



General Protocol for 2D-PAGE (24 cm IPG 4-7, 12% SDS-PAGE)

Sample preparation and in-gel rehydration

Protein sample lysates (created following the protocol on www.fixingproteomics.org) can be used directly for IEF or can be stored at -80 °C. When stored at -80 °C start with thawing the samples at RT, do not heat. Occasionally, some apparently insoluble material is present at the bottom of the tube after thawing. This will dissolve quickly once the sample is vortexed at RT.

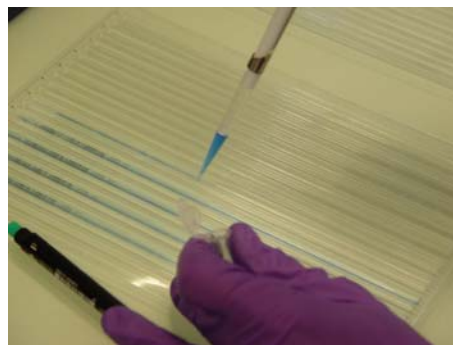
Vortex the thawed samples and dilute an amount corresponding to about 350 µg protein (usually 30-50 µL sample) to 480 µL with the standard lysis buffer according to Rabilloud. Mix thoroughly and centrifuge for 5 min at 13'000 g (max speed Eppendorf).

TIP

The optimal sample loading amounts vary depending on the sample and obviously on the staining. For DIGE one can load as little as 40 µg per sample, around 350 µg is required for the fluorescent dyes and for Coomassie we use 500 - 600 µg. All these amounts apply to a 1.5 mm thick gel of roughly 20 x 25 cm. For smaller and/or thinner gels less sample can be used.

Samples in DIGE buffer do not contain DTT or Pharmalytes. As soon as the amount of DIGE buffer exceeds 10% of the total sample volume, we add 2% Pharmalytes 3-10 (1 µl per 50 µl of DIGE buffer) and 1% DTT from a 20% stock in Rabilloud (2.5 µl per 50 µl of DIGE buffer) to keep the concentrations constant at 2% and 1%, respectively. See also sample preparation protocol for details on the buffers.

For each 1st dimension IPG strip, distribute 480 µL of the sample into a slot of the rehydration tray and apply the 24 cm pH 4-7 IPG strip on top. Passive in-gel rehydration of the strips should take at least 6 hours, but recommended and most practical is rehydration overnight at room temperature.

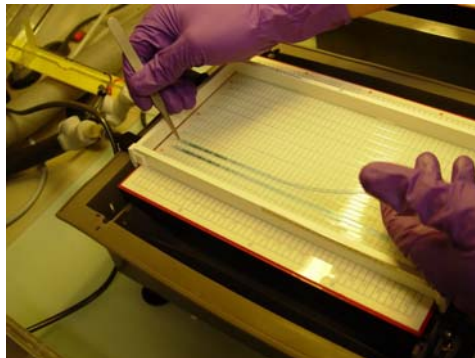


Running the first dimension

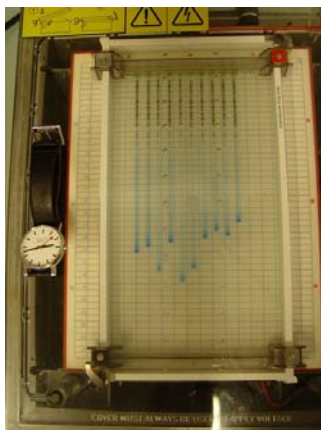
For running the IPG strips, we use the voltage gradient below on our favorite instrument, the Multiphor. For the pH 4-7 gradient, we tend to avoid higher voltages even on other instruments like IEF Cell or IPGPhor. For the pH 4-7 gradient (24 cm) we find that a total of 70-80 kVh running 'time' is optimal. Be aware that that can be quite different for other gradients and certainly for other strip lengths.

TIP

IEF should always be started at low voltage in order to remove salts etc. It is necessary to use paper wicks at the electrodes to collect proteins that are outside the pH range of interest. The paper wicks function as desalting tool as well; it is possible to exchange the paper wicks during the IEF. The paraffin oil prevents drying out of the strip and crystallization of the urea/thiourea present.



