Appendix : 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 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	Add 10% glycerol to the reswelling buffer
• " 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 electroendosmotic 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); or use DeStreak.
• 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	Start 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	Remove IPG strip after protein

surface of the horizontal SDS gel 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	transfer from the IPG strip onto the SDS gel 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