Western Blotting (or Immunoblotting)

Buffers & Reagents to prepare:

Abbreviations:
w/v= weight/volume
F/S= filter/sterilized
SDS= Sodium Dodecyl sulfate
PAGE= polyacrylamide gel electrophoresis
10'= 10 minutes

10% APS (w/v) [make ~5mL at a time]:
For 5mL:

Volume	Material
0.5g	Ammonium Persulfate
5mL	MQ- H_2O
F/S, store at 4°C	

1X M9 Liquid Media + 0.4% Sugar (F/S)

For 400mL of Mannitol

Volume	Material
80 mL	5X M9
16 mL	10% Mannitol
304 mL	MQ- H_2O
F/S store at Room Temp	

For 400mL of Glucose

Volume	Material
80 mL	5X M9
8 mL	20% Glucose
312mL	MQ- H_2O
F/S store at Room Temp	

SDS-PAGE 5x Sample Buffer:

Material	
250mM	Tris Buffer pH 6.8
10%	SDS
50%	Glycerol
0.5%	Bromophenol blue
store at Room Temp; **add 10% β-Mercaptoethanol to small volume of sample buffer when needed- before loading samples**	

SDS-PAGE Transfer Buffer:
For 1 Liter:

Volume	Material
3.0 g	Tris Base
14.5g	Glycine
200mL	Methanol
----------	Bring to1L with MQ- H_2O
Add all to 1000mL glass flask, store at 4°C	

10X Protein Electrophoresis Buffer:
For 500mLs

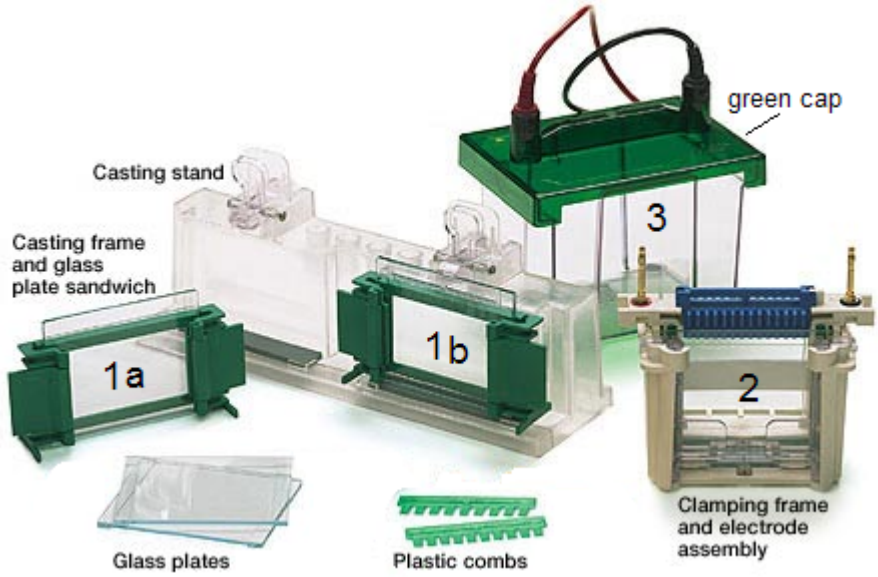
Volume	Material
5.0 g	SDS
15.0 g	Tris Base
72.0 g	Glycine
----------	Bring to 500mL with MQ- H_2O

Add all to 500mL glass flask, store at RT

---Dilute to 1X Protein Electrophoresis Buffer for use.

Western Blot Protocol:

Gel Prep (Day 1)



Wear goggles in the case of splashing of toxic reagents!
Wash glass plates, casting frame, and comb; then dry!

1. **Prepare** 10% SDS-PAGE Gel :

Volume	Material
2.5mL	1.5 M Tris Buffer pH 8.8
100 µL	10% SDS (w/v)
3.35 mL	PROTOGEL acrylamide *neurotoxin
4.1 mL	MQ- H_2O
*** STOP**	**Prepare apparatus: #1a then 1b in Figure above**
50 µL	10% (w/v) APS F/S (4°C) *should be used w/in 1 month
5 µL	TEMED (stinky)
Mix by gently swirling all the contents in a 50mL beaker	

2. **Pour** gel between the glass plates set up (Figure #1b) till a little below where the comb would be.
3. **Flatten** the gel immediately with 100µL of n-butanol
4. **Solidify** gel 30'-60'
5. **Remove** n-butanol using paper towel. **Rinse** the 3x with 600µL MQ-H_2O, remove w/paper towel.
6. **Prepare stacking gel**:

Volume	Material

0.625 mL	1M Tris Buffer pH 6.8
50 µL	10% SDS (w/v)
0.690 mL	PROTOGEL acrylamide *neurotoxin
3.6 mL	MQ- H_2O
*** STOP**	**Check apparatus before continuing to add**
25 µL	10% (w/v) APS F/S (4°C) *should be used w/in 1 month
5 µL	TEMED (stinky)
Mix all the contents gently in a 50mL beaker before adding to SDS gel	

