TMM

Two-Dimensional Gel Electrophoresis

There are 2 variants to run first dimension gels: Isoelectric focusing (IEF, O'Farrell, 1975) for neutral to acidic polypeptides and non-equilibrium pH gradient electrophoresis (NEpHGE, O'Farrell, 1977) for neutral to basic polypeptides. With IEF, the exact isolelectric point of a protein can be determined; with NEpHGE, this is not possible. Separation in second dimension is according to Laemmli or other systems.

1. Preparation of glass tubes for first dimension gels

Wash carefully and rinse finally in ethanol/ether. Treat with Repelcoat, rinse briefly with water, dry. Seal lower end with Nesco film (cut pieces 1 x 1 cm). Don't use tubes with broken edges, these will not seal.

2 A. Isoelectric Focusing (IEF)

Gel solution

5.7 g	Urea, ultrapure (final conc. 9.5 M),
1.33 ml	Acrylamide/Bis (final conc. 3.77 %, stock is made from 28.4 g A.amide and 1.6 g Bisacrylamide per 100 ml of water; filter sterile),
2 ml	10 % NP 40 (final conc. 2%; stock can be stored at 4°C for 1 month),
2 ml	water (high quality),

Dissolve by stirring (don't warm above 37 C!) and add:

0.2 ml Ampholines 4 - 6 (final conc. 0.8%)
0.2 ml Ampholines 5 - 7 (final conc. 0.8%)
0.1 ml Ampholines 2-11 (final conc. 0.4%)
10 µl 10 % APS (final conc. 0.1%)
10 µl TEMED (final conc. 0.07%)

Composition of ampholytes can be altered to achieve optimal separation in desired pH range. Using the mixture given above, proteins with an IP between 4 and appr. 6.8 can be resolved. It will be difficult to resolve proteins beyond pH 8.5.

Fill gel solution to same height in all glass tubes with long pasteur pipette, leaving appr. 6-8 mm to the top. This allows to load up to 150 µl sample. Remove air bubbles by tapping. Overlay gel carefully with 20 µl water. Stand tubes in vertical position during polymerisation. Polymerized gels can be stored dark for a week at room temperature. If urea cristallizes, discard.

IEF Sample buffer

9.5 M urea, ultrapure
2 % NP 40
10 mM DTT
0.8 % Ampholines 4 - 6
0.8 % Ampholines 5 - 7
0.4 % Ampholines 2 - 11
water up to 100 ml

Aliquot in 1 ml portions and freeze at -80°C. Don’t reuse after thawing.

Ampholine composition in gel and sample buffer should be identical (remember if altering ampholine composition). Sample buffer must be stored frozen in aliquots. Avoid warming above 37°C.

Overlay buffer for tube gels

This is required to overlay samples in tubes in order to avoid loss of sample and contact with electrophoresis buffer in upper reservoir.

4 M urea, ultrapure
0.4 % Ampholines 4 - 6
0.4 % Ampholines 5 - 7
water up to 100 ml

Equilibration buffer

This is required to equilibrate tube gels to the identical pH of upper gels (ie. pH 6.8) in second dimension:

60 mM Tris-Cl pH 6.8

2 %	SDS
10 mM DTT
10 %	Glycerol
water up to 100 ml

Can be stored for a few weeks in fridge. pH is absolutely critical and must be checked every time before use at RT!

2 B. Non-equilibrium pH gradient gradient electrophoresis (NEPHGE)

Gel composition is identical to IEF except that the amount of TEMED is doubled.

Sample buffer

9.5 M	urea, ultrapure
5 %	NP 40
10 mM DTT
2 %	Ampholines 2 - 11
water up to 100 ml
Store frozen at -80°C in 1 ml aliquots.

