Western Blot

RR-WB#

Samples:

Purpose:

Making the gels:

☐ Make up separating gel:

Volume for Different Percentages of Acrylamide					
Component	**6%**	**8%**	**10%**	**12%**	**15%**
lower gel 4X buffer	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
dH2O	5.9ml	5.4ml	4.9ml	4.4ml	3.65ml
40% Acrylamide	1.5ml	2.0ml	2.5ml	3.0ml	3.75ml
10% APS*	100.0µl	100.0µl	100.0µl	100.0µl	100.0µl
TEMED*	10.0µl	10.0µl	10.0µl	10.0µl	10.0µl
kDa Range:	***60-200***		***16-70***		***12-45***

*Add just prior to pouring the gel

☐ Pour into gel apparatus using a P1000 and quickly overlay with 1 mL of hydrated Butanol
(Leave ¼ space for stacking gel.)

☐ Let sit for 30-45 min

☐ After polymerization, remove the Butanol overlay via vacuum or by gravity

☐ Rinse with dIH_2O

☐ Make up stacking gel:

4% Stacking gel	
Component	**Volume**
upper gel 4X buffer	2.5ml
dH2O	6.4ml
40% Acrylamide	1.0ml
10% APS*	100µl
TEMED*	10µl

*Add just prior to pouring the gel

☐ Insert the comb and let sit to polymerize for at least 30 min

☐ Place the gel in the electrophoresis chamber, cover with running buffer, remove the comb and rinse the wells with buffer to remove unpolymerized acrylamide

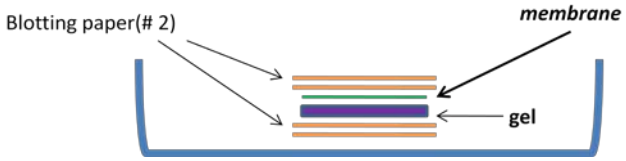
Western Blot

Preparing the samples:

- ☐ Add Laemmli buffer to aliquoted samples
- ☐ Incubate the sample at 95°C for 5 minutes to denature the proteins
- ☐ Mix by vortexing, blip spin and load onto the gel
- ☐ Add sample buffer into all empty wells for an even dye front
- ☐ Run gel at 180V until the dye front has emerged from the gel (about 1 hour)

Blotting:

- ☐ Remove stacking layer from the gel and clip the gel on the top, left corner by the marker
- ☐ Place membrane in tray filled with dH_2O
- ☐ Fill large tray with transfer buffer and assemble the blot:



**place the gel down so that the cut is on the top, RIGHT corner*
**place the membrane so that the shiney side is up and the matte side is down on the gel*
**use fingers to "walk" over gel, but do not rub*

- ☐ Flip the blot assembly over and place in semi-dry blotter (membrane-side down)
- ☐ Set to 100-300 mA, 15 V and 2-5 Watts (with a CONSTANT amp):

2 mini gels = 80 minutes
4 mini gels = 120 minutes
6 mini gels = 240 minutes

- ☐ Stain with 0.1% Ponceau S (in 1% acetic acid) in order to visualize protein bands by swirling the solution onto the blot for 5 minutes
- ☐ Rinse blot with dIH2O to remove background staining
- ☐ Scan or photograph the blot to confirm equal loading in each lane

**Ponceau S will come off during the blocking step.*
***Blot may be rinsed in dIH_2O and stored at RT *prior* to Ponceau stain.*
****Blot may be blocked, washed with 1X PBS and then stored at 4˚C. Use dIH_2O to wash if using an AP-conjugated secondary antibody.*

Blocking:

- ☐ Block in 5% non-fat dry milk in TBST at RT for 30 min or at 4˚C O.N.

Western Blot

Immunochemical Staining:

Primary Ab1: _____ *Dilution:* _____ *Species:*_____

Primary Ab2: _____ *Dilution:* _____ *Species:*_____

Secondary Ab: _____ *Dilution:* _____ *Species:*_____

☐ Incubate the blot with primary antibody diluted in Blocking buffer for 1-2 hours at RT ***or*** at 4˚C O.N. *(Use 5% BSA for antibodies that are to be recovered.)*

*[**Recover Ab Solution**: Recover the Ab solution in a 15 mL conical. Add 0.02% NaAzide to stem bacterial growth. The Ab solution may be used up to four times for diagnostic purposes. Do not recover HRP-conjugated antibodies as NaAzide acts as an inhibitor to the reaction.]*

☐ Wash blot with TBST for 3X 15 minutes

☐ Incubate the blot with secondary antibody diluted in Blocking buffer for 1 ½ to 2 hours at RT

☐ Wash blot with TBST for 3X 5 minutes

Antibody detection:

☐ Prepare ECL Plus reagents (2 mL solution A + 50 µL solution B)

☐ Remove blot to a flat surface on a transparency sheet and quickly pipette 1-2 mL ECL Plus

☐ Incubate for 5 minutes at RT

☐ Sandwich the blot between a second transparency and to film

**If this is a new antibody, make sure to also achieve an underexposed and overexposed setting in order to test the antibody parameters.*

Notes:

Western Blot

Label Blots: