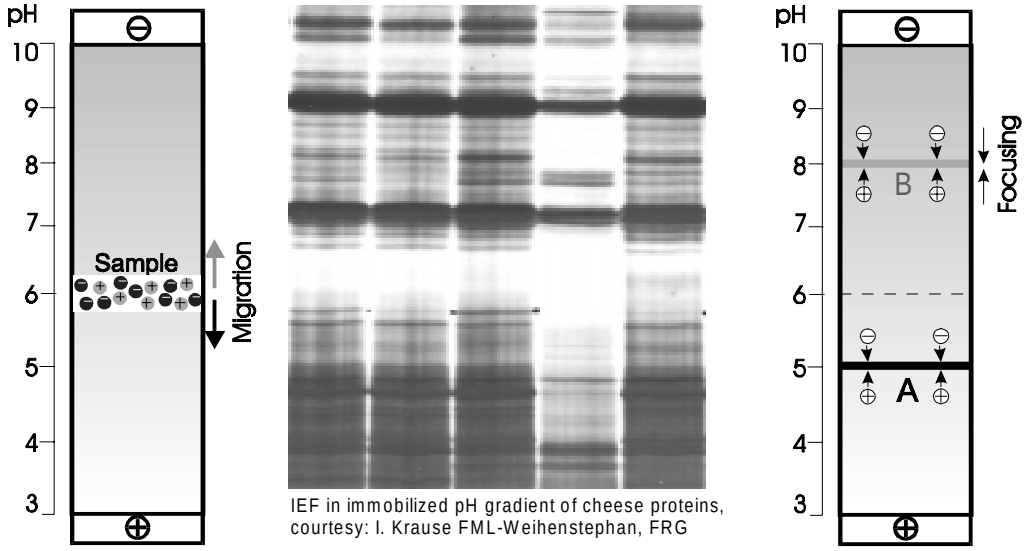
Guide to Isoelectric Focusing

Introduction

Isoelectric focusing methods are widely applied for the separation of proteins, peptides and enzymes. The principle: In a *pH gradient* the sample components migrate towards the anode or the cathode to the pH values, where their net charges are zero: their *isoelectric points* (pI). Should a protein diffuse away from its pI, it would gain a charge and migrate back: this is the *focusing* effect. Much more details of the theory and practice of isoelectric focusing can be found in the book by Righetti [1] .

The proteins are driven to their isoelectric points by the electric field. The isoelectric points of the proteins can be estimated with a calibration curve using marker proteins. Native or denaturing conditions can be chosen by omitting or adding high amounts of urea.

The proteins become highly concentrated at their pIs. This results in a high sensitivity for detection. Small charge differences can be differentiated. For improvement of resolution, narrow gradients can be employed. The method is also suitable for preparative applications, when high amounts of proteins - one hundred and more micrograms - have to be purified.



IEF in immobilized pH gradient of cheese proteins, courtesy: I. Krause FML-Weihenstephan, FRG

Different applications require different isoelectric focusing methods. Amersham Biosciences offers a wide range of products for isoelectric focusing. In the following technical note the various methods and products for isoelectric focusing are described.

Equipment

Isoelectric focusing (IEF) is mostly run in horizontal gels because of several reasons:
- As IEF separates only according to the charge, the gel matrix must contain large pore sizes. Such a soft gel can slide down between vertical glass plates. The gel should preferably be cast on a film support.
- Isoelectric focusing requires efficient cooling and exact temperature control (see below). This is optimally achieved on a horizontal ceramics cooling plate connected to a thermostatic circulator, or a peltier cooling plate.
- Samples have to be applied on a defined pH location within the pH gradient in order to avoid

aggregation and precipitation of some proteins. This is only possible on a horizontal gel with an open surface.
- Because sharply focused bands can only be obtained with a high field strength, high voltages have to be applied. Only horizontal equipment can meet the necessary safety precautions.

The power supply must provide enough voltage for obtaining sharply focused IEF zones and must preferably be programmable in order to run a multiphase IEF protocol automatically. For “normal sized” IEF (ca. 10 cm and longer separation distances) a modular system is available. IEF in “mini gels” (4 cm separation distance) can be run in an automated electrophoresis unit containing the separation chamber, programmable power supply and staining unit.

Multiphor II IEF unit	code no. 18-1018-06	
EPS 3500 XL Power Supply	code no. 19-3500-01	
MultiTemp III Thermostatic Circulator	code no. 18-1102-78	(230 V)
MultiTemp III Thermostatic Circulator	code no. 18-1102-77	(115 V)
PhastSystem all in one	code no. 18-1018-24	(230 V)
PhastSystem all in one	code no. 18-1018-23	(115 V)

Isoelectric Focusing with Ampholine® and Pharmalyte®

Ampholine® and Pharmalyte® are mixtures of 600 to 700 different homologues of amphoteric compounds with a spectrum of isoelectric points between 3 and 10 form a pH gradient under the influence of the electric field. These substances have high buffering capacities at their isoelectric points. They have molecular weights below 1 kDalton and do not bind to proteins, because they are highly hydrophilic. Their general name is “carrier ampholytes”. Mixtures with narrow intervals are available for higher resolution and the selection of defined isoelectric point ranges.

The difference between Ampholine® and Pharmalyte® is based on the chemistry of their production. Ampholine® are produced by reacting aliphatic oligoamines with acrylic acids, whereas Pharmalyte® are co-polymerisates of glycine, glycylglycine, amines and epichlorhydrin. Thus the isoelectric points and buffering properties of the individual homologues of the different products are slightly different.

Ampholine® bottles contain a 40 % w/v stock solution of carrier ampholytes; a 2 to 3 % w/v concentration is required in the gels for isoelectric focusing. Pharmalyte® bottles contain an similar amount of carrier ampholytes like Ampholine® bottles, it is not exactly defined. The buffering capacities of the individual homologues within a mixture are differing anyhow. According to the instructions they are used in a dilution of 1 in 16 volumes, which is equal to Ampholine®.

Ampholine® preblended can be used directly for agarose, Ultrodex or polyacrylamide gels and form linear gradients.

pH 3.5–9.5	code no. 80-1127-15	25 mL
pH 4.0–6.5	code no. 80-1127-17	25 mL
pH 5.0–8.0	code no. 80-1127-19	25 mL

Ampholine® broad range are used as the “backbone” for custom designed pH gradients, in order to obtain optimised resolution for certain samples.

pH 3.5–10.0	code no. 80-1125-87	25 mL

Ampholine® narrow range are narrow intervals, which are used to blend custom designed pH gradients.

pH 3.5–5.0	code no. 80-1125-89	25 mL
pH 4.0–6.0	code no. 80-1125-90	25 mL
pH 5.0–7.0	code no. 80-1125-91	25 mL
pH 5.0–8.0	code no. 80-1125-92	25 mL
pH 6.0–8.0	code no. 80-1125-93	25 mL
pH 7.0–9.0	code no. 80-1125-94	25 mL

Mixing table for Ampholine®, volumes for 30 mL monomer solution, examples:

pH range	pH 3.5–5.0	pH 4.0–6.0	pH 5.0–7.0	pH 6.0–8.0	pH 7.0–9.0
pH 3.5–5.2	0.9 mL	0.9 mL			
pH 4.5–7.0			1.35 mL		0.45 mL
pH 5.5–7.7				1.35 mL	0.45 mL

Pharmalyte® broad ranges can be used directly for agarose, Ultrodex or polyacrylamide gels and form linear gradients.

pH 2.5–5	code no. 17-0451-01	25 mL
pH 4–6.5	code no. 17-0452-01	25 mL
pH 5–8	code no. 17-0453-01	25 mL
pH 8–10.5	code no. 17-0455-01	25 mL
pH 3–10	code no. 17-0456-01	25 mL