Overlay buffer

4 M urea, ultrapure
1 % Ampholines 2 - 11
water up to 100 ml

3. Sample preparation

Proteins to be separated in 2D gels should be free of salt (below 50 mM). Is this is not the case, precipitate the protein sample with MeOH/chloroform or acetone and dry according to protocol. The

sample is then dissolved directly in 20- 100 μl of IEF or NEpHGE sample buffer. That might take up to 30 min (don't warm up!).
If dealing with soluble proteins (eg. in vitro translation assays), use 5-10 μl of protein sample and add 10 x volumes of sample buffer. Some proteins form oligomers even at 8 M urea, therefore it might be necessary to add a few small crystals of urea to have a final concentration of 9.5 M urea.
If the protein prep. contains high MW DNA/RNA, DNA/RNAse treatment is essential for good separation of proteins. In this case, treat sample with 10 u ultrapure DNAse I for 5-10 min at 37°C (this requires 10 mM $MgCl_2$) or 10 μg RNAse. After nuclease treatment, the sample should be precipitated with MeOH/chloroform or acetone and dried according to protocol. Then, dissolve in IEF or NepHGE sample buffer.

Before loading the sample on tube gel, spin 1- 2 min at full speed (RT), transfer spnt to fresh tube. Add 2-3 μg of each of the three marker proteins: BSA (IP 6.4, Mr 66.500), actin (IP 5.4, Mr 42.000), PGK (IP 7.3, Mr 43.000) which are dissolved in IEF sample buffer as well. Marker proteins are prepared in IEF sample buffer at 1 mg/ml at must be checked by SDS page for appropriate concentration. Never ruin your sample by adding bad marker or by forgetting it!

4. Running 1st dimension gels

Remove Nesco film and make sure that gel reaches lower end of tube. This might be achieved by carefully pushing gel downwards using a syringe with yellow tip attached. Seal lower end with a gaze strip and fix with Nesco film or rubber ring, otherwise the gel will slip out of the tube. Wash gel surface twice with distilled water and dry with Kleenex. Mount tubes in chamber.
IEF: Lower reservoir: 10 mM phosphoric acid. Upper reservoir: 20 mM sodium hydroxide. Before loading samples, add 20 μl sample buffer and fill up to the top with NaOH from upper reservoir. Pre-run gels 15 min 200, 15 min 300 and 30 min 400 V. After pre-run, empty upper buffer chamber, remove overlay buffer, rinse surfaces twice with distilled water and dry completely (use paper)!
NEPHGE: Lower reservoir: 20 mM NaOH. Upper reservoir: 10 mM phosphoric acid. Cathode at the bottom and anode at the top (i.e. the reversal of IEF !). For NEPHGE, there is no pre-run.

After loading samples, overlay with 20 μl of appropriate overlay buffer and top up with NaOH (IEF) and phosphoric acid running buffer (NEPHGE), respectively.
Run IEF for 18 h at 400 V.
For NEPHGE, run 10 min at 200, 10 min at 300 and 4- 5 h at 400 V. To achieve optimal separation of basic proteins, shorter running times might be used (test).

At the end of the run, remove gels with water-filled syringe from tube into 9 cm petri dish. Remove all water and add 10 ml of equilibration buffer. Incubate for 30 min at RT. Remove equil. buffer.
Gels can be frozen in sealed dish or applied on top of SDS-PAGE.

5. Running 2nd dimension gels

2nd dimension gels have no pockets. Pour upper gel up to the very top. A molecular size marker can be run if necessary. In this case, apply a single "tooth" to one side of upper gel and apply marker as usual.
Tube gels have to be fixed to gel surface with agarose. Prepare 20 ml of 0.8 % agarose solution in equilibration buffer. Place tube gel on top of SDS- PAGE and fix with a few ml of hot agarose solution, avoiding air bubbles. Stand for a few minutes. Add running buffer and a drop of a 1 % Bromophenol Blue solution to upper buffer reservoir. Run at 20 mA while sample runs through upper gel and at 30- 45 mA **per gel** through lower gel.
Stain or blot gel.

References

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P Z O'Farrell, H M Goodman and P O'Farrell (1977) Cell 12, 133-1142.
M J Dunn and A H M Burghes (1983) Electrophoresis 4, 97-116 and 173-189.