

## Western Blot Method

### Protein Electrophoresis

Prepare cell / tissue lysate(s) (see cell / tissue lysis protocol)

Make up lysates to a total volume of 10-20 ul with 4x SDS sample buffer (see recipes) containing a reducing agent (either 10% B-ME or 0.3M DTT in the 4x SB).

Prepare SDS-PAGE gel (see SDS-PAGE gel pouring). If pouring your own, this should be performed before starting the sample preparation. Numerous manufacturers sell a variety of pre-cast gel types and polyacrylamide concentrations with shelf life in the region of a year (Invitrogen, Biorad) and a corresponding sample buffer, running buffer and denaturant to go with this. This alternative to preparing your own is a time saving way. When considering what percentage gel to use, this depends on the protein of interest. For proteins whose MW is over 100 kDa, the percentage is 7%, for 50-100kDa, it is 10%, for 20-50kDa, it is 12%, and it is 15% for < 20kDa. Alternatively, a gradient gel (4-12%, 10-20%) is a good choice if you could not make sure the percentage or want to detect a range of proteins for multiple probing.

Place gel in running chamber and fill with 1 x SDS Running Buffer. Use 10x stock (see recipes).

Heat samples at 95-100C for 5 minutes.

Load 5ul of a control pre-stained protein ladder to track sample migration in the first lane of the gel.

This is also useful for the transfer step to verify the procedure is successful. These are widely available for most suppliers (Invitrogen, Biorad, Fermentas, etc).

Load boiled samples.

Electrophorese at constant 100V until the blue bromophenol dye reach the bottom of the gel.

### Protein Transfer

Place gel and blotting pads in transfer buffer solution.

Transfer gel to filter paper wetted in transfer buffer solution.

Assemble transfer sandwich by orientating cathode, filter paper, gel, membrane (nitrocellulose / PVDF), filter paper, anode so protein transfer goes in the direction of cathode to anode. These are typically coloured black (cathode) and red (anode). The choose of membrane (nitrocellulose / PVDF) depends on the protein studied and the antibodies used. Generally you will get the use instructions you need through antibody guidelines. PVDF is particularly useful if you are working with small or highly charged proteins. Remember to make PVDF soaked in methanol for 5 minutes prior to use.

Electrophorese for 90 minutes at 100V or overnight at 4°C at 20V. Proceed electrophoresis on ice or in a cold room as the transfer generates a lot of heat!

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Check membrane for transfer of control protein ladder. If using nitrocellulose, sample transfer and relative concentration can be assessed by staining the membrane with Ponceau S solution (0.1% ponceau, 5% acetic acid). This is a reversible stain which is removed by a simple wash in TBST.

### Protein Labeling

Incubate membrane in blocking solution for 1 hour at room temperature or at 4°C overnight with shaking. The blocking solution is normally composed of 5% non-fat milk in TBS-T, and some antibodies require BSA in place of milk. This is normally clear in the manufacturers instructions for the antibody for testing. To make TBS-T, using 10 x TBS, add Tween-20 to a final concentration of 0.05%.

Incubate primary antibody overnight or at room temperature for 2 hours prepared in 5% milk/TBST.

Remove antibody solution and wash membrane three times for 5-15 minutes in TBST.

Incubate membrane with an appropriate secondary antibody (peroxidase conjugated) for 1 hour at room temperature in 5% milk/TBST. For example if your primary antibody was raised in rabbit, a goat anti-rabbit HRP should be used as a secondary antibody.

Remove antibody solution and wash membrane three times for 5-15 minutes in TBST.

### Protein Detection

There are numerous commercial available chemiluminescence reagents (Amersham, Pierce, Invitrogen) and a range of sensitivities of detection levels is included. These typically take the form of two solutions which are combined and then incubated immediately on the membrane for 1 – 5 minutes. Make membrane exposed to X-ray film for 1 minute to 1 hour, depending on protein signal and chemiluminescence method.

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