Mixing table for Pharmalyte®, volumes for 30 mL monomer solution, examples:

pH range:	pH 2.5–5	pH 4–6.5	pH 5–8	pH 6.5–9	pH 8–10.5
pH 3–10	0.7 mL		0.5 mL		0.6 mL
pH 5–10			0.76 mL		1.14 mL
pH 2.5–8	1.14 mL		0.76 mL		
pH 4–9		0.8 mL		1.0 mL	
pH 5–9			0.83 mL	1.07 mL	
pH 2.5–6.5	1.14 mL	0.76 mL			
pH 6.5–10.5				0.9 mL	1.0 mL

Pharmalyte® narrow ranges are intervals for special dedicated applications in polyacrylamide gels, indicated in the following list:

pH 4.2–4.9	code no. 17-0562-01	25 mL (α1-antitrypsin)
pH 4.5–5.4	code no. 17-0563-01	25 mL (transferrin)
pH 5–6	code no. 17-0564-01	25 mL ( )
pH 6.7–7.7	code no. 17-0566-01	25 mL (hemoglobin)

Ampholine® and Pharmalyte® can also be blended with each other in order to obtain mixtures with more different homologues, if required.

Electrode solutions

To maintain a stable gradient, filter paper strips soaked in electrode solutions are applied between the gel and the electrodes, an acid solution is used at the anode and a basic one at the cathode. Should, for example, an acidiv carrier ampholyte reach the anode, its basic moiety would become a positively charged and it would migrate back towards the cathode. The electrode solutions are particularly important for long separations in gels containing urea, for basic gradients and short pH intervals. They are not necessary for short gels, like PhastGels®. Suggestions of different solutions are given in the following chapters for different gel types.

IEF electrode strips	code no. 18-1004-40	100 strips

Plateau phenomenon

In general, problems with carrier ampholytes can arise when long focusing times are necessary. For example, in the case of short intervals or in the presence of highly viscous additives such as urea or non-ionic detergents, the gradient slowly starts to drift in both directions but particularly towards the cathode. This leads to a plateau in the middle of the gradient with gaps in the conductivity. Part of the proteins leave the gel. It is thus important to limit the focusing time and to run similar experiments for exactly the same time for reproducible results.

Sample application

The samples are applied on the surface of the gels. Either they are pipetted on pieces from cellulose-cotton, which are placed on the gel surface at the optimal pH location, or a silicon applicator strip with sample holes is laid on the gel surface. The latter are available for 26 (up to 40 µL) or 52 (up to 20 µL) samples, the holes are arranged in the distances, or half the distances respectively, of the 96 well microtiter plates for multiple syringes . The mode of sample application is dependent on the sample composition and the IEF gel type. Because the gradient is sensitive to high salt and buffer concentrations, it is very helpful to desalt the samples before application. Desalting columns for sample preparation: PD-10 columns prepacked with Sephadex® G-25.

PD-10 columns	code no. 17-0851-01	30
IEF sample application pieces (20 µL)	code no. 80-1129-46	200
IEF/SDS sample appl. strip, 52 sampl.	code no. 18-1002-26	5 strips
SDS sample appl. strip, 26 samples	code no. 18-1002-74	5 strips

Special sample applicators are available for the PhastSystem®.

The procedure of an IEF run

As isoelectric focusing is in principle a nondenaturing method, the optimization of the running conditions is very important to prevent precipitation and aggregation of proteins, and to achieve good reproducibility.
The IEF running conditions should always be given in a protocol or a publication. Temperature control is important, because pIs are highly dependent on the temperature. Normally a prefocusing phase is performed, in order to establish the gradient. Samples are loaded on the optimized location with the optimized mode. It may be necessary to do a step test. Sample entry should be performed at low field strength to prevent aggregation. The separation time is a compromise between letting all proteins reach their pIs and keeping the gradient drift to a minimum. Often Volthour integration is used as a control. After the run, the proteins are fixed with TCA or by immunofixation and stained - or alternatively - detected with zymogram methods.

Isoelectric focusing in Agarose Gels

Separations in agarose gels are more rapid than in polyacrylamide gels. The pore size diameter of a 1 % agarose gel is ca. 150 nm. In addition macromolecules larger than 500 kDa can be separated since agarose pores are substantially larger than those of polyacrylamide gels. One oft the reasons to use agarose gels for isoelectric focusing is because they are not toxic and do not contain catalysts which could interfere with the separation. It is, however, important to use electroendosmosis free agarose to obtain a stable pH gradient. Mostly agarose gels are cast on GelBond film: a polyester film with a treated surface which binds to the agarose gel. Agarose gels can be cast by pouring it onto a GelBond film on a horizontal bench: oxygen from the air does not inhibit gelation. It is better to cast it in a vertical pre-warmed cassette, since this produces a more uniform gel layer. This cassette is made up by a glass plate with the film support and an U-frame, and is hold together with clamps.

Agarose IEF	code no. 17-0468-01	10 g
GelBond film	code no. 80-1129-32	12.4 × 25.8 cm (50)
Glass plates	code no. 80-1106-99	12.5 × 26.0 cm (2)
U-frame (0.5 mm)	code no. 80-1106-89	12.5 × 26.0 cm (2)
FlexiClamps	code no. 18-1013-73	6/pack

The best gels are obtained using 0.8 % agarose solution, which contains 2.7 % Ampholine® or Pharmalyte® and 10 % (w/v) sorbitol. Because sorbitol improves the mechanical properties of the gel and since it is hygroscopic, it works against the electro-osmotic water flow. The gel should be used not before one day after its preparation, because the matrix needs some time for complete formation. More details are found in reference [2].

The electrode strips must be cut shorter than the gel <25 cm). Electrode solutions for IEF in agarose gels:

pH Gradient	Anode	Cathode
3.5–9.5	0.25 mol/L acetic acid	0.25 mol/L NaOH
2.5–4.5	0.25 mol/L acetic acid	0.40 mol/L HEPES
4.0–6.5	0.25 mol/L acetic acid	0.25 mol/L NaOH
5.0–8.0	0.04 mol/L glutamic acid	0.25 mol/L NaOH

It is important to blot the strips with dry filter paper for about 1 min to remove excess liquid. Samples should never be applied with applicator pieces on an agarose gel.

Typical running conditions for an agarose IEF at 10 °C:

Prefocusing:	max. 1400 V	30 mA	8 W	30 min
Desalting:	max. 150 V	30 mA	8 W	30 min
Separation:	max. 1500 V	30 mA	8 W	60 min

During the run it is often necessary to interrupt the separation and blot the electrode strips with filter paper. These settings are valid for gradients from pH 3 to 10. For narrower gradients, e.g. pH 5 to 8, IEF should last about 2 h, since the proteins with a low net charge must migrate long distances.

Disadvantages of agarose IEF: Silver staining does not work as well for agarose gels as for polyacrylamide gels. In the basic area, electroendosmosis is particularly strong, which can lead to pronounced cathodal gradient drift.

Isoelectric focusing in Ultrodex® Gels

For preparative applications, IEF can be performed in a horizontal flat bed of a granulated dextrane gel: Ultrodex®. Ampholine® or Pharmalyte® is added to the dextrane slurry. Large sample volumes can be mixed with the original gel slurry from which the gel bed is prepared. Labile samples are applied at a defined zone of the gradient. The separation is run across the long distance of the Multiphor cooling plate at a constant power of 8 W for 14 - 16 hours at controlled temperature. pH measurements and prints on filter paper can be made directly on the surface. The sample fractions are collected by sectioning the gel and then eluting the proteins from these sections.

