

Buffer Recipes For 2DE

- Always use electrophoresis/molecular biology quality reagents or higher
- Always use 18.2 ohm water quality or higher (MilliQ or bottled)
- As indicated in individual buffers, some reagents have to be made fresh while others can be stored at -20°C . As a rule freshly made buffers are better, but time constraints may necessitate making up stock solutions of some reagents. If you do freeze reagents, aliquot the solutions into small amounts so that they aren't repeatedly freeze-thawed

Sample application method selection

Sample can be applied either by including it in the rehydration solution or by applying it directly to the rehydrated IPG strip via sample cups or sample wells. It is usually preferable to load the sample onto the IPG strip by including the sample in the rehydration solution. Advantages to this mode of application include the following:

- This method allows larger quantities of protein to be loaded and separated.
- This method allows more dilute samples to be loaded.
- Because there is no discrete application point, this method eliminates the formation of precipitates at the application point that often occur when loading with sample cups.
- This method is technically simpler, avoiding problems of leakage that can occur when using sample cups.

There are, however, cases when one might prefer to load the sample following rehydration, immediately prior to IEF:

- If proteolysis or other protein modifications are a concern, overnight rehydration with sample may not be desired.
- Better results are often obtained on pH 6–11 L IPG strips (AP biotech) when the sample is applied using a cup loading apparatus.

Rehydration solution preparation

- Just prior to use, slowly thaw the appropriate volume of stock solution. Add the appropriate amount of IPG Buffer, if it is not already included in the rehydration stock solution.
- Add sample. The rehydration stock can be diluted no more than 1/8 by sample addition [eg. 62 μl of sample for each 0.5 ml rehydration stock]. The amount of protein that can be added is dependent upon the length of strip, the pH range and the detection method to be used. Up to 1 mg can be added to a wide pH range 17 cm strip.
- *Note:* DTT and the sample must be added fresh, just prior to use.

Strip length (cm)	7	11	13	18
total volume per strip ¹ (ul) [min;max] ²	125;200	200;280	250;350	350;500

¹ this volume includes the sample

² The minimum volume rehydrates the strip to a gel thickness of 0.5 mm and a gel composition of 4%T/3%C. The maximum volume allows for larger protein sample loads, facilitates entry of larger proteins and minimizes protein solubility problems.

For most samples it is recommended to stay as close to the lower volume limit as possible.

Standard Rehydration Solution for IEF

8M Urea/ 4% CHAPS/ 50mM DTT or 2mM TBP/ 0.2% carrier ampholytes/ 0.0002% Bromophenol Blue

- This can be frozen without reductant (DTT or TBP) and carrier ampholytes. DTT and ampholytes should be added just before use
- Variations of detergent concentrations are common depending on your protein but should total 3-4%. Detergents must have no net charge for effective IEF. Common alternate detergents are Triton X-100, CHAPSO, SB 3-10, ASB-14, and/or octylglucoside.
- This will create a saturated solution. When preparing, allow several hours to slowly dissolve each component. The solution can be gently heated to dissolve, but must not be heated above 30C to prevent urea breakdown
- For basic proteins, it may help to add up to 10% isopropanol or glycerol to the solution

Enhanced Rehydration Solution

7M Urea/ 2M Thiourea/ 2% CHAPS/ 1% ASB-14/ 0.2% carrier ampholytes/ 0.0002% Bromophenol Blue

- As above
- A more recent recipe development suitable for membrane preparations and other potentially hydrophobic samples

Boiling Lysis Buffer

60mM Tris pH 6.8/ 2% SDS/ 10% glycerol

- An aggressive solubilization solution to dissolve difficult samples such as Gram negative bacteria and yeast. Preheat buffer to 95C and incubate in a heating block with sample for 5 minutes.
- Can be pre-made and stored at -20C indefinitely.
- This must be diluted with rehydration buffer after heating and cooling the sample. The final concentration of SDS in the IEF sample must be less than 0.2% for focusing to be effective. SDS will migrate out of the IEF strip so it is important that at least a ten fold excess of rehydration detergents are available to prevent protein precipitation during the first dimension.
- Hot SDS will inactivate most proteases, so it is generally not necessary to add protease inhibitors when using this solution.

After the sample has been dissolved in rehydration buffer it should be incubated at 30C for 1 hour to allow for complete sample reduction. The sample is then alkylated by the addition of iodoacetamide to a concentration of 2.5X the amount of reductant added. This mixture is allowed to incubate at 30C for 4 hours. The sample is then centrifuged at 21,000rcf for 15 minutes to remove any particulate prior to loading on the IPG strips

IPG strip rehydration

A - Prepare the focusing tray.

Select the focusing tray corresponding to the IPG strip length chosen for the experiment.

Important: Handle the trays with care, as the wires are delicate.

