**SDS-PAGE and Western blot**

SDS-PAGE

a) Set heat block to 99°C.

b) Heat samples for 10 minutes (boiling breaks disulfide bonds and separates beads from samples.

c) Set up SDS-PAGE apparatus with 700 ml 1X MOPS Running Buffer.
   1. Dilute 20x running buffer in ddH20.
   2. Remove pre-cast gels from top shelf of refrigerator, rinse in ddH20 to remove storage buffer. Remove tape on outside of gel.
   3. Place gels in electrode housing unit, writing side out; using grooved side if only one gel. Place dam on other side if only one gel.
   4. Place locking mechanism in chamber, check if gel tray is tight, then lock.
   5. Add 500ml 1X running buffer to outside of chamber. Inside chmaber should remain dry.
   6. Add 500ul NUPAGE antioxidant to remaining 200ml running buffer, fill center of chamber with this to top.
   7. Afetr filled, remove combs. Squirt air into wells with loading tips to wash out any non-polymerized acrylamide.
   8. Place gel rig on top of extra plate to facilitate turning if using two gels.

d) Crack lids carefully on boiled tubes to release steam pressure.
Centrifuge to bring down all liquid. Centrifuge again to pellet, and one more time after repositioning tubes to level pellet.
Load 18ul of sample supernantant, leaving beads behind. Load as fast as possible to avoid sample diffusion. Use 5ul SeeBlue 2 marker in first lane.

e) Run gel for one hour at 160v, max out current.

Western Blot

f) Prepare 2 liters of 1X Transfer Buffer.: Add 100ml 20X Transfer Buffer to 1500ml ddH20. Add 400ml methanol.
Place one liter in western tank, place ice block in other side of tank, cover and put in freezer. Always make fresh transfer buffer to use in tank, remaining warm buffer can be saved in refrigerator to use in wetting sandwich components in subsequent runs.
Keep other liter on counter top.

g) Cut 1 piece of nitrocellulose and 2 pieces of Whatman filter paper to size of transfer sandwich. Place on paper towel, DO NOT place on benchtop.

h) After SDS-PAGE run is complete, set up transfer:
   1. Soak 1 sponge in transfer buffer, place on black side of sandwich in left side of tray. Take out of buffer tray and put on kimwipe.
   2. Soak 1 piece of Whatman paper in transfer buffer, leave in buffer tray.
   3. Use gel cracker to carefully open gel. Keep small plate on top, leave gel on large plate (plate with writing). Cut off wells, cutting straight down

with razor blade; cut off bottom to right above slot. Clean off all gel pieces from plate. Place plate upside down and remove gel onto wet filter paper in buffer tray with bands at bottom of paper.

4. Manipulate/remove any air bubbles between paper and gel by lifting from bottom (stronger gel strength).
5. Carefully lift out gel+filter and lay on wet sponge.
6. Wet nitrocellulose starting from one edge in transfer buffer. Tap until wet.
7. Move and place on gel, do not move nitrocellulose once it is on gel.
8. Wet other piece of Whatman paper and place on top of nitrocellulose.
9. Roll out air bubbles with 2ml strippette, in both directions.
10. Wet last sponge, put on top. Gently close cassette, without securing latch.
11. Remove transfer tank from freezer.
12. Pick up cassette, keep horizontal to minimize dripping.
13. Hold cassette tightly together, lock and put in transfer tank. Add extra buffer to cover.
14. Put entire tank into ice bucket with ice, put on top of tank, pack with ice.
15. Set at 110 volts, 400 amps. Run 1.5 hrs.

i) Open sandwich and remove nitrocellulose, immediately rinse in ddH20 (~50ml) in glass corning dish. Drain off ddH20.

j) Stain with ~12-15ml Ponceau by incubating on rocker for 5 min.

k) Pour off Ponceau into 50ml conical, save for re-use.

l) Rinse 2-3x with ddH20.

m) Place nitrocellulose in ½ sheet protector and scan image on scanner.
-place image face down on scanner bed
-press button on front of scanner to launch software
-select :color photo, 8 bits, resolution 150dpi , 100% scaling
-drag/highlight regions to scan
-name image
-scan. Check lanes for straightness/evenness of loading.

n) Rinse nitrocellulose with 1X TBST until all of dye is gone.

o) Add ~15-50 ml 5% BSA in 1X TBST (cover bottom of tip box lid) and place on rocker for 1 hour (do not use timer on rocker) at "3" setting to block nonspecific binding of proteins to the nitrocellulose. Wash in same orientation as gel was run, top to bottom..

p) Pour off 5% BSA/TBST into 50ml conical to re-use. Dilute primary antibody 1:1000in 1% BSA/TBST (1/5 dilution of 5% stock from step o). Make enough of dilution (25-50ml) to cover membrane. Add to membrane.

q) Incubate on rocker 1.5 hrs at RT or overnight at 4$^{o}$C. Overnight is preferable, gives better result; increases signal to noise ratio.

Western Blot, continued (next day)

a) Pour primary antibody from step q) into 50ml conical tube to re-use.

b) Rinse nitrocellulose in 15 ml 1X TBST 2-3x
c) Wash with 50-100ml 1X TBST and incubate on rocker 8 minutes
d) Pour off TBST, repeat wash 2 more times.
e) Add 50 ml 1:10,000 secondary antibody (anti-goat conjugated HRP) in 1X TBST and incubate on rocker 30-45 minutes.
f) Repeat steps b – d (rinse and washes)

g) Develop Western in dark room
1. make developing solution: 1:40 dilution of B into A
i.e. 100ul B into 4mls A, or 175ul/7mls
2. Take everything to dark room (1/2 sheet protector, sample, film, scissors, film cassette, developing solution, 2ml strippette, paper towels, tape)
3. drain membrane and blot bottom edge.
4. put membrane in ½ sheet protector, blot and roll out excess buffer, add 4 ml developing solution. Incubate 4 minutes. Blot and roll out excess developing solution.
5. Turn on developer.
6. Tape membrane to top of film cassette(at the bottom edge if only one)
7. Place film on bottom of cassette. Turn on red light to open film box

9) Do the above steps quickly! Signal decrease with time, but don't leave wet.
10) Turn on red light to open film cassette.
11) Expose—. If the signal strength is unknown, put first exposures in developer and load new film for longer exposure while developing first set. Usually start with 5-10 seconds, 1 min, 5min exposure.