SDS-PAGE and Western Blotting for Chemiluminescence Entered by Vivian Fan
Janes Lab Protocols 4/19/10

I. SDS-PAGE:

1. Assemble glass sandwich
   a. Clean the short and 1.5 mm spacer plates with 70% alcohol before starting (one short and one spacer plate per gel).
   b. On a smooth level surface (i.e., the lab bench), place together the short and 1.5 mm spacer plates so that both plates are flush at the bottom.
   c. Slide the 2 plates into the casting frame with the smaller plate-facing front. Lock pressure cams to secure the glass plates.
      - *Be sure not to tilt the casting frame and glass plates when locking the pressure cams. This will cause the gel to leak.*
   d. Place a gray rubber gasket on bottom of gel assembly apparatus.
   e. Insert glass sandwich into the gel-casting apparatus: Place the glass sandwich on top of the gray casting stand gasket and engage the top of the spacer plate with the spring-loaded lever. The pressure of the lever will hold the spacer plate down so both plates are perpendicular against the gray rubber gasket.
      - *Do not push the glass plates down on the gray rubber gasket with more force than is provided by the spring-loaded lever. This will damage the gasket and cause the gel to leak.*
2. Prepare the separating gel solution.
   a. Consult the Sambrook table for the recipes using different volumes of separating gel (10 ml volume per gel) and percentages of acrylamide. Make sure to prepare the APS fresh and add it <u>last</u> to the separating gel solution.
   b. Swirl the separating gel gently after APS addition and use a disposable pipette to add the gel between the glass plates up to ~1 mm of the bottom of the green bar on the casting frame.
      - *Do not add the separating gel beyond the bottom of the green bar. This will cause the gel comb to push into the separating gel during Step #3.*
   c. Immediately after adding the separating gel, use a P-200 micropipet to add water-saturated butanol on top of the gel to smooth out the gel interface. Pull the butanol from the <u>top</u> layer of the butanol-water solution.
   d. Allow the separating gel to polymerize. Test the progress of the polymerization by squeezing the top of the used disposable pipette: if the solution has solidified, the polymerization has occurred.
   e. In the meantime, prepare the stacking gel. Consult the Sambrook table for the recipes using different volumes of stacking gel (~2 ml volume per gel, 5 ml minimum). Do not add the APS until Step #3.
   f. When separating gel has polymerized, pour off the butanol in the sink and rinse with tap water. Remove the water by capillary action with a paper towel.
3. Stacking gel
   a. Add the APS and use a disposable pipette to lay the stacking gel on top of the polymerized separating gel. Fill with separating gel up to the shorter gel plate. Insert a 1.5mm comb between the glasses while making sure no air bubbles are trapped between the teeth of the comb.
      - *When inserting the comb, keep your face <u>away</u> from the gel, because stacking gel commonly squirts out at this step.*
   b. In the meantime, prepare the running buffer by diluting the 5× Tris-glycine stock in H_2O (1 L running buffer per tank).
   c. When the stacking gel has polymerized, carefully remove the comb. Rinse and irrigate the wells with 1× running buffer thoroughly to remove unpolymerized acrylamide.
4. Remove the gel plates from the casting frames and place the plates in the electrophoretic apparatus with the gels firmly seated at the <u>base</u> of the electrophoretic apparatus and the <u>short plates</u> facing <u>inward</u>.
   - *Putting the plates in backwards will not allow current to flow through the gel, and it is very difficult to fix the plate orientation after the samples have been loaded.*
   - *If only running one gel, use the plastic buffer dam on the other side of the electrophoretic apparatus*

- *Be sure that the apparatus has two metal leads pointing upwards, that red-black on the leads matches red-black on the tank, and that only two gels are run per tank (Biorad says four can be run in the same tank, but this setup is troublesome).*

5. Fill the inner chamber full with running buffer. Fill the outer chamber up to the level specified on the tank.
   - *The liquid level in the inner chamber must stay above the short plates for current to flow through the gel. If the liquid level in the inner chamber is dropping, then there is a leak and the gel plates have not been properly installed.*
   - *If the leak remains after fixing the plates, fill the outer chamber up to the level of the inner chamber so that there is no fluid head between the inner and outer chambers.*
6. Load the samples:
   a. 5 μl volume for MW markers.
   b. 40 μl total volume for samples when using a 10-well comb (including 10 μl of 4× sample buffer). 20 μl total volume for samples when using a 15-well comb (including 5 μl of 4× sample buffer).
   c. Fill each well slowly—the goal is to get all of the sample solution into each well. Try to avoid bubbles in wells that may cause overflow of the solution into other wells
   d. When working with two gels, make sure to label on the outside of the container which gel is which.
7. Match the + and – electrode ends of the lid to the power supply, and set the apparatus to run ~1.25 hr at 130V constant voltage.
8. Check to make sure that the blue dye front has been run all the way to the bottom of the gel plate.
   - *For colorimetric staining of total proteins instead of Western blotting, stain for 1 hr in Coomassie Blue staining solution, destain periodically with destaining solution, and destain overnight with longer destaining solution*
9. Set up transfer apparatus
   a. Prepare 1L of transfer buffer (100 ml 10× transfer buffer stock, 800 ml H_2O, 100 ml methanol for most proteins; 100 ml 10× transfer buffer stock, 500 ml H_2O, 400 ml methanol for proteins less than ~20 kDa)
   b. Set up the transfer cassette
      i. Start with the clear half of the transfer cassette on the bottom
      ii. Fiber pad: -soak and squeeze out air bubbles in transfer buffer
      iii. Filter paper: -pre-wet in transfer buffer
      iv. PVDF membrane: -briefly soak in methanol (0.45 μm pore size for most proteins, 0.2 μm pore size for proteins less than ~20 kDa)
      v. Gel: -remove gel from glass plates by cracking plates open with a wet razor, gently cutting the stacking gel off, and laying the gel on top of the PVDF membrane using a wet razor; roll the gel with a wet 2 ml disposable pipet
      vi. Filter paper: -pre-wet in transfer buffer
      vii. Fiber pad: -soak and squeeze out air bubbles in transfer buffer
   c. Close together cassette and insert into transfer housing so that the black side of the cassette faces the black side of the housing.
10. Put in the ice block and surround the transfer tank in ice.
11. Set up the wet transfer to run for 1 hr at 100V constant voltage.

