The Fleischman Lab

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Title: Western Blotting

Introduction

Western blotting is a useful tool for examining protein expression in cells and tissues. This protocol is optimized for the analysis of cytoplasmic or cell-surface proteins from cells grown in culture or isolated from blood or bone marrow, and can be easily adapted to nuclear proteins as well. The NP40 lysis buffer selectively lyses the cell membrane while leaving the nuclear membrane intact. The lysate is then centrifuged to separate the cytoplasmic lysate from the nuclei. Protein concentration is determined via the colorimetric BCA assay and normalized, then proteins are denatured by heating in Laemmli buffer. Proteins are then separated by size via SDS-PAGE and transferred to a methanol-activated PVDF membrane for probing. After transfer, membranes must be blocked from nonspecific binding by incubation in 5% milk or BSA. Membranes are then probed with a primary antibody, followed by an HRP-conjugated secondary antibody. Finally, blots are exposed to a chemilluminescent substrate that emits light when cleaved by HRP, enabling protein detection and quantification.

Materials

1. NP40 lysis buffer
2. Protease inhibitor cocktail
3. Phosphatase inhibitor cocktail 2
4. BCA assay reagents (Chiu lab)
5. BSA standards (Chiu lab)
6. Plate reader (Meyskins lab)
7. Amersham full-range rainbow marker (Chiu lab)
8. 4x Laemmli sample buffer + β-mercaptoethanol
9. Heat block set to 70°C
10. Acrylamide (29:1, 30%)
11. Tris base, 1.5M pH 8.8 (36.33g Tris base, 200ml dH_2O, pH to 8.8 with HCl)
12. Tris base, 0.5M pH 6.8 (12.11g Tris base, 200ml dH_2O, pH to 6.8 with HCl)
13. 10% SDS
14. 10% APS (100µl aliquots in the -20°C)
15. TEMED
16. Gel casting apparatus, glass plates, combs
17. Gel running and transfer apparatus
18. Running buffer
19. Transfer buffer, chilled to 4°C
20. Tip boxes, for washing membranes
21. Tweezers
22. Immobilon-P membranes
23. dH_2O
24. Ethanol
25. Methanol
26. Blocking buffer (3-5% BSA or milk in TBST)
27. TBST
28. 1° and 2° antibodies
29. Pierce ECL 2 or West Dura kit (Chiu lab)
30. Digital ECL imager (Syngene G:BOX, Waterman lab)
31. 0.2N NaOH (diluted 1:50 from 10N stock)

