Western Blot

- Bio-Rad's Protein Blotting Guide

Step 01. Protein extraction

Sample buffer

Chemical	Stock Concentration	Addition	Final Concentration	Location
Tris (pH 6.8) α	0.5 M		120 mM	make yourself
Glycerol β			5%	V
SDS	10%		4%	III
DTT γ			5 mM	4°C (d)
Bromophenol Blue δ	10%		0.01%	VII
Total per 10 samples		200 µL		

α Prepare by yourself.

β Glycerol stock is too sticky to take with pipette tip. Aliquot some of it into a falcon tube and dilute to 50% with the same volume of ddH_2O.

γ DTT is a strong reductant which is used to break the disulfide bond. An alternative is 10% β-mercaptoethanol.

δ Bromophenol Blue sodium salt should be protected from light. DO NOT directly take from the stock vial for experimental use, keep your own stock aliquots (exp. 10%) instead.

Step 02. Protein purification (optional)

Step 03. Gel preparation

Important: Before starting the gel preparation, fill in some ddH_2O into the well to make sure the well is completely sealed.

Separating Gel (10% Acrylamide)

1. The stacking gel should be on top of the separating gel. Thus, fill in the separating gel first into the well.
2. Prepare the separating gel by adding the chemicals into a new tube following the order of the table below.

Chemical	Stock Concentration	Addition	Final Concentration	Location
ddH_2O	—	3.7 mL 3.2 mL 2.6 mL	—	—
Acrylamide $^{\alpha}$	30%	2.13 mL 2.67 mL 3.2 mL	8% 10% 12%	4°C (3 door)
Tris (pH 8.8)	1.5 M	2 mL	375 mM	make yourself
SDS	10%	80 µL	0.1%	III
APS $^{\beta}$	10%	80 µL	0.1%	4°C (d)
TEMED $^{\gamma}$	1000X	8 µL	1X	Yu-Chun
Total		**8 mL**		

$^{\alpha}$ Acrylamide/Bis is stored at 4°C (three door refrigerator, upper left corner). It should be protected from light. The stock solution should not be used directly. Take the solution as aliquots and keep your own.

$^{\beta}$ APS should be stored at -20°C for long term storage. This chemical is relatively unstable.

$^{\gamma}$ TEMED is an essential catalyst for polyacrylamide gel polymerization. It is used with ammonium persulfate (APS). Incresing the TEMED concentration (exp. increase from 1X to 5X) will speed up the gel solidification process.

3. After the TEMED was added, the gel starts to solidify. Thus, the gel solution should be thoroughly mixed and immediately fill in to the well.
4. After filling in the separating gel into the well, before the gel solidified, immediately add 100% isopropanol on top of the separating gel to flatten the gel surface.
5. After the separating gel was solidified, pour out the isopropanol and add some ddH_2O to rinse the gel.
6. Prepare the stacking gel in a new tube.

Stacking Gel (4% Acrylamide)

1. The stacking gel preparation is similar with the separating gel. Add the chemicals into a new tube following the order of the table below.

Chemical	Stock Concentration	Addition	Final Concentration	Location
ddH_2O	—	3 mL	—	
Acrylamide	30%	0.67 mL	10%	
Tris (pH 6.8)	0.5 M	1.25 mL	125 mM	make yourself
SDS	10%	50 µL	0.1%	
APS	10%	50 µL	0.1%	
TEMED	1000X	5 µL	1X	
Total		**5 mL**		

2. Before the stacking gel starts to solidify, immediately insert the comb.
3. After the stacking gel was completely solidified, mark down the position of each loading well. Otherwise, after pulling out the comb, the loading wells are difficult to be recognized.

Step 04. Gel electrophoresis

Running buffer [Tris (pH 6.8) + Glycine + SDS] (yellow color commercial solution, Yu-Chun bench)

Phase	Voltage (V)	Time (mins)
I (stacking gel)	~ 80	~ 30
II (separating gel)	~ 125	~ 90

Step 05. Transfer

- Bio-Rad's Transfer Buffer Formulations

Prepare 10X transfer buffer stock

We are using the Bjerrum Schafer-Nielsen Buffer. For convenience, make a 10X stock solution first, without the methanol. When in use, dilute to 1X with ddH_2O and do not forget to add the methanol.

Chemical	Stock Concentration	Addition	Final Concentration	Location
Tris base	—	58.2 g	480 mM	
Glycine	—	29.3 g	390 mM	
ddH_2O	—	~ 1 L	—	
Total		**1 L**		

Use 1X transfer buffer

Chemical	Stock Concentration	Addition	Final Concentration	Location
BSN buffer stock	10X	100 mL	1X	
Methanol	—	200 mL	20%	
ddH_2O	—	700 mL	—	
SDS (pH 9.2)	10% 37.5 mg	3.75 mL 37.5 mg	1.3 mM	
Total		**~ 1 L**		

Voltage (V)	Time (mins)
~ 100	~ 40

Step 06. Immunodetection

Step 07. Imaging

- Bio-Rad Molecular Imager® ChemiDoc™ XRS+ with ImageLab™ software

0. Pull out the lower door and load the membrane on the platform. The membrane could be placed on a clear plastic cover to avoid directly contacting the machine platform.
1. Open the `Imager Lab` software.
2. Create a new protocol by pressing `New Protocol`.
3. Switch to `1. Gel Imaging` section.

Take the **marker** image

4. Look in the ***Application*** panel, press `Select...` ⟶ `Custom` ⟶ `marker`. If the `marker` option was not found, then set the parameters as follow:

Marker Setting	Option
Light source	White epi illumination
Filter	Standard filter (Filter 1)
Image color	Gray
Binning	1 × 1

5. Press `Position Gel`
6. Make sure the bar, which is on top of the machine, already switched to ***Filter 1***.
7. Open the upper door, adjust the membrane position and orientation according to the alignment grids on the screen.
8. Press `Run Protocol` to take the marker image.
9. Save the marker image (optional), and minimize the marker image window.

Take the **membrane** image

10. Press `Select...` ⟶ `Blots` ⟶ `Chemi Hi Resolution`.
11. Look in the ***Image Exposure*** panel, select `Signal Accumulation Mode` and press `Setup`. Set the parameters case by case. For example:

Parameters	Values
First image time (sec)	30
Last image time (sec)	300
Total number of images	10

12. Press `Run Protocol`.

13. Switch the bar, which is on top of the machine, to ***No Filter***. Press `OK`.
14. Save the desired membrane images.

Merge marker and membrane image

15. Press `Open` to load in the desired membrane image.
16. Look in the ***Analysis Tool Box*** panel on the left hand side, press `Image Tools`.
17. Press `Merge` ⟶ `OK`. Make sure the chosen marker image is correct.
18. Export the merged image. Press `Export` ⟶ `Export for Publication...` ⟶ select `600 dpi` ⟶ `Export...` (save as TIFF file format is recommended).
19. Completed. Do not forget to unload the membrane and switch off the machine.