Wash each holder with detergent and the soft nylon brushes to remove residual protein. Rinse thoroughly with double distilled water. Use a cotton swab or a lint-free tissue to dry the holder or allow it to air-dry. Handle clean holders with gloves to avoid contamination.

Note: The holder must be completely dry before use.

B - Apply the rehydration solution.

Pipette the appropriate volume of rehydration solution into each well of the focusing tray. Deliver the solution slowly at a central point in the well. Remove any larger bubbles.

Important: To ensure complete sample uptake, do not apply excess rehydration solution.

C - Place the IPG strip.

Remove the protective cover from the IPG strip. Position the IPG strip with the gel side down and oriented the correct way (for AP strips the pointed end of the strip is the anode; for Bio-Rad strips the end with writing is the cathode). Lower the IPG strip onto the solution holding one end with tweezers. To help coat the entire strip, gently lift and lower the strip and slide it back and forth along the surface of the solution, tilting the strip holder slightly as needed to ensure complete and even wetting. Finally, lower the other

end of the IPG strip into the channel, making sure that the gel contacts the strip holder electrodes at each end. (The gel can be visually identified once the rehydration solution begins to dye the gel.) Be careful not to trap bubbles under the IPG strip.

D - Apply IPG Cover Fluid.

Apply IPG Cover Fluid (mineral oil) to minimize evaporation and urea crystallization. Pipette the fluid dropwise into one end of the well until one-half of the IPG strip is covered. Then pipette the fluid dropwise into the other end of the well, adding fluid until the entire IPG strip is covered.

E - Place the cover on the focusing tray.

Pressure blocks on the underside of the cover ensure that the IPG strip maintains good contact with the electrodes as the gel swells.

F - Allow the IPG strip to rehydrate.

Rehydration is usually done in the Protean IEF unit as a separate program or as the first part of a focusing run. This allows temperature control during the rehydration. Ensure that the holder is on a level surface. A minimum of 10 hours is required for rehydration; overnight is recommended. Note: do not rehydrate or run below 20°C if urea is present in the rehydration buffer.

G – Alternate Procedure If Using Disposable Rehydration Trays With Passive Rehydration

Follow steps A to C as above using the appropriately sized tray. Do not overlay the strips with mineral oil. Instead, seal the completed tray with the cover on in a ziplock bag with a wet paper towel. The humidity in the bag will prevent urea crystallization while improving the entry of hydrophobic proteins into the gel. Allow the strips to rehydrate for 21-24 hours on the bench at 20C. Rinse the IPG strips quickly with distilled water and gently blot off the excess liquid prior to the IEF. This removes any urea that may have crystallized on the surface of the strip.

Apply electrode pads.

This is strongly recommended and is done between the rehydration and focusing steps.

Under certain conditions, such as prolonged focusing, water may migrate toward one end of the IPG strip, causing the other end to begin drying out. This effect can be minimized by placing paper electrode pads between the IPG strip and each strip holder

electrode just before IEF. Electrode pads may also absorb ions that would otherwise accumulate at the ends of the IPG strip and possibly interfere with the separation.

A - Prepare electrode pads.

Cut two 3-mm-wide by 10-mm-long electrode pads for each IEF strip. Soak with deionized water briefly and remove excess water by blotting with filter paper.

Important: Electrode pads must be damp, not wet.

B - Position electrode pads.

Remove cover from focusing unit. Using forceps or tweezers, lift one end of the rehydrated IPG strip. Position an electrode pad over the electrode, then lower the IPG strip back into place. Repeat at the other end. Replace cover on strip holder.

C – Changing electrode pads.

Pause the focusing periodically during the first dimension run (recommended interval once per hour for the first four hours). Carefully remove the old pads and replace with new damp electrode wicks. This should remove contaminating ionic species, which accumulate at the anodic end of the strip and can cause streaking at low pH ranges.

IPG strip washing and storage

A- Washing.

Fill the wells of a rehydration tray with 1X SDS running buffer or other equivalent buffer. Place the strips, gel side up, into the tray and with tweezers gently move the strips back and forth in the buffer. Transfer to a fresh well and repeat until no visible sign of oil is present on the surface of the buffer [6-8 washings].

Note: the washing prevents streaks in the second dimension gel.

B - Storage.

Washed strips should be stored in a dry rehydration tray at -80°C.

Equilibration steps

Note: The second-dimension gel must be ready for use prior to IPG strip equilibration.

A - Prepare equilibration solution.

Prepare SDS equilibration buffer. This is a stock solution. Just prior to use, add 100 mg DTT **or** 250 mg iodoacetamide per 10 ml SDS equilibration buffer for the reduction or alkylation steps as appropriate.