Voltage gradient: 3 h 300 V. 5h: linear gradient 300-3500 V. 18 h at 3500 V.

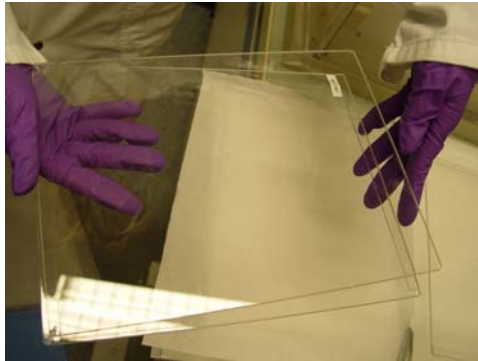


Uneven focusing is observed at the early stage, even when using same strips with same sample. This is normal and nothing to worry about

Casting gels for the second dimension

In labs with a medium to high throughput, electrophoresis chambers holding 10-12 large SDS-PAGE gels are used, such as the Dodeca system (or similar systems currently on the market) allowing a relatively large batch of slab gels to be run under identical conditions. Home-made gels are commonly used, but some ready-made gels are available on the market.

In the typical casting chamber 12-24 slab gels are prepared. The glass cassettes (with fixed spacers – either 1.0 mm or 1.5 mm - and a hinge) are assembled in the chamber and separated by a plastic sheet to prevent the plates sticking to each other. Each gel is given a unique serial identity number by placing a piece of printed Whatman paper between the glass plates prior to casting.



In a vacuum flask is prepared 920 mL ProtoGel (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide solution (37.5:1)), 575 mL 1.5 M Tris-HCl, pH 8.8 and 770 mL water. The mixture is degassed for 10 minutes before the SDS (2.3 g in 35 mL water) and catalysts (700 mg ammonium persulfate (APS) and 300 μ L TEMED) are added.





The gel casting chamber is filled from the bottom to a height of about 2 cm below the top of the glass plates. The gels are carefully overlaid with 1.0-1.5 mL buffer-saturated 2-butanol to allow for complete polymerization (at least one hour). For buffer-saturated 2-butanol, fill 100 ml gel buffer and 400 ml butanol in a flask and mix by inverting. After a little while the two phases will separate again, the butanol on top is now saturated. Gels can be stored up to one week at 4 °C.

TIP

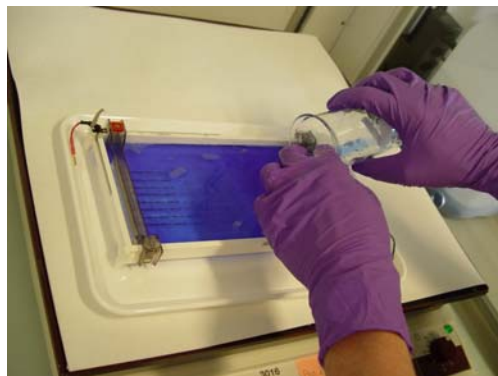
Casting a large number of gels requires some practice. The amount of catalyst that is used is minimal to prevent the gels from polymerization too rapidly, which leads to excessive heating of the casting chamber. Ideally, initial polymerization, which can be seen by the development of a distinct gel surface below the butanol layer, should take 30 – 60 minutes. Subsequently, gels can be washed, covered with gel buffer and stored at room temperature. During overnight residual polymerization will take place. The amounts of catalyst can be increased by 10% if necessary.

Equilibration of focused IPG strips

After completion of the IEF, remove strips from the IEF instrument and equilibrate 12-15 min with equilibration buffer (see below) to which DTT has been added to a final concentration of 2%, and then 6-15 min with equilibration buffer to which iodoacetamide has been added to a final concentration of 5%.

TIP

The two equilibration steps can be performed in the strip holder tray, without any strip handling. This avoids contamination or any other disruption of the strips. In our hands it is currently the best possible way for medium to high-throughput running of 2-D gels.

