Western Blot Protocol (Cell Lysate) and Creating Running Gel

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Abstract

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

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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.

Protocol

CELL PROTEIN PREPARATION PROCEDURE

Step 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.

Step 2.
Drug and stimulate cells as desired

Step 3.
Wash cells with ice-cold PBS

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

Step 5.
Incubate 5 min on ice

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

Step 7.
Centrifuge the samples at 10000xg for 5 minutes

Step 8.

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

GEL PREPARATION PROCEDURE (DAY 1)

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

Step 10.
Place plates into green casting frame

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

Step 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

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

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

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

Step 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)

Step 17.
Pour out ethanol above lower gel

Step 18.
Wash lower gel with distilled water once

Step 19.
Pour out distilled water above lower gel

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

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

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

Step 23.

Let upper gel sit for 10 minutes to solidify

1. During this time you can:
    1. Boil ladder (11uL Biotin ladder mixed with 11uL Opti Marker ladder) in water bath for 2 minutes – hold tube with tongs
    2. Boil proteins in heating block containing water at 90°C for 4 minutes

Step 24.
Carefully remove lane combs from the upper gel

Step 25.

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

RUNNING WESTERN BLOT PROCEDURE (DAY 1):

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

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

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

Step 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)

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

Step 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)

Step 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
    1. Roll out air bubbles gently (use a roller, 50mL conical w/o lid, etc.)

1. 2 pieces of Whatman paper
2. White sponge
3. Black sponge
    1. Roll out air bubbles firmly (use a roller, 50mL conical w/o lid, etc.)
4. Seal gel sandwich and place in transfer box (black side of sandwich chamber should face black side of transfer box)
5. Add an ice pack to the transfer box
6. Fill area around transfer box with packed ice
7. Fill transfer box with 1x Towbin transfer buffer

Step 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)

Step 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

Step 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.

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

Step 37.
Pour off excess Blocking Solution

Step 38.

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

Note: You can add the 1° 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)

Step 39.

Place 1° antibodies back in the stored 50mL conical and freeze for reuse

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

Step 41.

Add on 2° antibody dissolved in blocking solution into membrane Tupperware box and shake at room temperature for 1h

Step 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

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

Make ECL solution

Step 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)

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

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

Step 47.
Take picture using chemiluminescent machine in the anteroom

Step 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.