Western Blot Protocol

Sample Preparation

1. Prepare a lysate from cell culture or tissue source. Your choice of lysis buffer is dependent on the localization of your protein, as membrane bound proteins will require stronger lysis buffers than soluble proteins. Always use freshly made protease inhibitors to prevent degradation, keep samples on ice, and work quickly.

2. Determine the total protein concentration of your sample lysate by testing a small portion of the lysate with a protein quantitation assay such as the BCA, Lowry, or Bradford. This will assist you in loading equal sample across wells.

3. Prepare samples by diluting each in a reducing sample buffer such as Laemmli Buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 6.8).

4. Heat samples at 95°C for 5 minutes to denature the proteins.

Protein Electrophoresis

1. Prepare your SDS-PAGE gel by inserting it into the electrophoresis apparatus and filling with running buffer. Rinse the wells of the gel with running buffer and verify there are no bubbles.

2. Load the appropriate amount of sample into each well. If you are unsure of the amount to load, 10-30 µg of total protein is a suggested starting point.

3. Load pre-stained molecular weight ladders in the first and last lane in order to monitor the separation during electrophoresis, and subsequently verify protein sizes during analysis.

4. Run the gel until the loading buffer reaches the bottom. This is typically 45-60 minutes at 200 V (consult manufacturer's guidelines).

Membrane Electrotransfer (Wet Transfer)

1. Remove gel from the cassette and float in transfer buffer.

2. Choose either PVDF or nitrocellulose membrane for transfer (personal preference). PVDF needs to first be activated in methanol for 2 minutes. Incubate membranes in cold transfer buffer for 10 minutes.
3. Set up gel/membrane sandwich by placing the transfer cassette in cold transfer buffer. Create a sandwich stack by placing the following components from the cathode (negative black side) to the anode (positive red side) so that the negatively charged proteins move from the gel into the membrane:

   (- side) sponge, filter paper, gel, membrane, filter paper, sponge **(+ side)**

   Use a clean roller with each layer to gently roll out any bubbles present.

4. Lock the cassette and place in the transfer apparatus containing cold transfer buffer. Perform the transfer according to the manufacturer's instructions (normally 100 V for 30-120 minutes). In order to prevent heat buildup, it is beneficial to transfer with a cold pack in the apparatus, or in the cold room with a spinner bar.

Immunoblotting

1. Remove the membrane from the cassette and wash three times in ddH2O.

    1. Optional: Verify protein transfer by Ponceau red staining the membrane or Coomassie staining the gel.

2. Incubate the membrane with blocking solution for 1 hour at room temperature. Common blocking buffers include 5% non-fat dry milk, or 3% BSA, in TBS-T (1x TBS, 0.1% Tween-20). Do not use milk when probing with phospho-specific antibodies.

    1. Disclaimer: If you are using an antibody that is raised in goat, block including BSA or milk may cross react with the antibody. Use 5% rabbit or mouse normal sera in TBST for blocking and diluent buffer instead. Try to avoid using the sera from the host of your secondary as well.

3. Decant blocking solution and wash with TBS-T for 5 minutes.

4. Dilute the primary antibody in blocking buffer at the concentration recommended on the datasheet. Incubate membranes with primary antibody overnight at 4°C on with gentle shaking.
    1. Recommended: The use of a positive loading control antibody is encouraged. Doing so allows the user to verify equal amounts of total protein were loaded into each well, and aids in troubleshooting by removing any uncertainties with the western blot procedure.

5. Decant primary antibody and wash membrane with large volumes of TBS-T and vigorous agitation five times for 5 minutes each.

6. Dilute the secondary antibody in blocking solution and incubate the membrane for one hour at room temperature at the concentration recommended on the datasheet. The secondary should be directed against the host species of your primary antibody. E.g. If using a primary that was raised in rabbit, the appropriate secondary would anti-rabbit. Most secondaries are conjugated to an enzyme such as HRP in order to catalyze the electrochemiluminescent (ECL) reaction.

7. Decant secondary and wash membrane with large volumes of TBS-T and vigorous agitation five times for 5 minutes each.

8. Mix equal parts ECL reagents (1:1) according to the manufacturer's instructions. Incubate the membrane for 3-5 minutes without agitation.

9. Decant ECL mixture and use a laboratory wipe to wick off excess solution from the corner of the membrane. Place the membrane in a clear plastic wrap, such as a sheet protector, to prevent drying. Do not let the membrane completely dry out.

10. Expose the membrane to x-ray film and develop, or use an ECL capable imaging device.

11. Relative band densities can now be compared with commercially available software.