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Western Blot Protocol (Cell Lysate) and Creating Running Gel

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Protocol status: Working
I use this protocol to quantify proteins in all my western blot protocols. This protocol consistently develops.

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Abstract

A basic Western Blot protocol that has been optimized specifically for cell lysates.

Before start

You will need to optimize the weight of your running gel before you begin. Most proteins will be separated with 8%-12% acrylamide gels. If your protein runs off your gel, you will need to use a heavier gel. See step 12 for more information.

CELL PROTEIN PREPARATION PROCEDURE

1 Seed 2mL of cells at desired density in 6mL TC-coated petri dish

Note: Attached to this step is a document that has all the solutions described in this protocol.

western blot 2017.docx

2 Drug and stimulate cells as desired

3 Wash cells with ice-cold PBS

4 Aspirate the PBS and add 0.3mL ice-cold RIPA buffer

5 Incubate 5 min on ice

6 Scrape cells off the dish using a cold plastic cell scraper and transfer to a 0.5mL microcentrifuge tube

7 Centrifuge the samples at 10000xg for 5 minutes

8 Pipette the supernatant (containing the protein) into a fresh 1.5mL centrifuge tube, discard the pellet

GEL PREPARATION PROCEDURE (DAY 1)

9 Clean gel plates with distilled water (make sure they are “1.0mm” plates)

10 Place plates into green casting frame

11 Place casting frame (with plates) into the casting stand

12 Make lower gel.

If using a bigger protein, use a lower gel mix percentage

Guidelines for Acrylamide % in Gels
<60kDa = 12%
60-110 kDa = 10%
>110 kDa = 8%

IMPORTANT: Do **NOT** add TEMED until you're ready to start pouring the gel, as it will begin gel solidification

13 Have gel components (minus the TEMED) and ethanol ready

14 Add TEMED to gel components and immediately pour the lower gel between the plates

15 Add 100uL-1mL ethanol over top of the lower gel until it is even

16 Let the lower gel sit for 20 minutes to solidify.

During this time you can:
1. Make 1x Laemelli Running Buffer
2. Make 1x Towbin Transfer Buffer
3. **Make upper gel**
4. Get plastic lane combs (make sure they are 1.0mm)

17 Pour out ethanol above lower gel

18 Wash lower gel with distilled water once

19 Pour out distilled water above lower gel

20 Remove excess distilled water using the corner of a piece of Whatman paper (do NOT touch the gel)

21 Add TEMED to the upper gel components and immediately pour the upper gel overtop the lower gel until overflowing

22 Immediately add lane combs to the stacking gel, making sure there are no bubbles

23 Let upper gel sit for 10 minutes to solidify
1. During this time you can:
2. Boil ladder (11uL Biotin ladder mixed with 11uL Opti Marker ladder) in water bath for 2 minutes – hold tube with tongs
3.
4. Boil proteins in heating block containing water at 90^{o}C for 4 minutes
5.

24 Carefully remove lane combs from the upper gel

25 Wash once with distilled water by causing the water to overflow out of the casting frame

RUNNING WESTERN BLOT PROCEDURE (DAY 1):

26 Move gel plates to a clamping frame and place in a gel running box

27 Add 1x Laemmli running buffer just below the top of the plates, and to the appropriate line in the gel box

28 Make sure there are no bubbles where the gels are by aspirating the bubbles off

29 Add 10uL of sample to each gel lane, by slowly releasing sample until the first pipette stop (you will need to run a standard curve of various volumes to determine which volume to use; you want to make sure you can detect higher and lower protein concentrations and do not saturate the bands)

30 Run the gel at 160V (3.00A) at room temperature until the dye front begins to run off the bottom (usually 1h20min)

31 While gel is running:
1. Cut and label nitrocellulose membranes
2. Cut Whatman paper (if necessary)
3. Wash sponges in distilled water
4. Make 1x Towbin transfer buffer
5. Get a plastic tray to work in
6. Label transfer boxes

WESTERN BLOT TRANSFER AND QUANTIFICATION PROCEDURE (DAY 1)

32 Once gel is complete, make your gel sandwiches for protein transfer onto the nitrocellulose membranes

Gel sandwich (from black side to clear side)
1. Black sponge
2. White Sponge
3. 1 Piece of Whatman paper
4. Gel
5. Nitrocellulose membrane (with writing face down against the gel)
6. 1 piece of Whatman paper
7. Roll out air bubbles gently (use a roller, 50mL conical w/o lid, etc.)
8.
9. 2 pieces of Whatman paper
10. White sponge
11. Black sponge
12. Roll out air bubbles firmly (use a roller, 50mL conical w/o lid, etc.)
13.
14. Seal gel sandwich and place in transfer box (black side of sandwich chamber should face black side of transfer box)
15. Add an ice pack to the transfer box
16. Fill area around transfer box with packed ice
17. Fill transfer box with 1x Towbin transfer buffer

33 Run protein transfer at a current of 0.30A for 1h 15min (you will need to set a higher voltage but press run when current is selected)
Meanwhile, you can make 10mL Blocking Solution and store at 4oC (5% w/v Skim Milk Powder in TBS-T)

34 After protein transfer, move the membrane to a small membrane Tupperware box

Ponceau stain the nitrocellulose membrane to visualize transferred proteins and warm up camera

Take a picture of the stained transferred proteins with a 18-70mm lens

35 Wash membranes 3 times on a shaker with TBS-T for 5 minutes to remove Ponceau stain in shaker. Ensure there is no color left.

36 Add Blocking Solution to the membrane Tupperware box and shake for 1h

37 Pour off excess Blocking Solution

38 Add on 1^{o} antibody dissolved in blocking solution (usually 1:1000 dilution) into membrane Tupperware box and shake overnight at 4^{o} C. **STORE EMPTY CONICALS IN FRIDGE OVERNIGHT** as you can use the 1^{o} antibody for months.

Note: You can add the 1^{o} antibody for only an hour at room temperature, but your bands will be less clear and there may be more background bands.

WESTERN BLOT TRANSFER AND QUANTIFICATION PROCEDURE (DAY 2)

39 Place 1^{o} antibodies back in the stored 50mL conical and freeze for reuse

40 Wash membrane with TBS-T at room temperature for 1h on a shaker (add TBS-T every 10 minutes)

41 Add on 2^{o} antibody dissolved in blocking solution into membrane Tupperware box and shake at room temperature for 1h

42 Wash membrane with TBS-T at room temperature for 1h on a shaker (add TBS-T every 10 minutes) and turn on chemiluminescent camera at this time

Develop and Quantify Blots

43 Dry off membrane in Saran wrap and place in a new Tupperware container

Make ECL solution

44 Add 1mL light ECL reagents (per membrane) and 1mL dark ECL reagent (per membrane) into a 15mL conical

Important: Change tips to avoid contaminating ECL reagents)

45 With a 1mL pipette, add 2mL ECL solution over the membrane drop by drop until covered

46 Cover the membrane box (with aluminum foil) for 5 minutes

47 Take picture using chemiluminescent machine in the anteroom

48 Take picture at 70mm using the 18-70mm lens, using the aperture ring to get the correct exposure
OR the settings that are specific to the chemiluminescent machine available in your lab.