7. **Pour** stacking gel on top of the SDS-PAGE gel , place in comb slowly and in straight- DO NOT MAKE AIR BUBBLES!
8. **ADD COMB & DO NOT REMOVE IT.**
9. **Solidify** stacking gel w/ comb 30'-60'
10. Once solidified (check beaker), **rip** out a large piece of plastic wrap. **Place** two paper towels on the plastic wrap. **Soak** the paper towels with just enough 1X Protein Electrophoresis Buffer. Take plates with comb and gel still in between and **wrap** it in the wet paper towels tightly. Then wrap the plastic wrap completely around everything.
11. **Store** the wrapped gel in the refrigerator (4°C) overnight- shorter plate facing upwards.

Running Western Blot: Electrophoresis (Day2)

1. **Prepare** the sample buffer by adding 1/10 volume β-Mercaptoethanol.
2. **Add** ~1/4-1/2 volume of sample buffer to sample. Example: 10µL of sample + 5µL of β-MeSmpleBfr
3. **Incubate** tubes at 95°C, 10'. **Spin** briefly in picofuge for 2-3 seconds.
4. **Assemble** apparatus (#2 in Figure). Unwrap gel, wipe off excess residue. Tightly **seal** gel/plates with comb facing toward the inside and the buffer dam by clamping the green sides of clamping frame (#2). Then place entire electrode assembly into clear box (#3-without green cap).
5. **Pour** 1X Protein Electrophoresis Buffer in between the electrode assembly to the top. Check if there are any leaks in the 'reservoir' between the sealed gel and dam. If there are no leaks, fill the rest of the clear box with 1X Protein Electrophoresis Buffer until the "2 gel" line mark.
6. **Remove** comb. **Load** samples.
7. Put on green cap→ Match **black** to **black**; and **red** to **red**. **Turn on** the machine. Have the wires plugged in black to black; and red to red.
8. **Run** constant voltage: 200V, 40'.
9. **Empty** out the 1X Protein Electrophoresis Buffer into the sink.

Running Western Blot: Transfer

1. Get two filter papers, **cut** a piece of membrane (**slit** the top left corner of the membrane).
2. **Soak** the filter papers, membrane and sponges in a little bit of transfer buffer 5' before conducting the transfer.
3. **Prepare** the transfer sandwich: black→sponge→filter paper→gel→membrane (make sure to match the top left corner slit of the gel with the slit of the membrane)→filter paper→ sponge→ clear: CLAMP
4. Place the sandwich into the black-and-red box. NOTE: the black side of the sandwich should face the black side of the red-and-black box.
5. Place the black and red box with sandwich into the clear box (Figure- #3)
   - Make sure that black side is matched with the black of the clear box. Red with red.
6. Put in stir bar and ice box. Place clear box on stir machine.
7. **Fill** up box with 1X Transfer Buffer (retrieved from the fridge 4°C) until "Blotting Line."
8. **Cover** with green cap on clear box; black matches with black and red matches with red.
9. **Run** constant Amps: 300 mA (0.3 A) for 60', stirring.
10. Pour down 1X Transfer Buffer into hazardous waste beaker in the hood. (Do not pour Transfer Buffer down the sink because it contains methanol).
11. Use membrane for immunoblotting.
12. Gel can be saved in 1X Electrophoresis buffer for coomasie staining (~60') and destaining.
13. **Prepare** 10mL of TBST (1x TBS + 0.1% Tween) with 5% milk:

Volume	Material
0.5 g	**Dry powder milk**
----------	**Bring to 10mL with 1X TBS**
10 µL	**Tween**

14. **Block** for at least 60', gentle shaking, room temperature.
15. **Prepare** 45mL of TBST and **wash** 3x 5'.
16. **Prepare** 10mL of TBST + 5%milk + 5-10 µL of antibody.
17. **Incubate** for 60' gently shaking, RT.
18. **Prepare** 45mL of TBST and **wash** with 15mL increments 3x 5'.
19. **Rip** a piece of plastic wrap. **Pipette** the 1mL of DURAWEST (500 µL of brown bottle + 500 µL of white bottle)on to the middle of the plastic wrap.
20. **Place** the membrane (side with proteins) onto the 1mL of DURAWEST. Use a small tube to roll over the backside of the membrane to remove air bubbles and disperse the DURAWEST all over the membrane.
21. **Wait** for 5'. Remove excess DURAWEST by blotting with kimwipe.
22. **Rip** a new piece of plastic wrap, **place** the membrane right side up. **Close** up the sides of the plastic wrap tightly to avoid the membrane from drying out.
23. **EXPOSE!**