WESTERN BLOTTING OF PHOSPHO-PROTEINS PROTOCOL

Homogenize the cells or tissue of interest in lysis buffer made fresh and containing a cocktail of protease inhibitors (and phosphatase inhibitors when dealing with phosphorylated proteins).
As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added **fresh** to the lysis buffer.
Use a RIPA or NP40 buffer supplemented with fresh protease and phosphatase inhibitors:
Ready to use cocktails of inhibitors from well known suppliers are often used but you can make your own cocktail.

Remember to add phosphatase inhibitors to cocktails bought when investigating phosphorylation events.
Final concentrations of inhibitors: see table in WB protocol I

1. To a sample of protein solution containing 1-100 ng of the target protein (500 ug lysate), add an equal volume of 2x SDS-PAGE sample buffer. For reduced samples, the sample buffer should be supplemented with DTT or ß-mercaptoethanol. For non-reduced samples, the DTT or ß-mercaptoethanol is not added.

2. Denature the proteins by heating the sample to 95°C, or boiling, for 5 min.

3. Load the sample onto an SDS-polyacrylamide gel and run the gel under standard conditions.

4. Transfer the proteins to a PVDF membrane using semi-dry or wet transfer methods. Please note: for PVDF it is essential to pre-wet the membrane in methanol prior to transfer.

5. If required, the efficiency of transfer can be determined by staining the membrane briefly (10 s) in Ponceau stain. The stain can be removed by washing in PBST or TBST. We would recommend not washing blots in distilled water as this can strip off proteins in some circumstances.

6. Block the membrane with 5% w/v BSA in TBST. Incubate for 1 h at 4°C with agitation.

7. Dilute the primary antibody in TBST to the recommended dilution. We recommend incubating in a sealed bag, hybridization tube or 50 ml Falcon tubes (~2.5 ml primary antibody/blot). Incubate overnight at 4°C with agitation.

8. Rinse the blot in TBST three to four times for 5 min each at room temperature.

9. Dilute the horseradish peroxidase (HRP) labeled secondary antibody at the recommended dilution (1/5000 is usually a good working dilution although this needs to be optimized for the particular application) in TBST.

10. Rinse the blot in TBST three to four times for 5 min each at room temperature.

11. Perform ECL Plus detection.

Top tips for WB of phosphorylated proteins:
1. Keep the proteins in their phosphorylated state! Add adequate phosphatase inhibitors and keep samples on ice at all times.
2. Block the membrane in 5% w/v BSA (fractionV) NOT MILK (milk contains casein which is a phosphoprotein; This is why it causes high background because the phospho-specific antibody detects the casein present in the milk).
3. Remember the phosphorylation may need to be induced. Low signal or no signal may mean that the induction is not sufficient. Run the recommended positive control with your samples.