B – Equilibration and Reduction

Place the IPG strips in individual tubes with the support film toward the tube wall (screw-cap culture tubes work well). Add DTT-containing equilibration solution (100 mg per 10 ml solution) to each tube. Suggested volumes are 10 ml for 18 cm IPG strips, 5–10 ml for 11 cm or 13 cm IPG strips, and 2.5–5 ml for 7 cm IPG strips. Cap the tube or seal it with flexible paraffin film and place it on its side on a rocker. Equilibrate for 15 minutes.

C - Second Equilibration and Alkylation

A second equilibration should be performed with an iodoacetamide solution (without DTT). Prepare a solution of 250 mg iodoacetamide per 10 ml SDS equilibration buffer. Decant the first equilibration solution and add iodoacetamide-containing equilibration solution to each tube. Volumes and times are as in part B.

Note: This second equilibration step reduces point streaking and other artifacts.

D - Drain moisture from IPG strips.

After equilibration place the IPG strips on filter paper moistened with deionized water. To help drain the equilibration solution, place the IPG strips so that they rest on an edge. IPG strips can be left in this position for up to 10 minutes without noticeably affecting the spot sharpness. Alternatively, the IPG strips can be rinsed with SDS running buffer and gently blotted with moistened filter paper to remove excess equilibration buffer.

SDS equilibration buffer¹

Component	Final concentration	Amount
1.5 M Tris-Cl, pH 8.8	50 mM	6.7 ml
Urea (FW 60.06)	6 M	72.07 g
Glycerol (87% v/v)	30% (v/v)	69 ml
SDS (FW 288.38)	2% (w/v)	4.0 g
Bromophenol blue	trace	(a few grains)
Double distilled H ₂ O		to 200 ml

(50 mM Tris-Cl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 200 ml)

Store in 40 ml aliquots at –20 °C.

¹ This is a stock solution. Prior to use DTT or Iodoacetamide is added.

Loading strips onto second dimension

Prepare second dimension gels as required. Typically the percentage of acrylamide will be 10 or 12% for MW ranges of 20 to 200kDa. When pouring large format gels it is important to degas the monomer before adding the catalyst.

Separating Gels, in 0.375 M Tris, pH 8.8				
	7%	10%	12%	15%
distilled H₂O	5.1 ml	4.1 ml	3.4 ml	2.4 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
20% (w/v) SDS	0.05 ml	0.05 ml	0.05 ml	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2.3 ml	3.3 ml	4.0 ml	5.0 ml
10% (w/v) ammonium persulfate	0.05 ml	0.05 ml	0.05 ml	0.05 ml
TEMED	0.005 ml	0.005 ml	0.005 ml	0.005 ml
Total monomer	10.005 ml	10.005 ml	10.005 ml	10.005 ml

Ammonium persulfate must be made fresh as it decomposes in water. The other solutions can be pre-made and stored at 4C.

Pour the gels to within 1 cm of the top of the glass plate. A stacking gel isn't necessary unless liquid protein marker or sample is being added.

Wet the plastic back of the IPG strip with running buffer so that it slides easily on the glass. Place the equilibrated strip with the plastic backing on the glass and gently push the strip down onto the gel surface. It is important not to compress the soft acrylamide strip against the opposite side of the glass, which will interfere with the protein transfer. Ensure that there are no air bubbles between the IPG strip and the resolving gel interface. Add a molecular weight marker that has been blotted onto a small piece of filter paper if desired. Seal the strip and marker in place by overlaying with 1% agarose.

Once the overlay has solidified, place the gels in a buffer tank pre-cooled to 10C. The running buffer is 25mM Tris/ 192mM Glycine/ 0.1% SDS pH 8.3 for both the upper and lower reservoirs.

Always use fresh upper reservoir buffer (lower buffer can be re-used until gels start to run poorly). Run the gels at 24mA/gel for 6 hours or until the dye front just migrates off the bottom of the gel. Alternately, the gels may be run at 18mA/gel overnight (run time approximately 16 hours for large format gels). When the electrophoresis is finished,

open the gel cassette and remove the IPG strip from the top of the gel. Place the resolving gel in fixative or stain as required.

Contamination is a major issue in 2D electrophoresis. All equipment should be scrupulously cleaned with a high quality detergent and rinsed twice with MilliQ water immediately before use. Always wear clean powder free gloves when performing any manipulation. Ideally, gels should be run in a low traffic clean environment to minimize dust contamination.

The following websites are useful for both theory and technical procedures involving two dimensional electrophoresis:

[http://www5.amershambiosciences.com/applic/upp00738.nsf/vLookupDoc/172581038-R140/\\$file/80-6429-60AB_Version_May_2002.pdf](http://www5.amershambiosciences.com/applic/upp00738.nsf/vLookupDoc/172581038-R140/$file/80-6429-60AB_Version_May_2002.pdf)

http://www.bio-rad.com/LifeScience/pdf/Bulletin_2651.pdf

<http://proteomics.cancer.dk/procedures/procedure.html>

http://www.aber.ac.uk/parasitology/Proteome/Tut_2D.html