Two-Dimensional PAGE Protocol

A. 1st Dimension

1. Preparing tubes:

a) Tubes (16 cm made from 1ml graduated long glass pipettes) are cleaned by rinsing well, air drying, and incubating in chromic acid cleaning solution O/N (at least).
b) Always wash all the tubes that are in the acid to prevent dealing with the chronic acid numerous times.
c) Lift tubes out of chromic acid and let acid drain off.
d) Then place in tap H2O in plastic container. Rinse with several changes of tap H_2O.
e) Wash with ddH20 + ethanol (squirt ethanol into tube+ then ddH2O; do 3-4 sets).
f) Dry with vacuum hose (in 2D gel drawer; put wide end on vacuum).
g) Parafilm any tubes not to be used. Big parafilm over both ends as a group. For one set of samples, use the same kind of tube. There are some with a wide end and some without. The wide end allows loading of more sample and changes the effective focusing gel length (effective length = noodle plus sample height).

2. Preparing Gel Solution:

a) Meanwhile mix gel solution in 25mL flask - to provide surface area for degassing as described below (for 12 gels, +1 or 2 extra; make 14 or 7 at a time usually):
5.5g crystalline (not beads) urea (UltraPure; Life Technologies, Gibco BRL, Cat# 5505UX; on chemical shelf)
2.0ml 10% NP40 (Fluka, BioChemika 74385; 10% stock is in refrigerator; concentrated **NP40** in cupboard under chemical hood)- mix before use
2.25ml ddH2O
1.0ml Acrylamide/Bis stock (see Acryl/Bis recipe)-should be made fresh or frozen in 1 ml aliquots ≤ 1 week.
150 λ Ampholine pH6-8 (Pharmacia #80-1125-93; in refrigerator door)*
400 λ Ampholine pH3.5-10 (Pharmacia #80-1125-87; in refrigerator door)*
*create a pH gradient; mark the amount used on the 2D Gel sheet for the experiment.

Ampholines used and their amounts can vary if you want a different gradient. Double ampholines gives a little more stable and higher capacity gradient, for ex.

b) Parafilm flask, put in 37°C water bath for **~10-15 min** to dissolve urea; swirl gently every 4-5 min. Excess swirling or too long incubation can cause crystallization due to evaporation.

c) De-gas for **7 min**. Make sure urea doesn't crystallize out of solution. If so, may have to add more water (200 λ), re-dissolve, then de-gas for 2-4 min.

3. Final preparation of tubes:

a) Mark off 12.5 cm from the very bottom of the tubes (bottom = narrow end). Line the tubes up and mark in group.
b) Parafilm bottom ends of 1st dim. tubes. Make 5-6 layers; push on the end to make seal well (parafilm will be translucent when sealed).

4. Pouring gels:

a) Thaw 8 M Urea in the 37°C bath.
b) Polymerize 1st dim gel solution with
7 λ TEMED - add first
15 λ 10%APS (use new aliquot)- thaw in 37°C bath
Quickly: Swirl flask without aerating solution.
Pour contents into back end of 12 ml plastic syringe (with long skinny plastic tube or needle at tip end).
Put plunger on and invert so air can be pushed out.
Pour gel tubes by placing skinny plastic tube to bottom, then push slowly at first until solution covers tip of syringe by about 1/2 inch then push short, quick stroke so air bubble at bottom is dislodged and goes to the top.
Pour to 12.5cm line while raising needle as fill**.** Keep needle under solution at all times so your don't aerate the solution.

c) After pouring gel, do not rinse the syringe before overlaying the urea; squirt out unused portion of gel mix and then take up 8 M of urea through needle to overlay with. Overlay with a drop of 8M urea using syringe. See bubbles rise 2-3 mm. Make sure urea gets under bubbles. Save the remaining, unused urea in -30° C freezer. Rinse syringe with ddH2O.

5. Checking gels: After **10 min** invert tubes to make sure they are polymerized. You must check after 10 min or else you won't be able to tell if gel is usable. Bubbles will rise to top of the gel when gel is inverted. Gel top should be flat. Let sit **1-2hr** before loading samples.

