SOPS for General Surgery Labs:

Title: Western Blotting

Date: 6-2012

Last Updated: 8-2009

10X SDS Running Buffer/electrophoresis buffer

- Tris base 30.2g
- Glycine 144g
- SDS 10g
- Dissolve, place all in cylinder and dissolve with dH2O to 1000mL
- To use, make 1X dilution
    - 100mL 10X plus dH2O up to 1000mL

2X Transfer buffer

- Tris base 7.57g
- Glycine 36g
- Dissolve, add dH2O to 1000mL
- To use, dilute 2X buffer to 1X
    - Add 20% methanol

4% APS (ammonium persulfate)

- 0.4g APS in 10mL dH2O
- Keep in 4°C

10X TBS buffer

- Tris base 24.2g
- NaCl 80g
- Adjust pH to 7.6
- Add dH2O to 1000mL

Lower Tris buffer

- Tris base 90.85g
- SDS 2g
- Adjust pH to 8.6
- Add dH2O to 500mL

Upper Tris buffer

- Tris base 30.8g
- SDS 2g
- Adjust pH to 6.6-6.8
- Add dH2O to 500mL

3X Sample buffer

- In a 20mL conical tube, mix
  - Glycerol
  - SDS
  - Upper tris
  - 0.05% bromophenol blue
  - β- mercaptoethanol
- Vortex vial
- Add dH2O to 10mL
- Aliquot out 1 mL each and store at -20°C

Necessary buffers

- Upper Tris
- Lower Tris
- Sample buffer stock 3x (-20)
- Running buffer
- Transfer buffer

Making gels

- Clean glass plates, ceramic and plastic spacers (get 1.5mm and make sure they are the same size, generally the short fat ones are the ones you want)
  - If plates are not completely clean, gels will stick to plates
- Assemble plates (ceramic→ spacer→ glass) and place in plastic apparatus on benchtop and align
  - Make sure the curved side of the plastic apparatus is up
  - Use the black clamps on either side to lock the assembled cassette
  - Tighten middle screws, check bottom for alignment
  - Once alignment is satisfactory, tighten remaining screws
- Decide what % gel to use and how many you need to make
- Make 4% APS fresh (0.2g ammonium persulfate/5mL H2O)
- Make gels per protocol laminated sheets in 50cc conical vials (make extra so you can know when gel is hardened)
  - Start with lower gel first (lower tris for lower gel, upper tris for upper gel)

- Add APS and TEMED (4°C) last, these speed up the reaction and need to fill gels fast once these are added
- Fill gel using 5mL pipette, adding at total of 6-7mL
    - Gel should stop at the circle beneath the uppermost screw
    - Check for leaking
- Add 200uL t-amyl alcohol on top of gel
- Let dry 20-40 min
- Pour off alcohol into sink and use squirt bottle to wash lower gel with water
- Pour off water, blot with Whatman paper without disrupting gel
- Place in comb
    - Fat comb has 10 lanes and accommodates 30-40uL per well
    - Thin comb has 15 lanes and accommodates 20-30uL per well
- Make upper gel per protocol (again, make extra so that you know when gel has solidified
- Fill upper gel to top of ceramic plate using 5mL pipette
- Allow to dry
- Mark bottom of combs with marker

Preparing samples

- Choose a standard depending on the size of the band you are going to be probing for
    - Rainbow or kaleidoscope- if using kaleidoscope, record product ID as each kit differs some
- Calculate volume of protein/unknown needed per well
    - (and for each well multiply this by the number of gels which you are running to give you the total amount that you need)
- Calculate amount of 1x-3x sample buffer needed per well (and for each well multiply this by the number of gels which you are running to give you total amount you need)
- Make 1x or 2x sample buffer from stock 3x (i.e. make 1x by diluting 3x 1:2)
- Pipette protein and sample buffer into eppedorfs, labeling tops according to which will be in each well on the gel
- Pipette standard (10uL) plus sample buffer into eppendorf and label
- Boil samples for 2 min in 200cc water to denature protein
- Centrifuge quickly in the microfuge (10,000rpm) in order to bring down all the sample from the side of the tube