The method has a high loading capacity: up to gram quantities. It is much less sensitive to precipitation of proteins at their isoelectric points compared to preparative isoelectric focusing methods in a free liquid, because the precipitate is trapped within the gel bed. The recovery of the protein fractions from a granulated dextrane gel is much easier and gives a higher yield compared to compact gel media like agarose and polyacrylamide.

Ultrodex ®	code no. 80-1130-01
Preparative IEF Kit	code no. 18-1018-05

Anode solution: 1 mol/L phosphoric acid
Cathode solution:1 mol/L sodium hydroxid

Isoelectric focusing in Polyacrylamide Gels

Polyacrylamide gels have very low electroendosmosis, high mechanical and chemical stability and a clear background. Chemical polymerisation with the catalyst system ammonium persulfate and TEMED is preferably used instead of photopolymerization with riboflavin. The pore size is defined by the *T* value: the total monomer concentration (acrylamide + NN´methylenbisacrylamide) and the *C* value: the crosslinking factor. For IEF mostly a gel with 5 % *T* and 3 % C is employed, which has a pore diameter of 5.3 nm. More details are found in references [1] and [2].

Ready-made gels are available since many years: Ampholine®PAGplates (1mm thick, size: 12.5 × 26.0 cm) and PhastGel® IEF (0.35 mm thin, size: 5 × 4 cm) with different pH gradients.

Ampholine PAGplate pH 3.5–9.5	code no. 80-1124-80	5/pack
Ampholine PAGplate pH 4.0–6.5	code no. 80-1124-81	5/pack
Ampholine PAGplate pH 5.5–8.5	code no. 80-1124-82	5/pack
Ampholine PAGplate pH 4.0–5.0	code no. 80-1124-83	5/pack
PhastGel IEF pH 3–9	code no. 17-0543-01	10/pack
PhastGel IEF pH 4–6.5	code no. 17-0543-01	10/pack
PhastGel IEF pH 5–8	code no. 17-0543-01	10/pack

Polyacrylamide IEF gels can be cast in each laboratory using a cassette system. The Ampholine® and Pharmalyte® are included in the monomer solution. The cassette is made up by a glass plate with the film support and an U-frame, and is hold together with clamps. As support film GelBond PAG film is employed, which binds to polyacrylamide gels.

GelBond PAG film	code no. 80-1129-36	12.4 × 25.8 cm (50)
Glass plates	code no. 80-1106-99	12.5 × 26.0 cm (2)
U-frame (0.5 mm)	code no. 80-1106-89	12.5 × 26.0 cm (2)
FlexiClamps	code no. 18-1013-73	6/pack

Laboratories with very high sample throughput cast large formate gels (25 × 19 cm) and run them on the Multiphor II with two anodes and one common cathode in the center.

IEF electrode, anode	code no. 80-1106-61	1
GelBond PAG film	code no. 80-1129-37	20.3 × 26.0 cm (50)

Glass plates	code no. 80-1102-99	20.0 × 26.0 cm (2)
U-frame (0.5 mm)	code no. 80-1106-87	20.0 × 26.0 cm (2)
FlexiClamps	code no. 18-1013-73	6/pack

Acrylamide, NN´methylenbisacrylamide, catalysts and other additives can be found in high quality in the Amersham Biosciences **PlusOne** product family.

Typical running condition for a whole Ampholine PAGplate pH 3.5–9.5 at 7 °C [4]:

Max. 1700 V	50 mA	30 W	2 hours 15 min

For narrower gradients, e.g. pH 4.0 to 6.5, the separation time must be prolonged, since the proteins with a low net charge must migrate long distances.

Hydrophobic proteins need the presence of 8 molar urea to stay in solution. Because of the buffering capacity of urea, there is a light increase in the pH in the acid part of the gel. High urea contents in the gel lead to configurational changes in many proteins and disruption of the quaternary structure. The solubility of very hydrophobic proteins, such as membrane proteins for example, can be increased by the addition of non-ionic detergents (e.g. Nonidet NP-40, Triton X-100) or zwitterionic detergents (e.g. CHAPS, Zwittergent). Because the gels do not co-polymerize with the support films in the presence of non-ionic detergents, it is recommended to rehydrate a prepolymerized, washed and dried gel in the relevant solution (see below).

Electrode solutions for IEF in polyacrylamide gels:

pH Gradient	Anode	Cathode
3.5–9.5	0.5 mol/L H_3PO_4	0.5 mol/L NaOH
2.5–4.5	0.5 mol/L H_3PO_4	2% Ampholine pH 5–7
2.5–4.5	0.5 mol/L H_3PO_4	0.4 mol/L HEPES
3.5–5.0	0.5 mol/L H_3PO_4	2% Ampholine pH 6–8
4.0–5.0	0.5 mol/L H_3PO_4	1 mol/L glycine
4.0–6.5	0.5 mol/L acetic acid	0.5 mol/L NaOH
4.5–7.0	0.5 mol/L acetic acid	0.5 mol/L NaOH
5.0–6.5	0.5 mol/L acetic acid	0.5 mol/L NaOH
5.5–7.0	2% Ampholine pH 4–6	0.5 mol/L NaOH
5.0–8.0	0.5 mol/L acetic acid	0.5 mol/L NaOH
6.0–8.5	2% Ampholine pH 4–6	0.5 mol/L NaOH
7.8–10.0	2% Ampholine pH 6–8	1 mol/L NaOH
8.5–11.0	0.2 mol/L histidine	1 mol/L NaOH

Isoelectric focusing in rehydrated Polyacrylamide Gels

For a several reasons it is advantageous to use washed and dried polyacrylamide gels, which are rehydrated in a solution containing Ampholine® or Pharmalyte®:

-A few carrier ampholytes inhibit the polymerization of gels, particularly the co-polymerisation with theGelBond PAGfilm surface.

- Catalysts and unreacted monomers are removed from the gel. This allows IEF with fewer interferences, as is readily apparent in the straight bands also in the acidic gradient part. This is particularly beneficial for enzyme separations and zymogram detection techniques. In most cases the electrode strips, soaked in acid or basic buffer, are not needed.

- Chemical additives such as Triton, which allow the separation of many proteins but which would inhibit polymerization, can be added to the gel without any problems.

- Using ready-made dry gels, handling acrylamide monomers is not necessary and a lot of time and effort are saved.

- For applications requiring urea, this is the only ready-made gel, which is possible.

GelPool (tray for rehydration)	code no. 18-1031-58	1
CleanGel Dry IEF (12.5 × 26.0 cm)	code no. 18-1035-32	5
CSF Analysis Kit for PhastSystem	code no. 18-1039-14	10

When very acidic or very basic pH intervals are used in a CleanGel IEF, electrode strips with electrode solutions (see above) should be employed.

Typical running conditions at 10 °C for a whole CleanGel IEF PAGplates (0.5 mm thick, size: 12.5 × 26.0 cm) rehydrated with 10 % ethylenglycol, distilled water, 2 % Ampholine® pH 3.5–9.5:

Prefocusing:	max. 700 V	12 mA	8 W	20 min
Sample entrance:	max. 500 V	8 mA	8 W	20 min
Separation:	max. 2000 V	14 mA	14 W	90 min
Band sharpening:	max. 2500 V	14 mA	18 W	10 min

For narrower gradients, e.g. pH 5 to 8, the separation step must be prolonged, since the proteins with a low net charge must migrate long distances.

Isoelectric focusing in Immobilised pH Gradients

Immobilised pH Gradients are an alternative to carrier ampholytes generated gradients. There is no plateau phenomenon or gradient drift happening with these fixed gradients. Acrylamido buffers – "Immobiline®" – with carboxylic and tertiary amino groups are copolymerized with the polyacrylamide network. A linear gradient of monomer solutions has to be cast analogous to a porosity gradient. With 6 individuals of these Immobilines, all types of gradients can be produced: from wide to very narrow gradients. Immobilized pH gradient gels are cast on film support, washed with distilled water, dried down, and rehydrated before use with water or urea-detergent solution. All theoretical background and many hints for the practice are found in reference [3].

