**Basic Western Blot Protocol**
**Ezrin Tumor**

**Buffer Solutions:**

Running Buffer:
- 100 ml 10x TRIS/Gly/SDS
- 900 ml dH2O

Transfer Buffer:
- 100 ml 10x TRIS/Gly (40 ml of 25x and 760 dH2O)
- 700 ml dH20
- 200 ml methanol

TBS-T:
- 100 ml 10x TBS
- 900 ml dH2O
- 1 ml Tween 20

Blocking Buffer/Sln: 5% milk in TBS-T
- 3.5 g dry milk
- 70 ml TBS-T (100 ml TBS; 900 ml H20; 1 ml Tween)

**Protocol:**

I. Run gel:

1. Prepare samples for loading:
   a. Add 2x SDS loading buffer to sample at 1:1 (only if sample not already in SDS)
   b. Heat on heating block (100°) for 5 minutes
2. Use pre-made gel (in 4° behind Ling's bench)
   a. Remove tape, mark the bottom of wells with black marker for better visualization, and remove the comb (slowly so as not to break wells)
   b. Load gel in gel box and lock into place
   c. Fill inner chamber of gel box with running buffer … look for leaks
   d. Fill outer chamber ½ way or more
3. Add 10 ul of water to protein standard to resuspend then load (12-15 ul) into well
4. Load samples (max for a 10 well gel is 37 ul per well)

| Well | Lysate | Protein # | Lysate Vol | Result |
|---|---|---|---|---|
| 1 | Rainbow Marker | -- | 10ul | |
| 2 | Positive Control-A431 | ? | 30ul | |
| 3 | Cardiac | 4.904 | 24.5 | |

| | | | | |
|---|---|---|---|---|
| 4 | Liver | 9.378 | 12.8 | |
| 5 | Lung | 9.195 | 13.1 | |
| 6 | Spleen | 11.891 | 10.1 | |
| 7 | Kidney | 4.475 | 26.8 | |
| 8 | Pancreas | 4.561 | 26.3 | |
| 9 | Brain | 6.563 | 18.3 | |
| 10 | Large Intestine | 4.475 | 26.8 | |

5. Let gel run at 120V for 1-1.5 hrs.

II. Transfer to Nitrocellulose membrane

1. Soak red/black transfer block and two sponge sheets in transfer buffer
2. Prepare nitrocellulose membrane sandwich:
    a. Mark side of the nitrocellulose membrane that will contact gel with special marker (Ling's bench)
    b. Soak membrane and sandwich paper (x2) in transfer buffer
3. Remove gel from plastic panel, cut bottom just above protein front and at top just below the wells with ruler edge
4. Make Transfer "Sandwich": black on bottom, 1 sponge, 1 piece of sandwich paper, gel, nitrocellulose membrane (marked side down), 1 piece of sandwich paper, 1 sponge, red side on top
5. Place the red/black transfer block in the Owl transblot chamber
    a. Chamber should have stir bar in the bottom and be connected to the water coolant system next to it
    b. Black side facing black, red facing red (towards the wall)
    c. Fill chamber with transfer buffer to cover top of red/black transfer block
6. Run at 300mAmp for 1.5 hr (be sure the transfer gets to 300mAmp, may have to adjust voltage up to reach 280-300 mAmp mark)

III. Block

1. Carefully remove nitrocellulose membrane from red/black block
2. Cut membrane if necessary by staining to visualize desired band locations

*see antibody sheet to determine if dilute in 5% BSA or milk in TBS-T

a. Stain with 0.1% Ponceaus in 5% AAC (premade lab sln)
b. Wash excess stain off with dI water
c. Cut membrane at desired standard band
d. Rinse again in dI water (or TBS-T) to remove excess stain

3. Cover membrane with blocking solution and shake at RT for 1 hr
   a. 5ml for small container, 10ml for larger container

IV. Primary Anti-body

1. Pour off blocking buffer
2. Cover membrane thoroughly with TBS-T and place on shaker for 5 min. at RT repeat for a total of 3 washes
4. Pour primary antibody solution over membrane*
   a. Add 1:4000 dilution of p-Ezrint: 2.5ul of AKT to 10ml TBS-T (5% BSA)

5. Shake overnight at 4° or 1 hr RT

V. Secondary Antibody

1. Pour off and save primary antibody solution (store at 4°)
2. Cover membrane thoroughly with TBS-T and place on shaker for 5 min. at RT repeat for a total of 3 washes
3. Add secondary antibody and shake at RT for 1 hr
   a. Add 0.5ul of anit-mouse secondary to 10ml of TBS-T (1:20000 dilution of secondary antibody) to Ezrin

VI. Exposure

1. Discard secondary antibody
2. Cover membrane thoroughly with TBS-T and place on shaker for 5 min. at RT repeat for a total of 3 washes
3. Add 4 ml SuperSignal West Pico luminal enhancer and 4 ml SuperSignal West Pico stable peroxide solution to container and shake for 5 min. at RT
   *if doing a large number of containers, mix the enhancer and peroxide solution together just before adding to membranes
4. Remove membrane from solution, blot to dry, and wrap in plastic wrap
5. Expose membrane