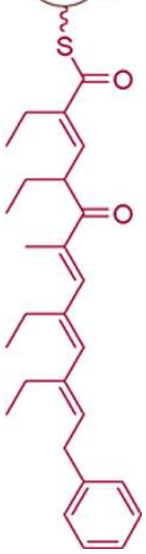

Western Blotting to identify his tagged proteins

Protocol

Required Solutions (Keep refrigerated at 4°C)

TBS (1L solution)

- 7.88g Tris-HCl
- 8.766 NaCl
- 1L H_2O
- pH adjust to 7.5

Transfer Buffer (1L)

- 3.03g Tris-base
- 14.41 g Glycine
- 200mL Methanol
- 800mL H_2O

Blocking Buffer (100mL)

- 5g of powdered milk
- 100mL TBS
- 100uL Tween20
- Mix thoroughly using stir bar

Wash Buffer (100mL)

- 100mL TBS

100uL Tween20

1. Run a protein gel using the pre-cast polyacrylamide mini gels.

2. Cut out the segment of the gel which will undergo western blotting using a clean razor. Place the gel in a petri dish and add 10mL of **Transfer buffer**. Equilibrate the gel for 1hr at room temperature while gently stirring.

3. Prepare the transfer sandwich. Be sure to wet all the components of the gel sandwich with **Transfer Buffer** before stacking. The gel sandwich is assembled in the following order:

- Cathode (---)
- Filter pad
- Filter paper
- Gel
- Transfer Membrane
- Filter paper
- Filter pad
- Anode (+++)

Cut the PVDF (or nitrocellulose) membrane with a razor to fit the dimension of the gel. This ensures an even flow of charge and allows for optimal protein transfer.

4. Once the sandwich is stacked, firmly close the cassette. No air bubbles should be present between the protein gel and the membrane. Gently rolling a glass test tube over the stacked sandwich can minimize air bubble interference.

5. The transfer is done using a Mini Trans-Blot ® Electrophoretic Transfer Cell. Place the cassette in the correct orientation (running to the anode). Fill the electrophoretic transfer cell with **Transfer Buffer** to the indicated "blotting" line. Place a frozen blue cooling unit inside the transfer cell to prevent heat build-up.

6. Initiate the transfer by running at 40V for 80 minutes. **Note:** These conditions apply for the pre-cast protein gels used in our lab.

7. Blocking step: After the transfer is complete, carefully remove the cassette. Using a clean forcep, remove the membrane and place it into a petri dish containing 15mL of **Blocking Buffer**. Orient the membrane so that the side containing the transferred proteins is always facing upwards.

Note: Residual gel that remains on the membrane after the transfer can prevent bands from being visualized. Gently scrape the gel off the membrane before the blocking step.

8. Blocking is done at 4^{o}C (in the cold room) overnight on a shaker. Ensure that the blocking buffer covers the entire membrane. Wrap the petri dish with parafilm to prevent leaking.

9. After blocking is complete, slowly decant the blocking buffer from the petri dish.

10. Incubate the membrane with the desired antibody. Add 5mL of **Blocking Buffer** to the petri dish. Next, gently add a desired amount of your antibody directly into the blocking buffer. A final concentration of 0.1-1.0 µg/ml of the antibody is sufficient. If using the HRP conjugated His-tag antibody, add 5uL. Incubate at 37^{o}C for 1hr with mild shaking (50 rpm).

11. Next, carefully decant the blocking buffer. Add 10mL of **Wash Buffer** into the petri dish and incubate for 5 minutes on the shaker at room temperature. Decant the washing buffer and this washing step is repeated three more times.

12. Once washing is complete, detection of the antibody (If it's HRP conjugated) is performed using the Millipore™ Immobilon™ Western Chemiluminescent HRP Substrate (ECL) kit. Mix equal volumes of the luminol reagent and the peroxide solution that are provided (2mL of each is sufficient). Allow the solution to reach room temperature.

13. Place the membrane in a new petri dish and add the ECL mixture. Equilibrate for 30 seconds. Place the membrane into the gel imager to see the results.

    Note: Filter #3 of the gel imager is used for Chemiluminescent detection. Using the UV filter will not detect any light and no bands will be seen.