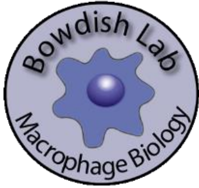

WESTERN BLOT

Updated by: Danny Ma Date: July 2019
Created by: Kyle Novakowski Date: November 9th, 2012
Bowdish Lab, McMaster University
Hamilton, ON, Canada
www.bowdish.ca

PROTOCOL

SAMPLE PREPARATION – BONE MARROW-DERIVED MACROPHAGE CELL LYSIS

1. Aspirate media off cells. Wash with 2 mL PBS. Remove PBS.
2. Mix 25 µL protease inhibitor and ½ tab of PhosSTOP™ (phosphatase inhibitor; Sigma) per 5 mL of RIPA lysis buffer. Note: the phosphatase inhibitor is required if you are probing for phosphorylated proteins.
3. Add ~100 µL lysis buffer solution to each well of the 6-well plate. Adjust accordingly depending on the density of your cells so that your samples are sufficiently concentrated.
4. Incubate plates at 4°C for 10 minutes. Grab ice bucket.
5. Use a cell scraper (Falcon) to lyse all cells in RIPA lysis buffer. Ensure that the plate remains on ice.
6. Transfer solution to Eppendorf tubes and freeze at -80˚C.

MEASURING PROTEIN CONCENTRATION OF SAMPLES W/ PIERCE BCA PROTEIN ASSAY KIT

1. Prepare diluted BSA standards for the standard curve. Use the following table as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard ampule (2 mg/mL) into Eppendorf tubes using RIPA buffer as that is the same diluent as the samples.

Vial	Diluent (µL)	Standard Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	25 of stock	2000
B	37.5	112.5 of stock	1500
C	75	75 of stock	1000
D	75	75 of vial B solution	750
E	75	75 of vial C solution	500
F	75	75 of vial E solution	250
G	75	75 of vial F solution	125
H	120	30 of vial G solution	25
I	25	0	0 = blank

2. Prepare the BCA working reagent (WR). 200 µl of WR reagent is required for each sample (including the standard curve samples). Use the following formula to determine the total volume of WR required:
3. (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required
4. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). Mix using a vortex. For example, combine 50 mL of Reagent A with 1 mL of Reagent B.
5. Pipette 10 µL of each standard or unknown sample into a 96-well microplate. Note: pipette standard samples in duplicate.
6. Add 200 µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
7. Cover plate and incubate at 37°C for 30 minutes.
8. Cool plate to RT. Measure the absorbance at or near 562 nm on a plate reader.
9. Using the standard curve, determine the protein concentrations of each sample.

MAKING GELS

1. Clean an appropriate number of 1.5 mm glass plates & front cover plates. Assemble the plates into the green holder. Place a grey rubber piece on the white 2-gel holder and add the plates. Test the seal by adding water between the glass plates and wait 2-5 minutes. Remove water and dry with a kimwipe if no leakage occurs.
2. Use the following table to create the separating gel, followed by the stacking gel. Ensure that 10% ammonium persulfate and TEMED are added to the mixture right before pouring into the mold.

	Volume (mL) to create 2x 10% separating gel	**Volume (mL) to create 2x stacking gels**
ddH_2O	8.0	5.2
30% acrylamide mix	6.6	1.54
1.5M Tris (pH 8.8)	5.0	0.0
1.0M Tris (pH 6.8)	0.0	1.0
10% SDS	0.200	0.080
10% APS	0.200	0.080
TEMED	0.008	0.008

3. Pour 10% separating gel into mold and top up with 70% ethanol. Wait approximately 40 minutes for the gel to polymerize.
4. Following polymerization, pour out the ethanol and wash with ddH_2O.
5. Pour the stacking gel into mold and insert comb. Wait approximately 10 minutes for the gel to polymerize. Note: gels can be made and stored for up to one month at 4°C.

SAMPLE PREPARATION

1. To determine the amount of sample required, refer to the BCA results from step I. The protein concentration will vary from sample to sample, requiring differing volumes of sample to be loaded into each tube. Ensure that an equal amount of protein is loaded between samples.

2. Each well on the gel can hold up to 40 µL. For a total volume of 40 µL, put 10 µL of 4x sample loading buffer into each tube.
3. Calculate the amount of water required: To bring each volume to 40uL, apply the following calculation to each sample: 40 µL – 10 µL loading buffer - ?uL sample = amount of water required.
4. Retrieve the tubes you prepared in step 1 and add the required amount of water to each sample. Always add the water first. (Reasoning: if you make a mistake, you can simply reprepare the loading buffer and start again. If you had added sample first, you cannot fix your mistake).
5. After the correct volume of water and loading buffer has been added to each tube, add the required amount of sample. As soon as you add sample to the tube, keep the tubes on ice.
6. Boil samples for 10 minutes at 99°C on the heat block.