<table>
<tr><th>Protocol</th><th>A. Sample preparation</th><th>Notes</th></tr>
<tr><td>1.</td><td>Prepare NP40 lysis buffer by adding protease inhibitor and phosphatase inhibitor cocktails:
<table>
<tr><th>Reagent</th><th>For 1 tube</th><th>For X tubes</th></tr>
<tr><td>NP40 lysis buffer</td><td>49 µl</td><td>49(x+1) µl</td></tr>
<tr><td>Protease inhibitor cocktail</td><td>0.5 µl</td><td>0.5(x+1) µl</td></tr>
<tr><td>Phosphatase inhibitor cocktail 2</td><td>0.5 µl</td><td>0.5(x+1) µl</td></tr>
</table>
Chill buffer to 4°C prior to use.</td><td>Use 1 ml lysis buffer for every 10^7 cells. The recipe in the table assumes 500k cells.</td></tr>
<tr><td>2.</td><td>Pellet cells in a 1.5 ml tube and aspirate supernatant.</td><td></td></tr>
<tr><td>3.</td><td>Resuspend cells in chilled lysis buffer.</td><td></td></tr>
<tr><td>4.</td><td>Incubate cells in the cold room with constant agitation for 30 minutes.</td><td>Agitate cells by securing a rack to the rocker or to a vortex on the lowest setting.</td></tr>
<tr><td>5.</td><td>Centrifuge at 12,000 rpm for 20 minutes.</td><td></td></tr>
<tr><td>6.</td><td>Transfer the supernatant to a fresh tube and discard the pellet. Store the lysate at -20°C to -80°C.</td><td></td></tr>
<tr><td></td><td>B. Protein assay and normalization</td><td></td></tr>
<tr><td>1.</td><td>Prepare standard dilutions:
<table>
<tr><th>Vial</th><th>Diluent (µl)</th><th>BSA (source) (µl)</th><th>Final BSA concentration (µg/ml)</th></tr>
<tr><td>A</td><td>0</td><td>300 (stock)</td><td>2000</td></tr>
<tr><td>B</td><td>125</td><td>375 (stock)</td><td>1500</td></tr>
<tr><td>C</td><td>325</td><td>325 (stock)</td><td>1000</td></tr>
<tr><td>D</td><td>175</td><td>175 (vial B)</td><td>750</td></tr>
<tr><td>E</td><td>325</td><td>325 (vial C)</td><td>500</td></tr>
<tr><td>F</td><td>325</td><td>325 (vial E)</td><td>250</td></tr>
<tr><td>G</td><td>325</td><td>325 (vial F)</td><td>125</td></tr>
<tr><td>H</td><td>400</td><td>100 (vial G)</td><td>25</td></tr>
<tr><td>I</td><td>400</td><td>0</td><td>Blank</td></tr>
</table></td><td>Standards should be diluted in NP-40 lysis buffer. Store prepared standards at 4°C.

Vial H may be omitted as the microplate method is not sensitive below 125 µg/ml.</td></tr>
<tr><td>2.</td><td>Prepare the appropriate volume of BCA working reagent:
<table>
<tr><th>Reagent</th><th>1 sample</th><th>Standards + x samples</th></tr>
<tr><td>BCA reagent A</td><td>400 µl</td><td>3600 + 400(x+1) µl</td></tr>
<tr><td>BCA reagent B</td><td>8 µl</td><td>64 + 8(x+1) µl</td></tr>
</table></td><td></td></tr>
<tr><td>3.</td><td>Pipette 10 µl of each standard and sample into a 96 well plate for a total of 2 replicates each. The ratio of sample to working reagent will be 1:20 and the working range will be 125-2000 µg/ml.</td><td>25 µl of sample/standard per well is ideal (ratio = 1:8; working range = 20-2000 µg/ml)</td></tr>
<tr><td>4.</td><td>Add 200 µl of prepared working reagent per well and gently mix.</td><td></td></tr>
<tr><td>5.</td><td>Cover plate and incubate at 37°C for 30 minutes.</td><td></td></tr>
</table>

<table>
<tr><td>6.</td><td>Measure the plate at 570 nm on the plate reader.</td><td>Plate reader password is 0000.</td></tr>
<tr><td>7.</td><td>Normalize all samples to the lowest concentration using NP40 lysis buffer.</td><td></td></tr>
<tr><td>8.</td><td>Add 4x Laemmli buffer to samples and incubate at 70°C for 5-10 minutes to denature proteins.</td><td></td></tr>
<tr><td></td><td>C. Casting gels</td><td></td></tr>
<tr><td>1.</td><td>Clean the glass plates used for gel casting in the following order:
Soap → tap H_2O → ddH_2O → MeOH
Scrape off any gunk with a blade.</td><td></td></tr>
<tr><td>2.</td><td>Prepare the gel casting apparatus and add ddH_2O to check for leaks.</td><td></td></tr>
<tr><td>3.</td><td>Prepare the appropriate volume of resolving gel.
<table>
<tr><th>14% Resolving Gel</th><th>10 ml</th><th>20 ml</th></tr>
<tr><td>dH_2O</td><td>2.6 ml</td><td>5.2 ml</td></tr>
<tr><td>30% Acrylamide (29:1)</td><td>4.7 ml</td><td>9.4 ml</td></tr>
<tr><td>Tris 1.5M pH 8.8</td><td>2.5 ml</td><td>5 ml</td></tr>
<tr><td>10% SDS</td><td>100 µl</td><td>200 µl</td></tr>
<tr><td>10% APS (fresh)</td><td>100 µl</td><td>200 µl</td></tr>
<tr><td>TEMED</td><td>10 µl</td><td>20 µl</td></tr>
</table></td><td>10 ml resolving gel is sufficient for 2 gels.

