WESTERN BLOT

Resolving Gel: (National Diagnostic Solutions)

- Add all of the following into a flask for the resolving gel:
  - 4.18 ml Protogel
  - 3.25 ml Resolving Buffer
  - 4.95 ml dH_2O
  - 125 µl 10%APS (Weezer)
  - 12.5 µl TEMED
    NB: Add the APS and TEMED last
- Swirl until thoroughly mixed
- Pour into cassette with pipette, fill to 2nd line (about ¼ inch below where the comb stops)
- Add water-saturated butanol (top layer) just to cover
- Set for ~15-20 min to polymerize
- Drain off Butanol

Stacking Gel: (National Diagnostic Solutions)

- Add all the following into a flask for the stacking gel:
  - 1.3 ml Protogel
  - 2.5 ml Protogel Stacking Buffer
  - 6.1 ml dH_2O
  - 50 µl 10% APS
  - 10 µl TEMED
    NB: Add the APS and TEMED last
- Swirl until thoroughly mixed
- Pipette Stacker into cassette on top of resolving gel
- Insert comb (gently!!)
- Set for 20 min to polymerize

Sample Prep:

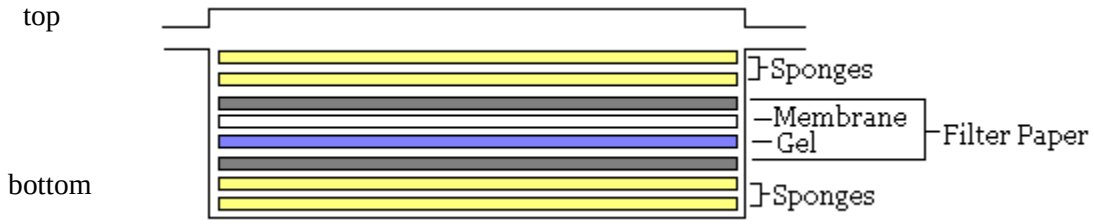
- Calculations: 25 µg Protein per sample
- Make working stock 4x WSB solution
  - 4x WSB in lab freezer
  - Add 15.5 mg DTT to WSB microfuge tube
- Add WSB so that it is 1x in sample and Q.S. with dH_2O to reasonable volume
- Put the samples in the heating block set at 100°C for 10 minutes (5S-19) or boil samples for 10 minutes before you load gel.

Set Up – Running Gel (Invitrogen Mini Gel System)

- Remove comb
- Remove white strip from cassette
- Add Western Running Buffer (1x Tris/glycine/SDS) to apparatus
- Set cassette in apparatus (chamber, blank cassette, spacer), place sample cassette front (cut-out) facing towards back
- Load 8 µl Rainbow Molecular Weight Marker (Weezer 73 B1)
- Load all of each sample into wells
- Hook up electrodes to the power supply
  - 15 milliamps constant current until protein runs through stacking gel
  - 30 milliamps for ~1 hour, or till dye front runs to bottom

Blotting

- Soak four sponges in western blotting buffer (cold in deli fridge)
- Soak Filter paper that surrounds membrane in Western blotting buffer
- Soak membrane in methanol for 15 sec, then place in Western Blotting Buffer with filter paper (don't touch membrane)
- Open cassette with gel knife, pry open around all edges
- Cut excess gel with razor blade (including bottom lip)
- Set up chamber as follows:



- To get gel off cassette, moisten gloves with buffer and lift off cassette gently. Can use the spatula to loosen the gel from the cassette.
- Place in apparatus with spacer
- Hook to power supply
  - 175 milliamps for 1 hour and 30 min

Blocking

- Remove sponges and filter paper, write NP near a corner to signify that side has no protein (make sure it is the side away from the gel)
- Take membrane out and place in plastic box top
- Add 1X Tris Buffered Saline with Tween 20 (TBST) solution, rotate for 5 min
- 2 more washes in TBST, 5 min
  NB: Optional stopping point – wrap in saran wrap and store 4°C
- Block with 5% non-fat dry milk (NFDM in 1xTBST) for 1 hour
- Wash 3 times with 1x TBS-T for 5 min each

Primary Antibody

- Add Primary Antibody (dilution depends on antibody), 3 hours at RT with rocking **OR** overnight @ 4° with rocking
- Wash 3 times in TBST 5 min each

Secondary Antibody

- Monoclonal HRP-conj. 1 hour (make sure that the Ab was raised in a different animal then the primary): typically use either anti-mouse HRP or anti-rabbit HRP 1:2000 dilution
- 3 washes in TBST 5 min each

Detection

- Mix together LumiGlo solution:
  - 9.5 ml dH_2O
  - 500 µl LumiGlo
- Mix Peroxide Solution:
  - 9.5 ml dH_2O
  - 500 µl Peroxide
- Combine Peroxide and LumiGlo Solutions together in a 50 ml conical tube.
- Pour into box top with membrane and rock for 1 minute
- Place saran wrap on bench
- Pick up membrane and blot corner with kimwipe
- Place membrane on Saran Wrap protein side down (you can read MW, facing you)
- Fold edges of Saran Wrap so that there is NO overlapping on the membrane and tape membrane in autorad with protein side facing up

Dark Room

- Things you need to bring with you
  - Film cassette holder (autorad), Timer, Keys
- When in dark room:
  - Turn off lights
  - Open top drawer with key and remove one piece of film
  - Close film box and place in drawer
  - Bend up one corner of film and place over membrane (with folded corner in upper right corner of cassette)
  - Close cassette and time for 30 seconds (time varies)
  - Open and remove film
  - Place film in developer machine
  - Turn lights on after machine beeps

Solutions Appendix

Gel Running Buffer: (1.5M Tris, 0.4% SDS solution, 10X) Dilute to 1x

Blotting Buffer:

- 18.1g Tris-base
- 86.4g Glycine
- Mix into 4L ddH_2O (~20-30min)
- Add 1200ml Methanol
- Check pH 8.3 (if need to adjust don't use HCl)
- Q.S. to 6L ddH_2O (top mark on jug)
- Store 4°C -

10x TBS: 24.2g Tris-base, 80g NaCl; adjust pH to 7.6 with HCl

Membrane Wash Buffer: Dilute 10x TBS to 1x TBS and add 10ml 10% Tween soln

Blocking Buffer: 5% Non-fat Dry Milk

- 150ml 1 x TBS-T
- 7.5g Non-fat Dry Milk