Loading gels

- Carefully unscrew plates
- Remove combs
- Place gel on gel running apparatus and snap in place with red clips (clip with one big edge and one small edge)

- Fill upper well (behind ceramic plate) to the level of the glass plate followed by the lower well using 1x running buffer
    - Do this to remove air bubbles from the wells
    - Do this before loading samples into wells to avoid spillage of samples
- Load samples and standard, fill remaining empty lanes with 1x sample buffer
- Attach cables making sure the black cable goes into the black knob on the power source and the red cable goes into the red knob and run at constant current 15mA per gel, then 20 once markers have made it past stacking gel

Transfer protein from gel to membrane

- Get one transfer cassette per gel (white side down, black up)
- Make sure you have ample amount of 1x transfer buffer
- Cut sized Whatman paper (2 per gel, 10x10cm) and membrane (8x6cm)- don't forget to label membrane with a pencil
- Moisten sponges with transfer buffer
- Moisten Whatman paper and membrane in transfer buffer
- Take gel off plates carefully using plastic spacer
- Cut off stacking layer
- Rinse gel in transfer buffer once
    - The transfer buffer in the box is reused, only adding more to reach the minimum level marked on the box. The buffer should be changed once a month or so, depending on how often the box is used. When changing the buffer, the used buffer should be drained into a waste container and disposed of as hazardous chemical waste.
- Make sandwich (white plastic down → sponge → Whatman paper → membrane (pour some transfer buffer over membrane now) → gel (oriented properly) → Whatman paper
- Roll with 5cc pipette to get air bubbles out
- Place last sponge, close black plastic lid
- Put in transfer apparatus, black side forward. Make sure transfer buffer is at adequate level in box (between min and max)
- Set appropriate current/voltage (10 volts/60mA) if transferring O/N, otherwise transfer for 2 hours at 30 volts/250mA

Block membrane

- Can block for 1-2 hours or overnight
- Make TBS-tween (800cc diH2O, 200cc 5x TBS, 0.5 tween)
- Make 5% milk (50cc, TBS-tween, 2.5 grams dry milk)
- Remove from transfer cassette
- Rinse membrane with TBS-tween once (pick up with forceps)

- Place membranes in purple boxes (2 membranes/box, proteins facing away from each other) and cover with 5% milk
- Decide to block for 2 hours or overnight depending on convenience and primary antibody requirements, placing on rocker in cold room

Primary antibody

- Make primary antibody dilution (check package insert recommendations)
    - 4cc TBS-tween, 1cc 15% milk, antibody (1:100 to 1:1000, i.e. for 1:1000 add 5uL antibody)
    - Make in 15cc conical tube
    - Keep antibodies on ice/cold and return to appropriate temp ASAP
- Mix by briefly vortexing
- Rinse membrane with TBS-tween once
- Cut plastic bags and label
- Place membrane along bottom edge of plastic bag and seal on two sides
- Add antibody mix, remove air along counter top edge and seal top
- Tape flat to Styrofoam, place on rocker for either one hour or overnight in cold room

Secondary antibody

- Obtain secondary antibodies from -20 across from Rick's desk
- Remove membranes from Styrofoam, put in purple box and rinse with TBS-tween 3 times for 10 min each on rocker (30 min total)
- While rocking, make 1:20,000 dilution of secondary antibody with milk
    - 16cc TBS-tween, 4cc 5% milk, 1uL antibody
    - Some antibodies may need a higher dilution
- Seal membrane in bag as before and add antibody mix
- Rock for one hour
- After one hour, rinse membranes three times with TBS-tween and rock for one hour
- While rocking, make chemiluminescence susbstrate (Pierce susbstrate in 4°C cold box) and mix 1:1 (5cc/membrane)
- Put membranes on saran wrap and dribble substrate over membranes- check to make sure membrane is right side up
    - Make sure membranes are flat and evenly covered
    - Let stand for 5 min
- Make sandwich with Whatman paper to dry
    - Pat
    - Air dry with 4-5 waves
    - Place membrane face down on saran wrap and completely wrap in back side
- Develop in cassette