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Western Blot for IP-TDMS Development

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ABSTRACT

This protocol can be used to determine relative quantities of protein target in IP fractions. Primary antibodies should be selected for the specific protein target.

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MATERIALS TEXT

Sample loading buffer with DTT
Microcentrifuge tube
Hot plate
Centrifuge
Gel box with power source
1 x MES running buffer
Invitrogen 4-12% BisTris gel (10, 12, or 17-well)
Protein MW Ladder
iBlot 2 Transfer Device
iBlot 2 PVDF transfer pads
Blot box
Razor blade or scalpel
Tweezers
5% BSA or milk diluted in 1 x TBST
1 x TBST
Gel shaker
Primary antibody (protein target-dependent)
Secondary antibody + HRP (dependent on host species of the primary antibody)
ECL reagent (Classico, Cresendo, Forte)
Cling wrap
iBright or comparable imaging device

1 Take aliquots from IP fractions and add sample loading buffer to a final concentration of 1X. Boil samples in hot plate at 95ºC for 5-10 min. Briefly, centrifuge samples to ensure sample entire sample is towards bottom of the tube.

2 Open gel and place into gel box. Add 1 x MES running buffer, ensuring that there are no bubbles within the wells of the gel. Carefully load samples, without loss of sample from the well. Make sure that the gel wells have a large enough capacity to contain the entire sample volume. Load at least one lane with 2-3 µL of protein MW ladder. Run the gel at 120-150 V for approximately 40 minutes, or until the sample dye has reached the bottom of the gel.

3 Remove the gel from the cassette and gently wash with deionized water. Cut off the bottom of the gel (where there is a ridge) and the lane dividers. Place gel into a dish with MilliQ H2O.

4 Prepare the PVDF stack. Place the filter paper into a dish of MilliQ H2O. Remove the top two layers of the stack, leaving the membrane layer exposed. Quickly add the protein gel to the stack and use a roller tool to remove the air bubbles (Note: 2 gels can fit into a full size stack; work quickly and do not let the membrane layer dry out or be exposed to air for long periods of time). Add the filter paper on top of the gel and roll out air bubbles. Add the top layer (rubber/silicon pad) and roll out air bubbles. Finally, add the felt layer with the metal tab aligned with the sensor on the iBlot device. Close the iBlot device and select the default "Program 3" that comes as a manufacture setting.

5 While the transfer is running, prepare a clean blot box with 5-10 mL of either 5% BSA or milk diluted in 1 X TBST (Note: prepare this blocking solution at least 3 hours prior to use). Once the transfer is complete, remove all layers above the membrane. Cut out the membrane where the gel had transferred. With a tweezers, pick up the membrane in a corner and transfer to the blot box (Note: do not touch the membrane with gloves or any other objects which may contain contaminating proteins). Make sure that the blot is fully covered in the blocking solution. Incubate at room temperature for at least 30 minutes with constant shaking.

6 Exchange the blocking solution for a fresh 5-10 mL of blocking solution. Add primary antibody to this solution according to the vendor recommendations. The final concentration will depend on the specific antibody. Incubate overnight at 4ºC with constant shaking.

7 Remove the primary antibody solution and wash twice in 5 mL of 1 X TBST for 5 min each time at room temperature (use gel shaker). Add 5-10 mL of either 5% BSA or milk diluted in 1 X TBST and add secondary antibody at a concentration according to vendor recommendations. Incubate at room temperature for at least 1 hr with constant shaking.

8 Wash blot 5 times with 5 mL 1 X TBST for 5 minutes at room temperature (use gel shaker). Treat with 1.5 mL of ECL and incubate at room temperature for no more than 2 minutes (strength of ECL and incubation time are dependent on the protein target concentration and antibody strength). Using the tweezers, place the blot onto a sheet of cling wrap. Seal the blot without obscuring the blot itself. Image using an iBright or comparable imager. Note: to be able to compare the efficacy of two or more conditions, they must be on the same blot together. Each blot will display procedural artifacts that could be misinterpreted as differences in IP efficacy.