SDS-PAGE AND TRANSFER TO PVDF MEMBRANE

1. Assemble the MiniPROTEAN cassette according to the manufacturer's instructions.
2. Load the gel into the insert by ensuring that the comb is also facing the gasket.
3. If running one gel, a buffer dam is required: place the buffer dam with the text facing the gasket (or inside of the chamber) into the insert.
4. Prepare 1X running buffer from the 5X running buffer solution. Add 240 mL of 5x stock to 1.2L of MilliQ water.
5. Pour the 1X running buffer in between the buffer dam and gel. Monitor the water level for 10 minutes to ensure it isn't leaking. This is very important and can impact the integrity of the way the gel runs.
6. After you have ensured it isn't leaking, add more 1X running buffer to the tank up to the line that says "4 Gels".
7. Remove the comb from the gel in a swift upward motion, careful not to move laterally and disrupt the wells. Do not remove the comb in a dry chamber; always make sure that the gel is in is running buffer before you remove it.
8. Load the wells of the gel with the samples. Plan the way you want to load the gel and record the loading order before you load the gel.
9. Load one well with 7.5 µL of Precision Plus Protein™ Kaleidoscope™ Prestained Protein Ladder (BioRad).
10. Run at 150V at room temperature. Run until the dye front runs off of the gel.
11. Prepare 1X transfer buffer. Add 100 mL of 10X transfer buffer and 200 mL of Methanol to a 1L graduated cylinder. Fill to 1L with ddH2O. Chill the transfer buffer at 4C before you transfer, if possible.
12. Prepare the transfer sandwich by retrieving the following: 1 transfer tank, the black and red insert, the cassette, 2 sponges, 2 Whatman paper, and 1 PVDF membrane.
13. Fill the Pyrex glass container with cold transfer buffer and begin preparing the sandwich.
14. Soak 2 pieces of Whatman paper (per gel) in transfer buffer along with the sponges
15. Soak PVDF membrane in methanol until wet and place in transfer buffer.
16. Place gel into transfer buffer. Remember to remove the wells (stacking gel portion) from the gel using a green cutter.
17. Assemble "sandwich": Black plastic → Sponge → Whatman paper → Gel → PVDF membrane → Whatman paper → Sponge → White plastic
18. Make sure there are no bubbles between the gel and PVDF membrane; roll with a 15 mL tube. 9. Place "sandwich" in MiniPROTEAN cassette.
19. Pour 1L transfer buffer into tank. Place cassette with transfer sandwich into tank. Ensure that the black part of the sandwich should face the black part of the cassette
20. Add small ice pack to tank. Run for 70 minutes at 70V and 4°C.

ANTIBODY STAINING

1. Incubate the membrane at room temperature on the shaker for 1 hour in 5% BSA to block.
2. After blocking, wash twice in TBS-t for 10 minutes each.
3. After blocking, the membrane can be cut to facilitate probing with multiple antibodies of differing molecular weights. This must be done carefully and after blocking using a razor blade and a glass plate.

4. Prepare the primary antibody of interest. All primary antibodies are prepared in in TBS-t with 0.5% BSA. 0.5% BSA is made using 1 mL of 5% BSA and 9 mL of TBS-t (1:10 dilution). Then, the appropriate amount of antibody is added (often 10 µL for 1:1000 dilution).
5. Incubate membrane with primary antibody overnight at 4°C in the cold room with motion (or for 1 hour at room temperature). Ensure that the membrane is covered.
6. After primary antibody incubation, retrieve the antibody; it can be frozen at -20°C and be reused. Be careful not to pour off the primary antibody.
7. Prepare the secondary antibody of interest. Secondary antibodies are prepared in in TBS-t (often 1:100,000 dilution; 1 µL in 10 mL TBS-t). I used the Donkey Anti-Rabbit IgG Polyclonal Antibody (IRDye® 800CW) and Goat Anti-Mouse IgG Polyclonal Antibody (IRDye® 800CW) from LI-COR Biosciences as we use the Odyssey® CLx Imaging System. Note that the Odyssey® CLx Imaging System can simultaneously two different antigens on the same blot and this may be of interest.
8. Incubate membrane with secondary antibody at room temperature for 1 hour with motion. Ensure membrane is covered with foil as the LI-COR secondary antibodies are light-sensitive.
9. Visualize membrane using Odyssey® CLx Imaging System if using secondary antibodies from Licor.

ANTIBODY STRIPPING

1. After membrane visualization, wash membrane 3 times for 5 minutes each in TBS-t. Ensure the membrane is not allowed to dry.
2. Incubate membrane for 30 minutes at 50°C in stripping buffer (with slight agitation). Stripping buffer can be prepared as follows: 6.25 mL of 1M Tris-HCl pH 6.8, 10 mL of 20% SDS and 700 µL β-mercaptoethanol. Bring to 100 ml with deionized H20. Make buffer fresh just prior to use.
3. Wash membrane 3 times for 5 minutes each in TBS/T.
4. The membrane is now ready to reuse. Start detection at Step 1 of "Antibody staining."

BUFFER RECIPES

5X Running Buffer

- 15.1g Tris base (25mM)
- 94g glycine (250mM, pH = 8.3)
- 50ml 10% SDS (0.1%)
- Top up to 1L with MilliQ H2O.

10X Transfer Buffer

- 30.2g Tris
- 144g glycine
- Top up to 1L with MilliQ H2O.

Making 1L 1X TBST:
10X TSB in large flasks by gel dock

- 100ml 10× TBS
- 900ml MilliQ H2O
- 1ml Tween 20
- Stir 5min.