reswelling buffer	Add 1% DTT to the sample buffer and 0.25% DTT to the reswelling solution
• Different oxidation forms of a single protein	Add sufficient amount of DTT; perform IEF under a protective layer of degassed silicone oil (flushed with argon or nitrogen)
• Depletion of DTT in IPGs exceeding pH 10 due to migration of DTT towards the anode	Add an "extra paper strip" soaked with 20 mM DTT near the cathode
• Wrong sample application area	Check whether anodic or cathodic sample application gives better results. Alternatively apply sample by in-gel rehydration
• Artifacts due to endogenous proteolytic activity in the sample	Inactivate proteases by TCA- acetone treatment, boiling with SDS and/or adding protease inhibitors
• Interference of atmospheric carbon dioxide	Perform IEF under a layer of silicone oil flushed with argon; add paper strips soaked with NaOH into the electrophoresis chamber to remove CO_2; seal the chamber air-tight
• Mr 68 and/or 55 kDa streaks: possible contamination due to keratin and/or albumin, or caused by mercaptoethanol	Use clean glassware only; filter all buffers (membrane filter!). Keep the lab dust-free. Use DTT instead of mercaptoethanol.
• Precipitation at the IPG strip application area	S tart IEF at low field strength (<10 V/cm)
• IPG strip rehydration time too short	Rehydrate > 6 hr (or overnight)

• Protein extraxt contains insoluble material which slowly re-dissolves during IEF	Thoroughly centrifuge the extract (40,000 g; 1 hr)

Vertical streaks on the SDS gel

Probable reasons	***Remedies***
• Horizontal SDS-PAGE: stacking gel length too short	Effective stacking gel length >25 mm
• Proteins insufficiently loaded with SDS	SDS-concentration in equilibration buffer > 1%; Equilibrate 2 x 15 min
• Glycoproteins	Use borate buffer instead of Tris buffer in SDS gel; use steep pore gradient; deglycosylate proteins
• Partial re-oxidation of free SH-groups leads to disulfide bonded aggregates	Add sufficient amount of DTT to equilibration buffer, alkylate proteins; use tributylphosphine instead of DTT and iodoacetamide
• Carbamylation trains	Never heat urea containing solutions > 37°C; deionize urea prior to use
• Endogeneous proteolytic enzymes have not been inactivated during sample preparation	Try to inactivate proteases by TCA-acetone treatment, boiling with SDS and/or adding protease inhibitors
• GelBond PAGfilm had not been washed and causes "spot streaking"	Wash GelBond PAGfilm prior to SDS gel casting
• "Point steaking" caused by dust particles or excess DTT	Filter all buffers (membrane filter); add iodoacetamide to the second equilibration step to remove excess DTT

Patterns partially distorted

Probable reasons	***Remedies***
• NP-40 or Triton X-100 concentration in the IPG strip too high	If possible, reduce amount of NP-40/ Triton or width of the IPG strip; use CHAPS instead of Triton / NP-40
• IPG strip has not been removed from the surface of the horizontal SDS gel after protein transfer from the IPG strip onto the SDS gel	Remove IPG strip after protein transfer from the IPG strip onto the SDS gel
• Electrode wick (or buffer strip) has not been moved forward to cover the former IPG strip application area	Cover former IPG strip application area with electrode wick after the IPG strip has been removed from the surface of the horizontal SDS gel

Uneven migration of bromophenol blue front

Probable reasons	*Remedies*
• Horizontal SDS-PAGE: large air bubbles trapped between GelBond PAGfilm and cooling plate; air bubbles trapped in cooling plate	Check for and remove air bubbles
• Horizontal SDS-PAGE: improper contact between electrode wicks (or buffer strips) and surface of the SDS gel	Check electrode contact
• SDS pore gradient gel: gel casting device had not been levelled horizontally during gel casting and polymerization	Level gel casting device

7 Silver staining

- Use pure chemicals (analytical grade) exclusively
- Use highly purified deionized or distilled water (conductivity < 2 µS)
- Use thorougly cleaned and dust-free vessels only
- Always wear gloves or use forceps. Never touch the gel with your fingers!

Observed problems:

No, or only few, proteins visible on the SDS gel

Probable reasons	*Remedies*
• Inappropriate sample extraction procedure (low protein concentration)	Perform protein assay (or SDS-PAGE) to estimate the protein concentration of the sample
• Insufficient sample entry into IPG strip	Start IEF with low field strength
• Acidic end of IPG strip facing towards the cathode; anode connected with the cathodic outlet of the power supply	Make sure that the orientation of the IPG gel strips on the cooling block of the IEF chamber is correct. Check proper connection of the electrodes with the power supply
• 2nd dimension: Large air bubbles between IPG gel strip and surface of SDS gel	Squeeze out air bubbles by pressing on the upside the IPG strip with forceps
• 2nd dimension: Poor protein transfer from IPG strip onto SDS gel	Perform protein transfer at low field strength (< 10 V/cm); use detergents other than NP-40, Triton or CHAPS, or use tributylphosphine (TBP) for improved protein solubilization
• 2nd dimension: IPG strip applied with GelBond side onto the SDS gel	The surface of the IPG strip must be in contact with the surface of SDS gel
• Erreaneous silver staining protocol	Check protocol
• Formaldehyde oxidized	Use fresh formaldehyde
• Improper pH of developing solution	Check pH of developer

• Insufficient volume of buffer solutions	SDS gel has to be completely covered with buffer solutions during the silver staining procedure

Low or high Mr proteins missing on the SDS gel

Probable reasons	***Remedies***
• Low Mr proteins not adequately fixed after SDS-PAGE	Use 20% TCA or glutardialdehyde as fixative instead of 40% alcohol and 10% acetic acid
• High Mr proteins missing due to proteolytic degradation	Inactivate endogenous proteases in the sample
• Poor transfer of high Mr proteins from IPG strip onto SDS gel	Perform protein transfer at low field strength (< 10 V/cm)

Diffuse background smear

Probable reasons	***Remedies***
• Endogenous proteases in the sample had not been inactivated	Inactivate proteases during sample preparation procedure
• Insufficient washing steps during silver staining procedure	Perform sufficient number of washing steps
• Complex formed between carrier ampholytes and SDS and/or other detergents	Fix the gel >3h or overnight and wash it intensively to remove SDS-carrier ampholyte complexes
• Poor quality of chemicals	Use analytical grade (or better)
• Poor water quality	Conductivity < 2 µS
• Reswelling tray or IPGphor strip holder may be contaminated with proteins	Clean reswelling tray and strip holders thoroughly after use

Negatively stained spots

Probable reasons	***Remedies***
• Inappropriate silver staining procedure	Change silver staining method
• Protein concentration too high	Reduce amount of protein to be loaded onto the gel or pre-stain with Coomassie blue

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