II. Western Blotting

1. Remove membrane paper from the transfer apparatus. Mark which side the gel proteins transferred onto by writing your initials and the date. Mark the MW markers and with a lab marker and indicate the pink "landmark" bands.
2. Make the blocking solution: 5% (w/v) nonfat skim milk in TBS-T (500 mg in 10 ml TBS-T per membrane)
   - *5% milk is a more-stringent blocking agent than 5% BSA*
3. Place the membrane in blocking solution for 1 hr @ RT with constant agitation.
   - *Shorter blocking times (as low as 30 min) are acceptable if necessary*

4. Wash the membrane for 5 min in TBS-T.
5. Place the membrane in 5 ml of primary antibody solution (dissolved in 5% [most Abs] or 1% [anti-tag Abs] BSA in TBS-T + 0.05% thymol microbicide) in a sealed bag overnight @ 4°C with constant agitation.
6. Pour primary solution back into its original test tube for reuse.
   - *The thymol will delay bacterial overgrowth and allow the primary antibody to be used several times*
7. Wash the membrane for 3 × 5 min in TBS-T.
8. In the meantime, prepare secondary antibody solution (1:10,000 dilution of HRP-conjugated goat anti-mouse or anti-rabbit [Jackson ImmunoResearch] in 5% (w/v) nonfat skim milk in TBS-T).
9. Place the membrane in secondary solution @ RT for 1 hr with constant agitation.
10. Wash the membrane for 3 × 5 min in TBS-T and place in Saran Wrap
11. Prepare homemade ECL detection reagent (see Janes_HomemadeECL.pdf)
12. Pipette the reagent mixture onto the blot, covering the entire blot surface.
13. Focus the camera on the AlphaEase and expose the blot for up to 5 min.
14. Snap a white-light image to capture the position of the molecular-weight markers
    - *These markers can be overlaid on the chemiluminescence image in Photoshop*
15. If needed, store the blot wrapped in Saran wrap with TBS-T at 4°C for stripping and reprobing.

III. Stripping & Reprobing

1. Place the blot in 10-15 ml of high-stringency stripping buffer in a sealed bag for 30 min @ 50°C
2. Wash the membrane for 3 × 5 min in TBS-T.
3. Go to Step #2 of Western blotting

- *Alternatively, strip the blot in 10 ml of low-stringency stripping buffer for 10 min @ RT, wash once with TBS-T, and go to Step #2 of Western blotting*
- *The low-stringency strip can partially preserve phospho-epitopes and will better retain low-abundance proteins but will not remove tight-binding primary antibodies*

Buffer recipes

- **4X sample buffer** Store @ -70 °C in 1 ml aliquots
  0.5 ml 2.5 M Tris, pH 6.8 (250 mM)
  2 ml 20% SDS (8%)
  2 ml glycerol (40%)
  0.2 ml 1% bromphenol blue in 10% EtOH (0.04%)
  (Add 400 mM DTT = 0.3084 g if reducing conditions are desired)
  Fill to 5 ml with H_2O (~ 0.3 ml)

- **5X Tris-glycine running buffer** Store @ RT
  15.1 g Tris base (125 mM)
  94 g glycine (1.25 M)
  5 g SDS (0.5%)
  Volume to 1 L (don't pH)

- **10X transfer buffer** Store @ RT
  29 g Tris base (240 mM)
  144 g glycine (1.9 M)
  3.75 g SDS (0.375%)
  Volume to 1 L (don't pH)

- **Coomassie blue staining solution**
  0.1% w/v Coomassie blue R250
  40% methanol
  10% glacial acetic acid

- **Destaining solution**
  30% methanol
  10% glacial acetic acid

- **Longer destaining solution**
  5% methanol
  7.5% glacial acetic acid
  5% glycerol

- **TBS-T**
  100 ml 10× Tris-buffered saline (0.2 M Tris-HCl [pH 7.5], 1.37 M NaCl)
  1 ml Tween-20 (0.1%)
  Volume to 1 L (don't pH)

- **High-stringency stripping buffer**
  3 ml 20% SDS (2%)
  0.75 ml 2.5 M Tris-HCl, pH 6.8 (62.5 mM)
  0.21 ml β-mercaptoethanol, 14.3 M stock liquid (100 mM)
  26.04 ml H_2O

- **Low-stringency stripping buffer**
  6 M guanidine-HCl in H_2O