6**.** Prepare samples for 1st dimensional gels:
a) Thaw dilution buffer. Make sure urea contained in dilution buffer is well dissolved upon thawing and well mixed before adding to sample. Add enough dilution buffer to double the volume. If sample is an IP, add the minimum of 2x bead volume of dilution buffer, unless you are doing prep 2D gels and multiple extractions of the beads.
b) Tap to mix. At this point, you can freeze your samples.
c) Saturate samples with urea beads to fully denature proteins: Use curved spatula to add beads. Open all tubes first. Add 4-6 beads/50 λ. For larger volumes, add ≈ 6 beads to start and when mostly dissolved, add more. Vortex full speed with closed caps for ~ 5 sec. Vortex smoothly in center of vortex so don't foam. Do one sample, then let sit, while do others in sequence. Before repeat sequence, add more beads if former ones have dissolved. Usually it takes about 15 min to saturate 12 samples. When beads no longer shrink (dissolve) on vortexing, stop so don't supersaturate (vortexing heats the sample so too much vortexing without a break for that sample will supersaturate it and it will solidify at RT or when loading. Urea will increase total volume by up to 50%. After sample saturates, let sample sit at RT for 30-45 min.

7. Setting up gels in apparatus and loading samples:
a) Make buffers
Upper chamber: base (700ml NaOH: 7ml 2N NaOH stock up to 700ml ddH2O)
Bottom chamber: acid (400ml H3PO4: 4ml 1M H3PO4 stock up to 400ml)
b) Cut parafilm off bottom of tubes one by one and insert into the apparatus. Check for bubbles at the end. Bubbles at the end are ok only if very small so can fill with buffers. You should get no bubble if gels are poured correctly.

c) Put tube into stopper and then into apparatus with stopper. Wet tube with water to insert stopper easily. Then cover bottom end with piece of gauze held by rubber band (bands are cut from rubber tubing with scissors). Wet gauze. The gauze will prevent the noodle from coming out during the run.
d) Push stopper into apparatus so sticks, and then move on by one until all tubes are done.
e) Using a ddH_2O squirt bottle, gently squirt out any bubble in bottom of gels. Add acid buffer to bottom chamber. Then put gel apparatus tube holder into bottom chamber of apparatus and push stoppers in very tightly or they will leak.
f) Tap a crystal of CaO (Aldrich Chemical Co), to absorb CO_2 produced, into top chamber. Don't get any in the tubes themselves. Pour part of the base buffer into the top chamber; check for leakage by lifting the top chamber & feeling for wetness on tubes below stoppers. Then add the rest of the base buffer to the top chamber. Cover tubes well so a small leak won't lower top buffer below top of tubes while it runs O/N. Tube tops should be ~ 1 cm below the surface of buffer.
g) Remove bubbles from tops of tubes by sticking needle of syringe used for pouring the tube gels into the tops of the tubes until bubbles rise. Make sure not to damage top of tube gels.
h) Suck up base buffer with pouring syringe and blow out urea gently from top of gel tubes. Do not shoot buffer in between tube gel and glass wall of gel tube.
i) If loading an IP, get sample from under beads so you don't take up beads (keep tip against bottom of sample tube). Load sample keeping tip under sample (watch from side as load). Pull out of sample (just above) for loading last bit so if a bubble comes out it doesn't disturb the sample. Use long round tips to load samples. Cut ~1cm from the tip because they are too narrow.
j) Overlay the samples with ~10µl of overlay (dilution buffer diluted 1:2 with ddH2O, + a grain of bromphenyl blue). *You can use the same tip each time to load the overlay if you don't contaminate it.*

8. Power supply settings:
a) Use the power supplies that have a voltage/amperage limit feature.
b) Current = 0.25 mAmp per gel, constant amps eg. 4 samples: 1mAmp
c) Power setting = 100W; voltage setting should be 500V although it will start ~125V and so go up with time.
d) Run overnight (O/N)
Check to make sure the voltage reaches 500V in AM (should reach in a couple of hours).