The reaction between APS and TEMED is what causes the gels to solidify. Do not add TEMED until you are ready to pour the gels.</td></tr>
<tr><td>4.</td><td>Load resolving gel into the space between the glass plates (up to the imaginary line at hinges).</td><td>Ensure there are no air bubbles.</td></tr>
<tr><td>5.</td><td>Gently add a layer of dH_2O up to the green line.</td><td>The H_2O layer levels the resolving gel and prevents over-drying.</td></tr>
<tr><td>6.</td><td>Allow gel to solidify for 15 min.</td><td></td></tr>
<tr><td>7.</td><td>Pour off the H_2O layer. Use a kimwipe to remove all remaining H_2O.</td><td>Throw away any gels that have air bubbles.</td></tr>
<tr><td>8.</td><td>While the resolving gel is setting, prepare the appropriate volume of stacking gel.
<table>
<tr><th>4% Stacking Gel</th><th>10 ml</th><th>20 ml</th></tr>
<tr><td>dH_2O</td><td>6 ml</td><td>12 ml</td></tr>
<tr><td>30% Acrylamide (29:1)</td><td>1.33 ml</td><td>2.66 ml</td></tr>
<tr><td>Tris 0.5M pH 6.8</td><td>2.5 ml</td><td>5 ml</td></tr>
<tr><td>10% SDS</td><td>100 µl</td><td>200 µl</td></tr>
<tr><td>10% APS (fresh)</td><td>100 µl</td><td>200 µl</td></tr>
<tr><td>TEMED</td><td>10 µl</td><td>20 µl</td></tr>
</table></td><td>10 ml stacking gel is sufficient for 3-4 gels.

Do not add TEMED until you are ready to pour the gels.</td></tr>
<tr><td>9.</td><td>Clean green spacer combs with EtOH.</td><td></td></tr>
<tr><td>10.</td><td>Pour stacking gel between the glass plates. Allow to overflow and gently insert the comb.</td><td></td></tr>
</table>

11.	Allow gel to solidify for 15 min. Once solidified, scrape off any excess gel from the outside of the glass plates.	*Solidified gels can be wrapped in saran wrap and stored at 4°C.*
	D. Running gels	
1.	Load gels into the apparatus with the shorter glass plate facing inwards.	*Use the buffer dam for an odd number of gels.*
2.	Remove the combs and clear the wells with running buffer and a transfer pipette.	*Be careful to avoid breaking or deforming the wells.*
3.	Add running buffer to fill the interior of the apparatus (between the two gels). Allow to overflow until the exterior of the apparatus is filled to the correct volume for the number of gels being run.	
4.	Pre-run the gel at 150V for 10 minutes prior to loading samples.	
5.	Using gel loading tips, load 5 µl of the Amersham protein ladder (located in the Chiu lab's freezer) and normalized samples into the wells.	*Use the gel loading guide if you have difficulty seeing the wells.*
6.	Fill with running buffer and run the gel at 150V for 1.5 to 2 hours, or until the sample buffer is at the bottom of the gel.	
	E. Transfer	
1.	Prepare the membranes while the gel is running by washing in old tip boxes. 1st wash: MeOH 15 seconds 2nd wash: ddH2O 2 minutes 3rd wash: Transfer buffer	*Transfer buffer should be cold. Leave the membranes in transfer buffer until ready to use.* *Always use tweezers when handling the membrane.*
2.	Soak the 4 black fiber pads and 4 large filter papers in a tray of cold transfer buffer. Begin building your sandwich by placing a filter paper on top of a black fiber pad.	*Trim filter papers to the same size of the sponges.*
3.	Use a blade to separate glass plates and cut off the stacking gel. Run the blade down the sides of the gel and gently remove from the plate.	
4.	Lay the gel onto the filter paper such that the ladder is on the right. Smooth out any ruffles in the gel.	
5.	Carefully lay the prepared membrane over the gel. Do not adjust the membrane after it is laid onto the gel.	*Always use tweezers when handling the membrane.*
6.	Cover the membrane with a filter paper and use a broken glass pipette to gently roll out any bubbles between the gel and the membrane.	*Roll out bubbles without pressing down on the sandwich.*