IEF gels with immobilised pH gradients can be cast in each laboratory using a cassette system and a gradient maker. The needed Immobiline, 0.2 mol/L stock solutions, are added to the two monomer solutions, the acidic and the basic solution. The cassette is made up by a glass plate with the film support and an U-frame, and is hold together with clamps. As support film GelBond PAG film is employed, which binds to polyacrylamide gels.

GelBond PAG film	code no. 80-1129-36	12.4 × 25.8 cm (50)
Glass plates	code no. 80-1106-99	12.5 × 26.0 cm (2)
U-frame (0.5 mm)	code no. 80-1106-89	12.5 × 26.0 cm (2)
FlexiClamps	code no. 18-1013-73	6/pack
Gradient maker	code no. 18-1013-72	1
Immobiline II pK 3.6	code no. 80-1255-70	10 mL
Immobiline II pK 4.6	code no. 80-1255-71	10 mL
Immobiline II pK 6.2	code no. 80-1255-72	10 mL
Immobiline II pK 7.0	code no. 80-1255-73	10 mL
Immobiline II pK 8.5	code no. 80-1255-74	10 mL
Immobiline II pK 9.3	code no. 80-1255-75	10 mL

Acrylamide, NN´methylenbisacrylamide, glycerol, and catalysts can be found in high quality in the Amersham Biosciences **PlusOne** product family.

As the casting procedure requires a lot of skill, it is recommended to use ready-made gels, Immobiline DryPlates (size: 12.5 × 26.0 cm), which can be rehydrated with water or additive solution either in a vertical reswelling cassette or – much easier – in the GelPool.

	Reswelling Cassette	code no. 80-6371-84	1
or	GelPool (tray for rehydration)	code no. 18-1031-58	1
	Immobiline DryPlate pH 4.0–7.0	code no. 80-1128-28	5
	Immobiline DryPlate pH 4.2–4.9	code no. 80-1128-28	5
	Immobiline DryPlate pH 4.5–5.4	code no. 80-1128-28	5
	Immobiline DryPlate pH 5.5–8.5	code no. 80-1128-28	5
	Immobiline DryPlate pH 4.0–5.0	code no. 80-1128-28	5

No electrode solutions are needed for Immobiline gels. Either the filter paper strips are soaked in distilled water, or no strips are used at all. No prefosucing phaase is applied, because the gradient exists already.

Typical running condition for a whole IPG DryPlate pH 4–7 rehydrated with distilled water 10 °C:

Max. 3500 V	1.0 mA	5 W	5 hours

For narrower gradients, e.g. pH 4.5 to 5.4, the separation time must be prolonged, since the proteins with a low net charge must migrate long distances.

Isoelectric focusing in gel strips with Immobilised pH Gradients

Immobilised pH gradients (IPG) are particularly advantageous for IEF as the first step in 2-D electrophoresis. The dried IPG gels on film supports are cut to 3 mm thin strips. Those are rehydrated to the original thickness of 0.5 mm in a solution of 8 mol/L urea, 1 % (w/v) CHAPS, 0.2 (w/v) Dithiothreitol, and 0.25 % (w/v) IPG buffers (special carrier ampholyte mixture) overnight before they are used in the first dimension. The samples are either applied with applicator cups at the anodal or the cathodal end of the rehydrated strips, or the dry strips are rehydrated with the sample solution. This in-gel sample application is particularly beneficial when high volumes have to be loaded for micropreparative applications.

A novel system - the IPGphor - saves an entire working day, because rehydration, sample application, and isoelectric focusing are performed over night without attendance. The base of the IPGphor is a Peltier cooling plate, which is divided into a short cathodal and a long anodal area. It contains also a programmable high voltage power supply, which generates up to 8,000 Volts.

The IPG DryStrips are rehydrated and focused in individual strip holders. Different lengths are available. A strip holder is made up from a narrow thermally conductive ceramics tray with built-in platinum electrodes and a transparent lid. The sample is pipetted into the tray, the IPG strip id laid gel side down onto the solution and overlaid with 200 to 300 µL paraffin oil. The strip holders are placed onto the IPGphor platform, with the acidic side of the IPG strip on the anodal area, the basic side on the cathodal area. Up to twelve strip holders can be applied.

IPGphor Focusing apparatus	code no.	
IPG stripholders 7, 11, 13, and 18 cm see catalogue		
IPG DryStrip Reswelling tray	code no. 80-6371-84	
Multiphor II IEF unit	code no. 18-1018-06	
EPS 3500 XL Power Supply	code no. 19-3500-01	
MultiTemp III Thermostatic Circulator	code no. 18-1102-78	(230 V)
MultiTemp III Thermostatic Circulator	code no. 18-1102-77	(115 V)

IPG DryStrip Kit code no. 80-1004-30
IPG DryStrips 7, 11, 13, and 18 cm see catalogue

Temperature

Since the pK values of the gradient forming buffers like Immobilines®, Ampholine® and Pharmalyte®, as well as of the substances to be analysed are temperature dependent, IEF must be carried out at a constant controlled temperature, usually 10 °C. For the analysis of the configuration of subunits of specific proteins, ligand bindings or enzyme-substrate complexes, cryo-isoelectric focusing at temperatures below 0 °C have been used. In order to increase the solubility of cryo-proteins (like IgM), which precipitate at low temperatures, isoelectric focusing in agarose gels is performed at + 37 °C.

pH gradient measurement

Measurement of the pH gradient with electrodes is a problem since these react very slowly at low temperatures. CO_2 diffusing into the gel from the air reacts with water to form carbonate ions. Those form the anhydrid of carbonic acid and lowers the pH of the basic area. To prevent errors which can occur during the measurement of pH gradients, it is recommended to use marker proteins of known isoelectric points. The pIs of the sample can then be measured with the help of a pH calibration curve.

Marker proteins for various pH ranges are available. These proteins are chosen so that they can focus independently of the point of application. Standard marker proteins can not be used in urea containing gels, because their conformations are changed, and thus their isoelectric points (pI).

Broad pI Kit	pH 3.5–9.3	code no.	17-0471-01
Low pI Kit	pH 2.5–6.5	code no.	17-0472-01
High pI Kit	pH 5–10.5	code no.	17-0473-01

Staining of Isoelectric focusing gels

Isoelectric focusing gels require special staining procedures: the proteins have to be efficiently fixed, whereas carrier ampholytes have to be washed out. A high detection sensitivity is obtained only with the procedure described below.

Coomassie Blue Staining of Agarose gels:

*Fixing:*30 min in 20% (w/v) TCA;
Washing: 2 × 15 min in 200 mL fresh solutions of 10% acetic acid, 25% methanol;
Drying: place 1 layer of moistened filter paper and 3 layers of dry filter paper on the gel, a glass plate, and a 1 kg weight on top. Remove everything after 10 min and finish drying in the heating chamber;
Staining: 10 min in 0.5% (w/v) Coomassie R-350 in 10% acetic acid, 25% methanol: dissolve 3 PhastGel Blue R tablets (1 tablet = 0.4 g of Coomassie Brilliant Blue R-350) in 250 mL;
Destaining: in 10% acetic acid, 25% methanol till the background is clear;
Drying: in the heating cabinet.

Staining tray, stainless steel	code no. 18—1018-08	
PhastGel Blue R tablets	code no. 17—0518-01	40 tablets

Quick Coomassie Blue Staining of Polyacrylamide gels:

Stock solutions:

TCA: 100% TCA (w/v) 1 L
A: 0.2% (w/v) $CuSO_4$+ 20% acetic acid
B: 60% (v/v) methanol
C: dissolve 1 tablet of Phast Blue R in 400 mL of H_2O_{dist}, add 600 mL methanol, stir 5 to 10 min.