B. **2nd Dimension- the next morning**

1. Initial Preparations: Turn up 1st dimensions to 1000V in AM (do not up the amperage limit). Let go for 3 hours. During this time, set up plates. Record the time you changed the voltage on the 2D gel sheet.

2. Prepare gel solutions: In Erlenmeyer flasks mix running gel and stacking gel solutions. (Recipes are posted above bench top.) Wait before adding APS and Temed. Record % acrylamide of gel on gel sheet. Choose flasks that are appropriate size, i.e., too small will affect de-gassing. (For example, use 125ml flask for 1 running gel and 50ml flask for 1 stacking gel).

3. Degas, etc:

a) Check oil in de-gasser pump. Degas running gel for **7 min** while you seal the plates.
b) Thaw 10% APS (ammonium persulfate) in water bath.
c) Mark 2.5cm from top center of eared plates.

4. Seal plates: with 1.3% agar (door of frig; microwave to melt with cap loosed or will explode; do not over-microwave) using Pasteur pipette pipette (If you make a new solution of agar, you must microwave to almost boiling, swirl to dissolve, and microwave again and swirl several times to completely dissolve the agar). Do not use agarose for sealing. Extrude bubbles from the pipette before applying agar. Apply on one side, bottom, then other side. Especially at bottom junctions of spacers.

5. Pour plugs:
a) Pull out 2ml of running gel solution from degassing gel solution per gel. Put in Falcon tube or small flask. *Continue to de-gas the rest of the running gel on ice if doing many gels so it won't polymerize too fast later when pour the gels.*
b) Add Temed (2λ/ml) and APS (8λ/ml).
c) Quickly swirl and pour. If you're preparing more than one slab gel, add 2ml at a time with a pipette into a corner so it goes down the inside of a side spacer. Use a pipette.
d) **Squeeze** bottom of the plates to release bubbles. Double check for slow streams; if so, give quick squeeze then leave alone. Don't try to get every bubble out. Move quickly because plug polymerizes fast.
e) Let plug sit for 4min; check to make sure it polymerized.

6. Pour running gels:
a) Add temed and APS (use chart for values).
b) Quickly swirl gel solution and pour down middle of tipped back plate up to the 2.5cm mark. *All the gels should be at the same level relative to the 2.5cm mark. This insures equal sized gels and thus allows comparison of proteins run on two different gels.*
c) Return flask to ice.
d) Overlay gels with ddH2O. Use long Pasteur pipette with drawn out end (in flame) so ddH_2O comes out slowly. Technique: Apply steady and constant stream of water, but not too much at one time or it will mix in with the gel mix.
e) Let gel polymerize for at least **45min**.

C. **Extruding 1st dimensions, equilibration, and sealing onto second dimensions.**
1. Cover bench area with upside down bench papers.

2. Set out reagents and supplies needed:
a) 15ml Falcon tubes (one for each tube gel); or 50ml Falcon tubes for prep 2D gels.
b) Rim Buffer: 0.1%SDS + ddH_2O; 4°C frig
c) 6 or 12 ml syringe
d) long rim needle (custom made by Vita Needle Co., Needham, MA 02192; 3 inches; 27 gauge)
e) clean plastic beaker

3. Prepare tubes for equilibration:
a) Label 15ml Falcon tubes for each gel and set in rack with loose caps.

b) Add 6ml Equilibration Buffer (stored at 4^oC) to each Falcon tube. Do this in the hood. Rinse pipette with cold H_2O before setting in the wash or it will stink up the room.

4. Remove tubes from gel apparatus:
a) Squirt H_2O on the bench top and anything used during this process because the noodles stick to dry surfaces. Make sure gloved hands are also wet.
b) Dump buffer from 1st dimension gel apparatus into sink.
c) Remove gauze and rubber bands and stoppers. Easy to do when wet.
d) Lay tubes in order--do not mix up (alternatively, do tubes one at a time after removing each from stopper, etc).

