Western Blotting Protocol

Protein Isolation:

*Always make fresh working solutions before isolation of proteins

1.) Dilute 50X PI solution to 1X PI in TM buffer keeping the solution on ice
2.) Weigh certain amount of tissues and chop them into small pieces
3.) Keep the tissue on dry ice
4.) Add 1X PI in TM buffer to the tissue at 2.5mL per gram of tissue and put in ice for 5 minutes
5.) Homogenize the tissue for 20 seconds and then put on dry ice for 15 seconds
6.) Homogenize the tissue again for 20 seconds (and a third time if necessary)
7.) Rotate the homogenized tissue at 4^{o}C for 20 minutes
8.) Centrifuge at 11,000 rpm at 4^{o}C for 20 minutes
9.) Collect the supernatant
10.) Determine the protein concentration of the total proteins

Reagents for SDS-PAGE

Sample Buffer (SDS reducing buffer)(store at room temperature)

Deionized Water	3.8mL
.5M Tris-HCL, pH 6.8	1.0mL
Glycerol	0.8mL
10% (w/v) SDS	1.6mL
2-mercaptoethanol	0.4mL
1% (w/v) bromophenol blue	0.4mL
	8.0mL Total

Dilute samples at least 1:4 with sample buffer and heat at 95^{o}C for 5 minutes

5X Electrode Running Buffer, pH 8.3 (enough for 10 runs)

Tris Base	9g	(15g/L)
Glycine	43.2g	(72g/L)
SDS	3g	(5g/L)
to 600 mL of Water		

Store at 4^{C}C. Warm to room temperature before use if precipatation occurs.
Dilute 60mL 5X stock with 240mL deionized water for one electrophoresis run

Load samples into gel
Run gel at 80-100 Volts through the stacking part of the gel
Turn the volts up to 150V after the proteins have gone through the stack and are migrating through the resolving gel
Allow migration to continue until the blue dye front is at the end of the glass plates, but does not migrate off the gel.

Western Blot
Materials you will need:

The SDS-PAGE gel that was just ran
1 L transfer buffer: 10% MeOH, 0.84g NaBicarb, 0.32g NaCarb
Nitrocellulose paper
Biorad Mini transfer apparatus
Fiber pads
Chromatography paper
TBS/Tween: 20nM Tris pH 7.4, 0.15 M NaCl, 0.1% Tween
Dry Milk
1^o Antibody
2^o Antibody-HRP
Substrate for detection (Pierce Dura Substrate)

1.) Remove gell from electrophoresis apparatus
2.) See preparation for blotting section 2.2 #3 for preparing the sandwich
    a.) Remember to "build on black"--- meaning, on the black side of the cassette holder, lay the first fiber pad, then the chromatography paper on top, THEN THE GEL on top of the chromatography paper, then the nitrocellulose membrane, then the chromatography paper, then the next fiber pad.
    b.) MAKE SURE TO SMOOTH OUT ANY BUBBLES using a glass stirring rod or a pipette.
3.) Transfer for 2hrs at 50V in 4^oC
4.) Block in 5% milk/TBS/Tween overnight at 4^oC
5.) 1^oAb= 1 hour at room temperature, shaking in 5%milk/TBS/Tween
6.) Wash 3 times for 10 minutes each: 2X with TBS/Tween and the 1X with 5% milk/TBS/Tween
7.) 2^oAb= 1:2500 in 5% milk/TBS/Tween 1 hour
8.) Wash- 3X for 10 minutes in TBS/Tween
9.) Add 2mL of substrate and expose

Coomassie staining of SDS-PAGE gel

1.) Remove the gel and pour 100-200mL of Coomassie stain on gel
    a.) Note: recipe for Coomassie Stain

Vf= 1L

250mL Isopropanol
100mL Acetic Acid
0.5g Coomassie Brilliant Blue R

b.) Let gel stain for 10 min to check for total transfer of proteins