Staining:
Fixing: 10 min in 300 mL of 20% TCA;
Washing: 2 min in 300 mL (mix A and B 1:1);
Staining: 15 min in 300 mL staining solution (mix A and C 1:1) at 50 °C while stirring;
Destaining: 15 to 20 min (mix A and B 1:1)
Impregnating: 10 min in 200 mL 5% glycerol, 10% acetic acid;
Drying: air-dry.

Hoefer Automated Gel Stainer	code no. 80-6395-02	
PhastGel Blue R tablets	code no. 17-0518-01	40 tablets

Silver Staining of Ampholine PAG plates acc. to Wurster [4]

Staining solutions:

A. Fixing sol. I, 20 % TCA: 30 g TCA, dissolve in distilled water and make up to 150 mL.
B. Fixing sol. II, 50 % methanol, 10 % acetic acid: Add 75 mL methanol and 15 mL acetic acid and makeup to 150 ml with distilled water
C. Fixing solution III, 5 % methanol, 7 % acetic acid: Add 7.5 mLmethanol and 10.5 ml acetic acid and make up to 150 ml with distilled water.
D. Fixing solution IV, 2.5 % glutardialdehyde: 15 mL glutardialdehyde, make up to 150 mL with distilled water.
E. Ammoniacal silver reagent: a) dissolve 180 mg silver nitrate in 0.75 mL distilled water
   b) To 3 ml 1 mol/1 NaOH add 1.1 ml NH_3. Make up to 150 mL with distilled water.
   c) With the help of a pipette slowly add solution a) $AgNO_3$ to the vigorously vortexed solution b) ammoniacal sodium hydroxide. Any brownish precipitate should immediately disappear, otherwise the concentration of the ammonia solution is too low and should be checked.
F. Developing solution: For the preparation of 0.05 % (w/v) citric acid dissolve 125 mg monohydrate in 250 mL distilled water. Immediately before use dilute 15 mL of the citric acid solution to 150 mL and add 200 µL of formaldehyde.
G. Stopping solution, 10 % ethanol, 1 % acetic acid: ad 200 mL ethanol to 20 mL acetic acid and make up to 2L with distilled water.
H. Preserving solution: To 60 mL stopping solution G add 15 mL glycerol and make up to 150 mL with distilled water.

Step	Solution	IN-port	OUT-port	Time (min)
1	20 % TCA	7	7	45
2	Distilled water	0	7	0.5
3	50 % methanol, 10 %acetic acid	1	9 (waste bottle)	40
4	5 % methanol, 7% acetic acid	8	9	20
5	Glutardialdehyde 2.5 %	2	2	30
6	Distilled water	0	7 (change to drain)	10
7	Distilled water	0	7	30

8	Distilled water	0	7	30
9	Distilled water	0	7	360
10	Distilled water	0	7	360 (hold)*
11	Distilled water	0	7	60
12	Silver Solution	3	3	40
13	Distilled water	0	7	0.5
14	Distilled water	0	7	8
15	Developing solution	4	9	5
16	Stop solution	5	9	5
17	Stop solution	5	9	5
18	Stop solution	5	9	5
19	Stop solution	5	9	60
20	Preserving solution	6	9	60

Adjust the pumped volume to 160 mL to ensure complete delivery of the 150 mL solution volumes. To assure free floating of the gel do not place the gel into the tray, before TCA has been pumped in (pause in step 1 and remove glass lid to submerge the gel). The stainer stays in the HOLD* position after over-night water washing and must be restarted the next morning. Thus the silver solution can be prepared fresh which gives a lighter background. Alternatively the silver solution may be provided the day before together with all other solutions. In that case the HOLD option must be canceled and staining will proceed automatically until the end. In accordance with safety regulations toxic solutions (TCA, glutardialdehyde) are pumped back to their dispensing vessels, as is the silver solution, to allow separate disposal. All other solutions are gathered in a waste flask connected to port 9. Distilled water is directly emptied into the drain.

The stained gels are air-dried over night. The sticky surface is then covered by a rolled-on Mylar sheet. The gels can be punched, filed and stored for several years without fading.

Hoefer Automated Gel Stainer code no. 80-6395-02

References

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[2] Westermeier R. Electrophoresis in Practice. VCH Weinheim (1993).
[3] Righetti PG. In: Burdon RH, van Knippenberg PH. Ed. Immobilized pH gradients: theory and methodology. Elsevier, Amsterdam (1990).
[4] Wurster U. Isoelectric focusing of oligoclonal IgG on polyacrylamide gels (PAG plates pH 3.5 – 9.5) with automated silver staining. Amersham Biosciences Application Note (1998).

Trouble Shooting

Isoelectric focusing with carrier ampholytes

Gel characteristics:

Symptom	**Cause**	**Remedy**
Gel sticks to glass plate.	Glass plate too hydrophilic.	Clean the glass plate and coat with Repel Silane.

Incomplete polymerization.		See below.
No gel or sticky gel, insufficient mechanical stability.	No or incomplete polymerization:	
	Poor water quality.	Always use double-distilled water!
	Too much oxygen in the gel solution (radical trap)	Degas thoroughly.
	Acrylamide, Bis or APS solutions too old.	Maximum storage time in the refrigerator: Acrylamide, Bis solution, 1 week; APS solution 40%, 1 week.
	Poor quality reagents.	Only use analytical grade quality reagents.
	Photochemical polymerization with riboflavin.	Chemical polymerization with APS is much more effective.
	The pH value is too basic (narrower basic pH range).	Rehydrate the prepolymerized and dried gel in a carrier ampholyte solution.
Gel peels away from the support film	Wrong support film was used.	Only use GelBond PAG film for polyacrylamide gels not GelBond film (for agarose).
	Wrong side of the support film was used.	Only cast the gel on the hydrophilic side of the support film, test with a drop of water.
	Support film was incorrectly stored or too old.	Always store the GelBond PAG film in a cool, dry, and dark place (< 25 °C), check the expiry date.
	Insufficient polymerization.	See above.
	Gel solution contains non-ionic detergents (Triton X-100, Triton X-100).	Rehydrate the prepolymerized and dried gel in a carrier ampholyte/detergent solution.

Problems during IEF:

Symptom	**Cause**	**Remedy**
No current.		
	Safety turn off, “ground leakage” because of massive short circuit.	Turn off the power supply, check the

separation unit and cable. Dry the bottom of the	separation chamber, cooling coils and	laboratory bench, turn the power on again.
Too low or no current.	Poor or no contact between the electrodes and electrode strips.	Make sure that the electrode strips are correctly placed; if a small gel or part of a gel is used, place it in the middle.
	The connecting cable is not plugged in.	Check the plug; press the plug more securely into the power supply.
Current rises during the IEF run.	Electrode strips or electrodes mixed up.	Acid solution at the anode, basic solution at the cathode.
General condensation.	The power setting is too high.	Check the power supply settings. Guide value: at most 1 W per mL of gel.
	Insufficient cooling.	Check temperature, if focusing is carried at a higher temperature e.g. 15 °C, reduce the power. Check the flow of the cooling fluid (bend in tubing?). Add kerosene between the cooling plate and the support film.
Condensation on the sample applicator.	Excessive salt concentration in the sample (>50 mmol/L) which causes local overheating.	Desalt the sample by gel filtration (NAP column) or dialyze against 1% glycine or 1% carrier ampholyte (w/v).
Gel swells around electrode strips.	EEO causes a flow of water in direction of the electrodes (especially the cathode).	Normal phenomenon. It is not a problem unless it interferes with the run. It may help to occasionally blot the electrode strips.
		After soaking, blot the strips with filter paper.
	There is too much electrode solution in the electrode strips.	
	Electrode solution is too concentrated.	Use electrode solution at the specified concentration, dilute if necessary.
Condensation along the electrode strips. Electrode strips reversed. Local condensation.	Acid solution at the anode, basic solution at the cathode.	

