Western Blotting

Solutions

4x Separating Buffer (500mM Tris pH 8..8)

151.67g Trizma base in 400ml water- adjust pH to 8.8 with concentrated HCl then adjust volume to 500ml and autoclave

4x Stacking Buffer (100mM Tris pH 6.8)

30.25g Trizma base in 400ml water- adjust pH to 6.8 with concentrated HCl then adjust volume to 500ml and autoclave

10% SDS

10g in 100mL dH_2O. Sterile filter.

10% APS (ammonium persulfate)

1g in 10mL dH_2O. Sterile filter. Store at 4ºC for 1 month or aliquoted at -20ºC.

6xSMASH Buffer (Loading Buffer)

125mM Tris.HCl (pH 6.8)		2.4mL (1M Tris.HCl)
20% Glycerol		3.4mL
4% SDS	1.2g	
1mg/mL bromophenol blue		a little
286mM β-mercaptoethanol		3ml
H_2O		1.2mL

5x Running Buffer

final concentration	amount to add from stock	conc 1x
125mM Tris	15.1g Trizma base	25mM
968.5mM glycine	72g	193.7mM
2.5% SDS	25ml 20% stock	0.05%

make up to 1litre with water- on day of use dilute to 1x

5x Transfer Buffer

final concentration	amount to add from stock	conc 1x
240mM Tris	29g Trizma base	48mM
195mM glycine	15.5g	39mm
0.185% SDS	18.5ml 20% stock	0.037%

make up to 1litre with water- on day of use make 1x with 20% methanol

TBS

Final concentration	amount to add from stock
100mM Tris pH7.5	100ml of 1M stock
0.9% NaCl	9g

TBST – TBS + 0.1% tween

Blocking Solution – TBST + 5% non-fat milk

Separating Gel **(volumes calculated for 15mL)**							
	5%	6%	7%	8%	10%	12%	15%
Acrylamide	**2.5**	**3.0**	**3.5**	**4.0**	**5.0**	**6.0**	**7.5**
4xSeparation Buffer	**3.75**	**3.75**	**3.75**	**3.75**	**3.75**	**3.75**	**3.75**
10% SDS	**150µL**	**150µL**	**150µL**	**150µL**	**150µL**	**150µL**	**150µL**
10% APS	**150µL**	**150µL**	**150µL**	**150µL**	**150µL**	**150µL**	**150µL**
TEMED	**15µL**	**15µL**	**15µL**	**15µL**	**15µL**	**15µL**	**15µL**
Water	**8.45**	**7.95**	**7.45**	**6.95**	**5.95**	**4.95**	**3.45**

Stacking Gel **(volumes calculated for 15mL)**			
	4%	5%	6%
Acrylamide	**2.0**	**2.5**	**3.0**
4xStacking Buffer	**3.75**	**3.75**	**3.75**
10% SDS	**150µL**	**150µL**	**150µL**
10% APS	**150µL**	**150µL**	**150µL**
TEMED	**15µL**	**15µL**	**15µL**
Water	**8.95**	**8.45**	**7.45**

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Gel

1. Clean glass plates and spacers. Set up gel plates with BioRad clamps. Place in casting stand. Mark position of bottom of wells.
2. Prepare separating gel using table below. Combine all but TEMED.
3. Add TEMED to resolving gel mix (amounts can be increased 25%). Swirl gently. Pour it and overlay with isopropanol. Will polymerize in 15-20 minutes.
4. Pour off unpolymerized resolving gel mix. Wash with dH_2O several times (squirt bottle). Place on side to drain dH_2O completely or clean with filter paper.
5. Prepare stacking gel using table below. Combine all and poor immediately.
6. Introduce clean, dry comb at an angle to avoid trapping bubbles.
7. Clamp gel to electrode stand. Place in tank and add running 1x Running buffer.
8. Remove comb slowly. Wash out wells with reservoir buffer using syringe.
9. Load 5-10 µl of sample on bottom of well. Use lanes on outer edges last. To prepare samples, put those 5 min at 100^0C and then vortex for 5 min at maximum speed.
10. Run gel at 50-80V until the sample has cleared the stacking gel (~20-30minutes). Increase voltage to 100-150V and run until loading dye has run to the bottom

Semi-Dry transfer

1. Cut 2 pieces of 3mm paper and 1 piece of PVDF transfer membrane the same size as the gel
2. Wet the membrane in methanol for 1 minute
3. Pour methanol out and add water- hold the membrane under the water until it stays
4. Pour water out and add transfer buffer
5. Take gel out of apparatus and put in transfer buffer
6. Soak 3mm paper in transfer buffer
7. Assemble everything on the transfer apparatus from bottom to top: 1 piece 3mm paper, membrane, gel, other piece of 3mm paper
8. Get rid of bubbles by gently rolling a pipette over the top
9. Mop up excess liquid
10. Transfer at 15V for 45 minutes

Blot

1. Disassemble apparatus
2. Block membrane in blocking solution for 1 hour, shaking
3. Incubate with primary antibody overnight at 4^{o}C, shaking

4. Wash with blocking solution- 1 rinse then 3x 15minutes, shaking
5. Incubate with secondary antibody in blocking solution for 1 hour at room temperature

6. Wash with blocking solution- 1 rinse then 2x 15minutes, shaking
7. Wash with TBST for 15 minutes shaking
8. Wash with PBS- 1 rinse then 5 minutes with shaking

ECL

1. Mix together equal volumes of the two ECL detecting reagents (total volume 500µl per membrane)
2. Place membrane face down in the mixture, ensure there are no bubbles, and incubate for 1 minute
3. Place membranes on a glass plate or celofane paper
4. Mop up the liquid using 3mm paper
5. Cover plate with Saran wrap
6. Expose film for an appropriate time and put film in machine