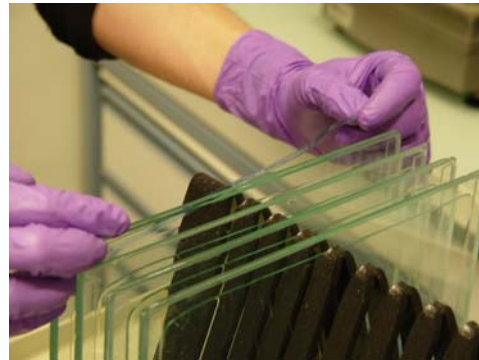
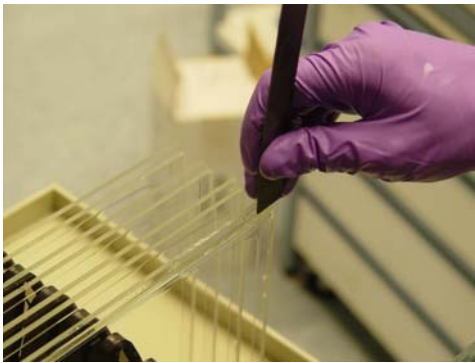


Transfer of IPG strips to SDS-PAGE gels

In order to ensure good contact between the strip and the gel an agarose solution is added. The agarose not only keeps the IPG strip in place, but also ensures good electrophoretic transfer between the IPG strip and the gel. The agarose solution is kept at 70 °C and added first on top of the gel. Immediately after, the equilibrated strip is placed on the gel.

TIP

The agarose overlay is prepared in such a way that it remains fluid at relative low temperatures. In order to realize this a mixture of low melting agaroses is used (0.4% (w/v) standard low Mr from Bio-Rad Laboratories (162-0100) and 0.1% (w/v) type VII-A-low gelling temperature from Sigma (A0701), dissolved in Running Buffer, see below). A trace of bromophenol blue can be added as tracking dye.



If you are using a large tank it is important to fill it with the appropriate volume (up to 23 liters!) of Running Buffer beforehand and start the cooling at 15 °C. It will take a few hours until the temperature is reached.

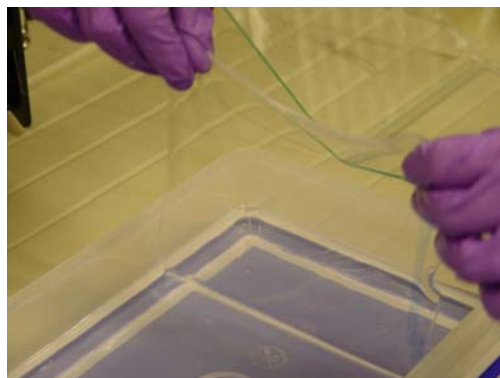
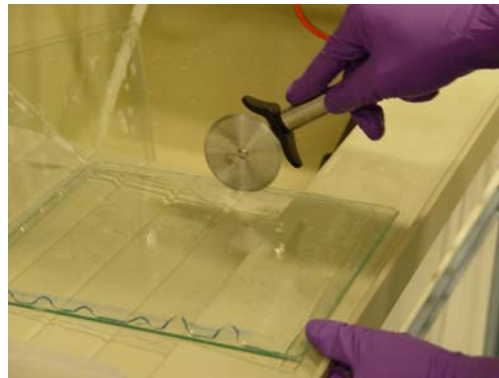
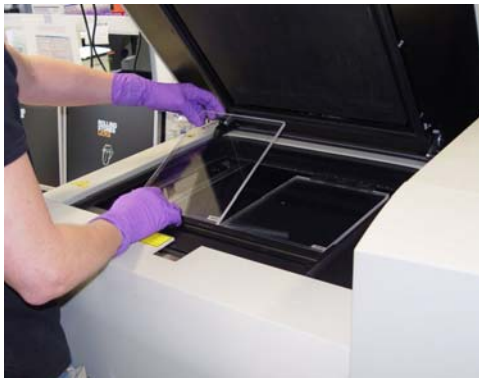


Running the second dimension

The preferred gel height for the 2nd dimension is 20 cm. The second dimension should be run overnight at 15 °C on 1.5 mm thick Tris-glycine/SDS gels with 12 % T, 2.6 % C in a Laemmli Tris/Glycine/SDS running buffer (detailed composition see below). Running conditions are 1 h at 5 – 10 mA constant current per gel followed by 16 mA per gel until the bromophenol dye front reaches the end of the gel. In our Dodeca system this second phase usually takes around 15 h.