Localized hot spots due to bubbles in the insulating fluid.	Remove the air bubbles, avoid them from the beginning if possible.	
	Cooling not effective enough.	Replace old MultiphorII cooling plate by the new ceramics cooling plate.
Condensation over the basic half of the gel.	Electro-osmotic water flow in direction of the cathode.	Use a better or fresher acrylamide solution. Reduce focusing time as much as possible, for narrow pH ranges pH>7, blot the liquid which collects.
Lines of condensation over the whole gel.	Hot spots, conductivity gaps because of plateau phenomenon. Too long focusing time, especially for narrow pH ranges.	Fill the conductivity gaps by adding carrier ampholytes with a narrow pH range. Keep the focusing time as short as possible, or use IPG.
Sparking on the gel.	Same causes as for condensation, next stage (dried out gel).	Remedy as for condensation. Take measures as soon as condensation appears.
Sparking along the edge of the support film.	Electrode strips hang over the edge of the gel.	Cut the electrode strips to the size of the gel.
	High voltage and ions in the insulating fluid.	Use kerosene or DC-200 silicone oil, not water.

Separations:

Symptom	**Cause**	**Remedy**
The pH gradient deviates from that expected.	Gradient drift (Plateau phenomenon).	
	Acrylic acid polymerized in the gel.	Only use analytical grade quality reagents.
	Acrylic acid polymerized in the gel because the acrylamide, Bis stock solutions were stored too long.	Maximum storage time in the refrigerator, in the dark: 1 week. The storage life can be prolonged by trapping the acrylic acid with Amberlite ion-exchanger MB-1.

Temperature dependence of the pH gradient (pK	values!).	Check the focusing temperature.
	Too long focusing time.	Reduce the focusing time as much as possible, especially in narrow basic pH intervals; or else use IPG.
	Gel stored too long.	Gels with narrow alkaline pH intervals have a limited storage life, use rehydratable gels.
	Gel contains carbonic acid ions.	Degas the rehydration solution (removal of CO_2); avoid the effects of CO_2 during IEF (particularly in basic pH ranges): seal the separation chamber, flush with N_2, trap CO_2: add 1 mol/L NaOH to the buffer tanks.
Partial loss of the most basic part of the pH gradient.	Oxidation of the carrier ampholytes during the run.	Reduce the influence of CO_2 as much as possible: see above.
	Oxidation of the electrode solutions.	See above.
Wavy iso-pH bands: 1. no influence of the sample.	Too much APS was used for polymerization.	Use a polymerized, washed and dried gel. Increase viscosity of the gel by adding 10% (w/v) sorbitol to the solution or urea (< 4 mol/L, not denaturing in most cases).
	Urea gels stored too long, urea degraded to isocyanate.	Use urea gels immediately after preparation or else rehydrate the gel shortly before use
	Bad electrode contact.	Check the electrode contacts, especially the anode; if necessary put a weight on the electrode support.
		Unevenly or excessively wetted electrode strips.

	Soak the electrode strips completely with electrode	solution and blot them with filter paper.
	Wrong electrode solutions.	Use the electrode solutions recommended for the pH range in the correct concentrations.
	Gel too thin.	Ultrathin gels <μm are sensitive to protein overloading, varying protein concentrations, buffer and salt ions as well as diffusion of electrode solutions (compression of the gradient).
Wavy iso-pH lines. 2. induced by the sample. (A) Protein concentration Strongly varying protein concentration of the sample.	Either dilute the highly concentrated samples or apply in order of increasing or decreasing concentration. Prefocuse. Decrease the field	strength at the beginning: (< 40 V/cm).
	Samples applied too far apart.	Apply samples closer to one another (1–2 mm)
	Highly concentrated samples applied at different places in the pH gradient.	It is not possible to proceed another way with the step trial test; but otherwise apply the samples as close as possible in the pH gradient. Prefocus; field strength at the beginning: <40 V/cm.
	Overloading, – protein concentration too high.	Dilute the samples (2 to 15 μg per band) or use a thicker gel. Ultrathin-layer IEF: use gel thickness >250 μm or IPG.
(B) Buffer, salt concentration.	The buffer or salt concentrations in the samples vary a lot.	As for (A); if necessary, desalt highly concentrated samples. High buffer or salt concentration in the samples.

	As for (A); make sure that the samples are applied close to one another, at the same level in the gradient. Prefocus;	field strength at the beginning (<40 V/cm; at first for 30 min at <20 V/cm let salt ions migrate out of the gel or use IPG.
	Buffer or salt concentrations in the samples too high - desalting too risky or not possible because of eventual protein losses.	Cast sample application strips in polyacrylamide (*T* = 10% ca.) or agarose (ca. 2%) containing salts in the same concentrations as the samples and place over the whole width of the gel. Apply the samples in the wells; let the salt ions migrate out for about 30 min at E = V/cm, the salt load will then be the same over the whole width of the gel, so individual shifts in the pH gradient will be compensated. Alternatively use IPG.
Streaking or tailing of the sample.	Precipitate and/or particles in the sample.	Centrifuge the sample.
	The applicators retain the proteins and release them later.	Remove the applicators after about 30 min of IEF or use applicator strips.
	Old or denatured sample.	Check sample preparation, carry it out shortly before the separation. Store samples at < −20 °C.
	High molecular weight proteins have not reached their pI yet.	Focus longer or use agarose gels.
	Poorly soluble proteins in the sample.	Focus in urea (if necessary with non-ionic or zwitterionic detergents) or 30% DMSO.
	Protein overloading.	Dilute sample or apply less.

	Protein aggregation during sample entry.	Set a lower current (mA) limit for the sample entry phase. This reduces the field strength in the beginning.
Diffuse bands.	Diffusion during IEF, low molecular weight peptides.	It is preferable to focus oligopeptides with molecular weight <2 kDa in IPG where diffusion is less marked.
	Diffusion after IEF, inadequate or reversible fixing.	Check the fixing and staining methods.
	Urea IEF: Urea precipitation in the gel.	Run urea gels at 15–20 °C.
	Focusing time too short.	Focus for a longer time.
	Marked gradient drift.	See above.
	Influence of CO_2 on the basic bands.	See above.
Individual bands are diffuse.	See above.	See above.
	The focusing time for the individual proteins is too short (large molecules and/or low net charge).	Optimize the sample application point with a concentration test or titration curve analysis. Apply the sample on the side of the pI where the charge curve is steeper.
Missing bands.	Concentration too low or detection method not sensitive enough.	Apply more sample or concentrate the sample. Use another detection method (e.g. silver staining of the dried gel, blotting).
	The proteins are absorbed on the sample applicator.	Use sample application strips.
The proteins precipitate at the point of application.	Application too close to the pI.	Apply sample further away from its pI (step trial test, titration curve)
	The field strength is too high at the point of sample entry.	Reduce the voltage at the beginning (E< 40 V/cm).

