Western Blot Protocol

Gel run

Resolve 20-30ug of protein sample by SDS-PAGE and electroblot. There are many commercially available setups for this, please follow the manufacturer's recommendations.

Stain/Destain (optional)

If desired, the blots can be stained for 5 min with 0.1% Amido Black (in 25% isopropanol + 10% acetic acid + DI H2O) and then destained with 25% isopropanol + 10% acetic acid + DI H2O. The blot can be blocked immediately or allowed to air dry.

Staining helps monitor the efficiency of transfer, but is not required. If you feel the staining may interfere with the immunoblotting, this step can be skipped. Be sure to use a prestained molecular weight marker if the blot will not be stained.

Blocking

Without letting the blot dry, block for 15-30 min. Shake gently while blocking. If the blot has dried, wet it for a few seconds with 100% methanol, wash with TBST, and begin blocking. Which blocking buffer to use depends whether the target is a phosphoprotein or not.

a. Non phosphoprotein detection: use 5% nonfat dry milk in 1X TBST (Tris buffer + 0.1% Tween 20).

b. Phosphoprotein detection: block with 5% BSA in 1X TBST. Milk contains the phosphoprotein casein, and non-specific binding of the primary antibody to phospho motifs may cause high background.

These are general blocking buffer recommendations. It has been seen that some primary antibodies are not affected by the presence of casein. If high background is seen using one blocking buffer, try the other to see which performs best.

Primary Antibody

Incubate blot with primary antibody in 1% milk or 1% BSA + 1X TBST overnight at 4oC. Follow the manufacturer's recommendation for concentration/dilution of primary. If a range is recommended, start high and titer down as needed. This prevents 'below the detection threshold' from being interpreted as 'negative.' Wash the blots 3-5 times, 5 min per wash, with 1X TBST.

Secondary Antibody

Incubate blot with secondary antibody-HRP conjugate in 1% milk or 1% BSA + 1X TBST for 60 minutes at 4oC, shake gently. Follow the manufacturer's recommendation for concentration/dilution of secondary. Wash the blots 5 times, 5 min per wash, with 1X TBST without sodium azide.

Do not use sodium azide in the HRP conjugate diluent or subsequent wash. Azide is an inhibitor of HRP and will cause the immunoblotting to fail.

Substrate

Thoroughly drain away wash buffer and add chemiluminescent substrate, rock gently. Follow the manufacturer's recommendation for how long the blot remains in the substrate solution.

Exposure and Development

Expose the blot to appropriate X-ray film for various amounts of time. Generally, there is a short exposure of ~15 seconds, a medium length exposure of 1-2 minutes and a long exposure of 10-15 minutes.