Detecting proteins – Staining the gels

After electrophoresis is completed the gels can be taken out of the glass plates and processed for staining. In case of DIGE, gels can be scanned directly within the low fluorescence glass plates.



Handling the gels prior to staining or in the case of DIGE gels, directly scanning them within the low fluorescence glass plates

Staining with the fluorescent dye *SYPRO* Ruby (Invitrogen)

Step	Solutions	Time
1. Fixing ¹	40% (v/v) ethanol, 10% (v/v) acetic acid	3 hours
2. Washing	Distilled water	3 x 30 minutes
3. Staining	<i>SYPRO</i> Ruby ready-to-use solution (150 mL/gel)	O/N
4. Washing	Distilled water	2 x 30 minutes

Staining with the fluorescent dye Flamingo (Bio-Rad)

Step	Solutions	Time
1. Fixing	40% (v/v) ethanol, 10% (v/v) acetic acid	3 hours
2. Washing	Distilled water	3 x 30 minutes
3. Staining	Flamingo 10x diluted (150 mL/gel)	3 hours – O/N
4. Washing	Distilled water	2 x 15 minutes

Staining with colloidal Coomassie Blue

Step	Solutions	Time
1. Fixing	50% (v/v) ethanol, 3% (v/v) phosphoric acid	3 hours
2. Washing	Distilled water	3 x 30 minutes
3. Staining – first step	34% (v/v) methanol, 3% (v/v) phosphoric acid, 17% (w/v) ammonium sulfate	1 hour
4. Staining – second step ²	Coomassie Blue G-250 (350 mg/L into solution 3)	1 – 5 days ³
5. Washing	Distilled water	3 x 30 minutes ⁴

Staining with MS compatible silver

Step	Solutions	Time
1. Fixing	40% (v/v) ethanol, 10% (v/v) acetic acid	3 hours
2. Washing	30% (v/v) ethanol	2 x 20 minutes
3. Washing	Distilled water	20 minutes
4. Sensitizing	0.02% (w/v) sodiumthiosulfate	3 minutes
5. Washing	Distilled water	3 x 1 minute
6. Staining	0.2% (w/v) silvernitrate	30-60 minutes

7. Washing	Distilled water	3 x 1 minute
8. Developing ⁵	3% (w/v) sodium carbonate, 0.05% (v/v) formaldehyde solution (37%)	2 x 5 – 10 minutes
9. Washing	Distilled water	1 minute
10. Stopping ⁶	1.5% (w/v) Na ₂ EDTA	10 minutes
11. Washing	Distilled water	3 x 10 minutes

Notes

General: in all steps we use 5 – 6 gels per plastic tray in a volume of approximately 1 liter, except during development in silver staining (gels are developed one by one).

¹ Fixing times are minimal for large format gels (20 x 25 cm). Gels can be left overnight in fixing solution. Up to 5 – 6 gels can be processed simultaneously during the staining procedure.

² Coomassie Blue G-250 (also available as Serva Blue G-250) is added as solid to the solution and forms immediately small colloidal particles. The gel boxes can be sealed with tape to prevent evaporation.

³ After overnight staining spots will be visible, but complete end-point staining will be reached after 4-5 days.

⁴ The gels are sufficiently washed when no solid Coomassie particles are present anymore.

⁵ During the developing process it is best to limit the number of gels at one during processing. As soon as the developing solution becomes yellow it should be replaced with fresh solution. Development time is experimentally determined, the background should not be too high.

⁶ Stopping the development in a Na₂EDTA solution prevents the gel spots from bleaching from brown/black into yellow.

Reagents:

Reagent	Composition
DIGE buffer	30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, pH 8.5
Rabilloud buffer	7 M urea, 2 M thiourea, 1% DTT, 4% (w/v) CHAPS, 2% Pharmalytes 3-10, + a trace of bromophenolblue
Equilibration buffer	50 mM Tris, pH 8.8, 6 M urea, 2% SDS, 30% Glycerol, 0.01 % Bromophenolblue.
Laemmli system	
Gel composition	12% acrylamide (from a 30:0.8 acrylamide/bisacrylamide stock), 0.375 M Tris pH 8.8, 0.1% SDS
Running buffer	25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3. Almost always prepared from a commercial 10x solution. Do not adjust pH with acid or base, in doubt just check that it is in the right range

The final result!