	The molecule is too large for the pores of the gel.	Use agarose instead of polyacrylamide.
	The proteins form complexes.	Add urea (7 mol/L) to the sample and the gel; add EDTA to the sample; add non-ionic or zwitter-ionic detergents to the sample and the gel.
	Protein unstable at the pH of site of application.	Apply the sample at another point (step trial test, titration curve).
	The protein is unstable at the temperature used.	Change the focusing temperature.
Individual bands focus at the wrong place.	The proteins form complexes.	See above. If it is suspected that complexes form with the carrier ampholyte, check with IPG.
	The proteins have lost ligands.	Check with titration curve analysis.
"One" protein focuses in several bands.	The protein exists in various states of oxidation.	Check the sample preparation; eventually focus under N_2.
	The protein has dissociated into subunits.	Do not focus in the presence of urea.
Urea IEF: Carbamylation by cyanate.	Check sample preparation and gel casting with urea.	
	Different conformations of a molecule.	Focus the protein in the presence of urea (>7 mol/L).
	Different combinations of oligomers of a protein or of subunits.	Natural phenomenon.
	Different degrees of enzymatic phosphorylation, methylation or acetylation exist.	Check the sample preparation procedure.
	Various carbohydrate moieties of glycoproteins.	neuramidase for example, to verify.
	Natural phenomenon.	
	Partial proteolytic digestion of a protein.	Treat the sample with Add inhibitor (e.g. 8 mmol/L PMSF).
	Check the sample preparation procedure.	

	Complex formation.	If complex formation with the carrier ampholytes is suspected, verify with immobilized pH gradients.

Isoelectric Focusing in Agarose Gels

When agarose IEF is used, it should be remembered that the matrix is not as electrically inert as polyacrylamide, since sulfate and carboxy groups are still bound to agarose which is of natural origin, and they give rise to electroendosmotic phenomena.

Gel properties.

Symptom	**Cause**	**Remedy**
Insufficient gel consistency.	Incomplete solidification of the gel.	Let the gel solidify>1 h. It is best to remove it from the cassette after 1 h and store it overnight in a humidity chamber at +4 °C (maximum storage time: 1 week).
	The agarose concentration is too low despite the fact that the agarose was precisely weighed out, the agarose has absorbed water.	Store the agarose in a dry place out of the refrigerator. Close the package well.
	Urea gel: urea disrupts the structure of agarose	Use a higher agarose concentration (2%); let the gel solidify longer or use rehydratable agarose gels.
The gel comes off the support film.	Wrong support film used.	Only use GelBond film for agarose, not GelBond PAG film (for polyacrylamide gels).
	The wrong side of the support film was used.	Cast the gel on the hydrophilic side of the support film.
	The gel was cast at too high a temperature.	The temperature should be kept between 60 and 70 °C during casting.
	The solidification time was too short.	See above.

Problems during the IEF run:

Symptom	**Cause**	**Remedy**
Flooding on the surface.	The gel surface was not dried.	Always dry the surface of the gel with filter paper before IEF.
	The solidification time was too short.	See above.

	The wrong electrode solution was used. In general it is recommended to use: at	the anode: 0.25 mol/L acetic acid; at the cathode 0.25 mol/L NaOH.
	The electrode strips are too wet.	Remove the excess liquid. Blot the electrode strips so that they appear almost dry.
	EEO. Natural phenomenon.	Blot the electrode strips every 30 min. or replace them by new ones. chemicals: 0.8% agarose IEF with 2.7% Ampholine.
Strong EEO.	Always use double-distilled water; use an ideal combination of	
	There are no water binding additives in the gel.	Add 10% sorbitol to the gel solution.
Water build-up at the cathode.	EEO, cathodic drift.	Dry the cathode strips more often and carefully; only focus as long as necessary.
Water build-up at the sample application site.	EEO because of the material used for sample application.	Only use sample application strips or masks, do not use paper or Paratex for example.
	The protein or salt concentration is too high.	See polyacrylamide gels.
Formation of a ditch in the gel.	Advanced cathodic drift because of EEO. Insufficient gel consistency	See above. Focus at 10 to 15 °C. See above.
Formation of small hollows near the sample application site.	The power was too high during sample entrance.	Set the power at 5 to 10 W at most for the first 10 to 15 min (for a 1 mm thick gel, 25 cm wide × 10 cm separation distance, use correspondingly lower settings for smaller gels.
	Sample overloading.	See under polyacrylamide gels.
The gel dries out.	Advanced EEO. The gel was irregularly cast.	See above. Position the leveling table exactly when casting horizontal gels or else use the vertical technique (“clamp” technique in prewarmed molds).

Heat source in the proximity.	During agarose IEF do not place the separation	chamber beside a thermostatic circulator.
	The air is too dry.	When the ambient humidity is too low, pour a small volume of water in the electrode tanks.
Sparking	Advanced stage of the effects listed above.	See above, if possible take measures before this occurs.

Separation results:

Symptom	**Cause**	**Remedy**
Bands too wide.	Too much sample solution was applied.	Reduce the sample volume.
Diffuse bands.	Focusing time too long (gradient drift) or too short (the proteins have not reached their pI yet).	See above and under polyacrylamide gel.
	Because of the larger pore size, diffusion is more marked in agarose than in polyacrylamide gels.	Check the fixing and staining procedures. Dry the gel after fixing and then stain (this is also valid for silver staining).
Missing bands.	See above.	See above.
Missing bands in the basic part of the gel.	Part of the gradient is lost because of a cathodic drift (more pronounced in agarose than in polyacrylamide gels).	Add a carrier ampholyte with a narrow basic range; focus for a shorter time.
Distorted bands at the edge of the gel.	Fluid has left the gel or the electrode strips; fluid has run along the edge of the gel and forms L-shaped “electrodes”.	Blot the gel or electrode strips regularly when water oozes out.
	The samples were applied too close to the edge.	Apply the samples about 1 cm from the edge.
Wavy bands in the gel.	As for polyacrylamide gels.	See under polyacrylamide gels.
	Irregularities in the surface of the gel.	Degas the gel solution properly. Use a humidity chamber for storage.
Bands are diffuse, disappear or do not appear.	Diffusion	Always dry agarose gels after fixing and before staining them.
The gel comes off the support film during staining.	The fixing solution was not completely removed from the gel before drying.	Rinse the gel in twice for 20 min each time in the destaining solution containing 5% glycerol.

	A mistake was made during gel casting.	See above.

Immobilized pH gradients

Gel properties:

Symptom	**Cause**	**Remedy**
The gel sticks to the glass plate.	The glass plate is too hydrophilic.	Clean the glass plate and coat it with Repel Silane.
	The gel was left too long in the mold.	Remove it from the cassette 1 h after the beginning of polymerization.
	The gel concentration is too low.	Do not use glass when T< 4%, use acrylic glass (Plexiglas) instead.
	Incomplete polymerization.	See below.
No gel or sticky gel.	Always use double-distilled water!	
Poor water quality.	APS solutions is too old.	Maximum storage time in the dark in the refrigerator: 40% APS solution: 1 week.
	Poor quality reagents.	Only use reagents of analytical grade quality.
	Too little APS and/or too little TEMED were used.	Always use 1 µL of APS solution (40% w/v) per mL of gel solution and at least 0.5 µL of TEMED (100%) per mL of gel solution.
	The pH value was not optimal for polymerisation.	For wide (>1 pH unit) and alkaline (above pH 7.5) pH ranges: titrate both gel solutions with HCl 4 mol/L respectively NaOH 4 mol/L to about pH 7 after TEMED has been added. The precision of pH paper is sufficient.
	The polymerization temperature was too low.	Let the gel polymerize for 1 h in a heating cabinet or incubator at 50 °C or 37 °C respectively.
One half of the gel is not or insufficiently polymerized.	The APS solution has not mixed properly with the gel solution (usually the dense solution: the APS solution overlayers it	because of the glycerol content). After adding the APS solution stir vigorously for a short time. Make sure that the drops of

APS solution are incorporated in the gel	solution.	
	One of the solutions was not titrated to pH 7.	See above.
The surface of the gel is sticky, swells during washing and detaches itself from the support film.	Oxygen has inhibited polymerization of the surface.	Overlay the surface of the gel with about 300 µL of double distilled water immediately after casting; do not use butanol.
The gel detaches itself from the support film.	The wrong support film or the wrong side of the support film were used or else the support film was stored incorrectly.	See under polyacrylamide gels.