7.	Place a second fiber pad on top of the filter paper to complete the sandwich.	
8.	Load into the cassette such that the clear side is on the top of the sandwich (membrane side) and tighten the latch.	*See diagram in the Trans Blot instructions.*
9.	Place the assembled cassettes into the Trans Blot apparatus and load into the tank. Add the frozen blue cooling block.	*The black side of the cassette should be facing toward the electrodes.*
10.	Pour chilled transfer buffer into the "blotting" mark on the tank.	
11.	Run at 100V for 1 hour, or 30V overnight in the cold room.	
	F. Probing the membranes	
1.	Remove membranes from the transfer cassette and transfer to a tip lid. Wash in TBST for 5 minutes on a rocker.	
2.	Discard TBST and add about 20 ml of blocking buffer. Block membranes by rocking for 1 hour.	
3.	Prepare 1° antibody solution in 1:1 blocking buffer:TBST.	*Concentrations typically range from 1:100 to 1:1000.*
4.	Discard blocking buffer and pour 1° antibody solution over membranes. Place on a rocker and incubate for 1 hour at room temperature or overnight at 4°C.	
5.	Discard the 1° antibody solution and fill trays with TBST. Wash on a rocker for 10 minutes, 3x.	*Replace with fresh TBST after each wash.*
6.	Prepare 2° antibody solution in TBST.	*Concentrations typically range from 1:1000 to 1:5000.*
7.	Discard TBST and pour 2° antibody solution over membranes. Place on a rocker and incubate for 1 hour at room temperature.	
8.	Discard the 2° antibody solution and fill trays with TBST. Wash on a rocker for 10 minutes, 3x.	
9.	Transfer the membranes to plastic wrap or a plastic sheet protector. Ensure there are no air bubbles between the plastic and the membrane.	
10.	Prepare ECL substrate. For ECL 2, the ratio is 40:1 substrate A: substrate B For SuperSignal West Dura, the ratio is 1:1 stable peroxide solution: luminol/enhancer solution.	*Prepare roughly 750 µl of substrate per blot. Always probe with the less sensitive substrate first.*

11.	Pipet the prepared ECL substrate over the front of the blot (ladder to the left) and incubate for 1 minute.	
12.	Cover the membrane with the plastic wrap or sheet protector, again ensuring that there are no air bubbles between the membrane and the plastic.	
13.	Image on the Waterman lab imager using the MLW program at the bottom of the list. Save images to the lab Dropbox or to a USB drive.	
14.	Membranes may be stored in plastic wrap at room temperature.	
	G. Stripping the membranes for re-probing	
1.	If the blot is dried, rehydrate by washing in TBST for 5-10 minutes.	
2.	Transfer the blot to a tip lid and add 20 ml 0.2N NaOH. Wash on a rocker for 20 minutes, 2x.	*Replace with fresh NaOH after each wash.*
3.	Discard NaOH and fill tray with TBST. Wash on a rocker for 5 minutes, 5x.	*Replace with fresh TBST after each wash.*
4.	Membranes are now stripped and can be re-probed, beginning with the blocking step (see step F.2 of this protocol).	*Membranes will lose some protein with every stripping. Do not strip and re-probe more than twice.*