5. Rimming tube gels:
a) Fill syringe with rim buffer from back end; put plunger on end and invert; push out air.
b) Want bevel (slanted part of tip) of needle facing gel when rimming.
c) Rim over clean plastic beaker (in case gel slips out into beaker while running; if beaker is not clean, can get proteolysis). Dump noodle into labeled 15mL Falcon tube after pull out needle. If dump before, needle may retain other end and gel will break.
d) When rimming using the long rimming needle, push the buffer out constantly while inserting the needle in top end of tube. Constantly roll tube back and forth about a quarter turn as insert needle all the way in (eventually). If at anytime you insert or pull out without pushing rim buffer out, you may tear the gel. After rim top half of gel, insert in bottom and rim other half until whole tube gel is freed. Hold tube horizontally while rim.

6. Equilibration: Place Falcon tubes with noodles on shaker. Let equilibrate while shaking gently for 30-45min. More time would result in proteins diffusing out of the noodle.
Note: If can't run all 2nd dimensions on this day, you can freeze the noodles as follows:
a) Equilibrate for only 20min in 1ml 100%glycerol + 6ml equilibration buffer.
b) Freeze at -20°C with Falcon tubes almost on their sides but noodles not touching cap).
c) When ready to run on 2nd dimension, thaw completely without moving (float in cold water) so there are no ice crystals when start shaking or will break the tube gel when shake. When thawed, equilibrate for another 20 min in the same equilibration buffer before loading onto 2nd dimension.

7. Pouring stacking gel:
a) De-gas stacking gel for 5-7 min while 1st dimension gels are equilibrating.
b) Add Temed and 10% APS and swirl to mix.
c) Pour off the ddH20 from step 6.
d) Pour. Ok if stack overflows; let it brim over.
e) Overlay with brisk sweep of water.
f) Let stack polymerize for 15 min to 45 min.

8. Sealing:
a) Mark plates with gel number from 2D Gel sheet and mark gel sheet in lab so no one uses the same #'s.
b) Thaw markers

c) On upside down bench paper, place 2 rubber-coated chromic acid bottles full of H_2O. Tip/lean a plate against each bottle at ~ 60° angle from table, with eared plates facing you.
d) Set out 2 clean spatulas.
e) Set out a clean plastic beaker.
f) Microwave the blue sealer until it melts + is quite hot, but don't boil over.
g) In the chemical hood, pour equilibration buffer from the first Falcon tube into the plastic beaker. Pour out buffer using cap to keep noodle in Falcon.
h) Pour noodle onto spatula.
i) Spread out using bottom of Falcon. The end with dye and/or bumps should be on the right, i.e. acid side.
j) Pour water off slab gel onto bench.
k) Fill pasteur pipette with sealer. Remove bubbles from tip of pipette, then overlay with pipette-full of sealer.
l) Invert noodle from spatula onto gel.
m) Tap down with spatula end to remove bubbles.
n) Cover again with pipette-full of sealer and suck off any bubble from below noodle.
o) Repeat for 2nd noodle.
p) Allow sealer to solidify (3-5 min. depending on how hot the sealer was). Then move and lean two more gel plates and repeat for remaing samples.

9. Inserting gel into slab gel apparatuses for running:
Put plates into apparatuses. Ear plates face inward. Pour running buffer into top well and then into bottom well. Use curved pasteur pipette to blow out bubbles at the bottom of the gel.

10. Load Markers:
On acid end ("lane 1 side"), dig small hole into sealer with pasteur pipette ~1/8 inch from edge of gel. Hole should go down into overlay until reach stack. View from top instead of side to help see hole. Sometimes a piece of glass from pasteur pipette will get stuck in gel. Leave it there.
Load 5-7.5λ marker with long skinny round tip and pipetteman.

11. Run gels O/N: (~16 hrs) at 6-7 mAmps each; 500V max.; 100W max. Next morning turn up to 40mAmps each to finish quickly (optional). Can take an additional 1-2 hours to finish depending on how many hours it has been running; a total of three hours to run all the way. Stop gels when dye gets to the top of the plug. Gels run at 10 mA each will reach top of plug in approx. 14 hrs.

12. Transfer gels: onto nitrocellulose, PVDF, etc. (for Western blotting) if want.