Effects during washing:

Symptom	**Cause**	**Remedy**
The gel has a “snake skin” structure in certain areas or all over.	This is normal. Because of the fixed buffering groups the gel possesses slight ion-exchanger properties and swells.	The gel surface will become normal again when it is dry.
The gel becomes wedge shaped.	This is normal. The buffer has different concentrations and properties which results in different swelling characteristics within the gradient.	Dry the gel after washing; rehydrate it in the reswelling cassette (the cassette prevents it from taking a wedge shape) or in the GelPool with defined liquid volume.

Effects during drying:

The support film rolls up.	The gel pulls in one direction.	Add 1% to 2% of glycerol to the last washing, this makes the gel more elastic.

Effects during rehydration:

The gel does not swell or only partially.	The reswelling time is too short.	Adapt the reswelling time. If the gel was stored for a long time at room temperature or if the use-by date is expired, prolong the reswelling time.
	Gel was dried too long or at too high a temperature.	Dry the gel with a fan at room temperature, the air-flow should be parallel to the gel surface and the gel should be dried in a dust free atmosphere

	The gel was stored too long at room temperature or higher.	Use the gel immediately after drying or store it hermetically sealed at <– 20 °C.
The gel sticks to the reswelling cassette.	The surface of the glass is too hydrophilic.	Coat the surface of the gel within the gasket with Repel Silane.
The gel sticks to the support glass plate.	The gel surface was by a mistake applied on the wet glass plate.	Pull it away gently under water in a basin.
Effects during the IEF run:		
No current.	The cable is not plugged in.	Check the plug; insert the plug more securely in the power supply.
Low current.	This is normal for IPG. The gels have a very low conductivity.	Standard setting for whole IPG gels: 3500 V, 1.0 mA, 5.0 W. Regulate IPG strips with the voltage setting.
Localized condensation over specific areas.	Salt concentration in the samples is too high.Salt ions form arcs when leaving the sample wells, spots with very high salt concentration result where two fronts meet.	Apply samples with high salt concentration close to one another, if samples must be applied at different areas within the pH gradient. Separate the traces by cutting strips or scraping out troughs.
Local sparking at specific points.	See above; next stage.	See above; if focusing is carried out overnight, do not apply more than 2500 V and turn up to 3500 V the next day.
Sparking along the edge of the gel.	High voltage and there are ions in the contact fluid.	Use kerosene as contact fluid between the cooling plate and the film.
Sparking at an electrode.	The gel has dried out because of EEO. This occurs in narrow pH gradients at extreme pH intervals (<pH 4.5; > pH 9).	Either add glycerol (25%) or 0.5% non-ionic detergent to the reswelling solution.
	The electrode solutions are too concentrated.	Soak both electrode strips in double-distilled water. The conductivity is sufficient; in addition, the field strength decreases at the beginning of IEF for

improved sample	entrance .	
	Gel insufficiently polymerized.	See above.
A narrow ridge develops over the whole width of the gel and slowly migrates in direction of an electrode.	This is a normal phenomenon during IPG: it is an ion front at which a jump in the ionic strength and a reversal of the electroendosmotic effect occur.	Wash the gel thoroughly. Apply the sample so that the front comes from the furthest electrode. Add 2 mmol/L acetic acid to the reswelling solution for samples applied at the anode and 2 mmol/L Tris to the samples applied at the cathode.
The ridge does not migrate any further.	The gel contains too many free ions, the difference in conductivity within the gel is so large that the voltage is not sufficient to carry the ions further.	Wash the gel thoroughly. Use a power supply with a high voltage (3500 V are sufficient). Focus for a long time, overnight if necessary.

Separation results:

Symptom	**Cause**	**Remedy**
The bands and iso-pH lines are curved.	The gel polymerized before the radient had finished leveling.	Cool the casting cassette in the refrigerator before casting (this delays the onset of the polymerization). Use glycerol and not sucrose (its viscosity is too high) to make the acid solution denser.
	The catalyst was not properly washed out.	See above
The bands are diffuse.	Focusing time too short.	Focus for a longer time, overnight for example.
	The field strength is not sufficient when the pH range is narrow or the separation distance is long (10 cm).	High voltages are necessary for narrow pH ranges and long separation distances: use a 3500 V power supply.
	There are problems with polymerization, for example the acrylamide	or Bis solutions are old; see above. Use fresh stock solutions.
The bands in the basic part of the gel are diffuse. Influence of CO_2.	Trap CO_2 during IEF: seal the chamber, add	soda lime or 1 mol/L NaOH to the buffer tanks.
No bands are visible. The pH gradient is wrongly orientated.	Place the gel on the cooling plate with the acid side towards the anode and the basic side	towards the cathode; the basic side has an irregular edge and the support film sticks out.

The proteins have stayed at the site of application. The field strength was too high at first.	Do not prefocus (the pH gradient already exists). Keep the field strength low at the beginning.	
	The proteins have aggregated at the site of application because their concentration was too high.	Dilute the sample with water or water/non-ionic detergent; it is preferable to apply a large sample volume than a concentrated solution.
	Some proteins have formed complexes and obstructed the pores.	Add EDTA to the sample. Add urea to the sample and rehydration solution; complex formation is prevented by a urea concentration of 4 mol/L but most enzymes are not denatured yet.
Conductivity problems.	Apply the sample to the other side, or direct the	ionic front as described above.
	The salt concentration in the sample is too high.	Dilute the sample with water and apply a larger sample volume.
	High molecular weight proteins are unstable when the ionic strength is low.	Prepare a gel matrix with large pores so that the protein can penetrate the gel before it has completely separated from the low molecular substances.

As emergency measure it is recommended to add 0.8% (w/v) carrier ampholyte to the sample and 0.5% (w/v) carrier ampholyte from the corresponding pH range to the rehydration solution.

The pI of the proteins lies outside of the gel.	Narrow pH range: the focusing was carried out at the wrong temperature.	Focus at 10 °C and/or widen the pH range.
The pI obtained by carrier ampholyte IEF is shifted in comparison to the one obtained by IPG.	Use a wider or different pH range.	The immobilized pH range is not correct or not present.
	Immobiline was not stored correctly. The acrylamide or Bis solutions are too old. Pipetting mistake.	Follow the recipes for Immobiline and casting instructions exactly; otherwise: see above.
	The focusing time is not sufficient.	Lengthen the focusing time, if necessary focus overnight.

Some bands are missing, are diffuse or are at the wrong place.	Oxygen sensitive proteins have oxidized in the gel (Immobiline gels trap oxygen from the air during drying).	Add a reducing agent to the rehydration solution when working with proteins which are sensitive to oxygen.
The separation lanes are curved and run from one another.	The conductivity of the gel is much lower than the conductivity of the sample (proteins, buffer, salts).	Direct the ionic front as described above; apply the samples beside one another; separate the lanes by cutting the gel or scraping out troughs.

Specific staining problems with IPG:

Symptom	**Cause**	**Remedy**
There is a blue background after Coomassie staining.	Basic Immobiline groups tend to bind Coomassie. Use a solution with 0.5% Coomassie; or, even	better, use colloidal staining: no background staining!

Abbreviations

APS	ammonium persulfate
Bis	NN' methylen bisacrylamide
DMSO	dimethylyulfoxide
EEO	electroendosmosis
IEF	isoelectric focusing
IPG	immobilised pH gradients
PMSF	phenylmethyl-sulfonyl fluoride
TCA	trichloro acetic acid