Cell Team Western Blot Protocol for Detecting AXL in PC9 and PC9 AXL KO cells

This protocol was followed by last year's Cell Team during their Western Blotting lab.

Background Information:

This experiment examines the AXL receptor on PC9 cells. **PC9** is a cell line derived from human lung carcinoma, and it is often employed in the testing of novel cancer treatments and the study of metastasis. **AXL receptor tyrosine kinase** is a cell surface receptor that passes signals from the extracellular matrix into the cytoplasm. The AXL gene is an oncogene that has been associated with numerous types of aggressive, metastatic cancers.

Researchers have observed that AXL activates **Epithelial-Mesenchymal transition**, where differentiated epithelial cells revert to their migratory stem cell form. During cancer progression, this cell transition initiates metastasis.

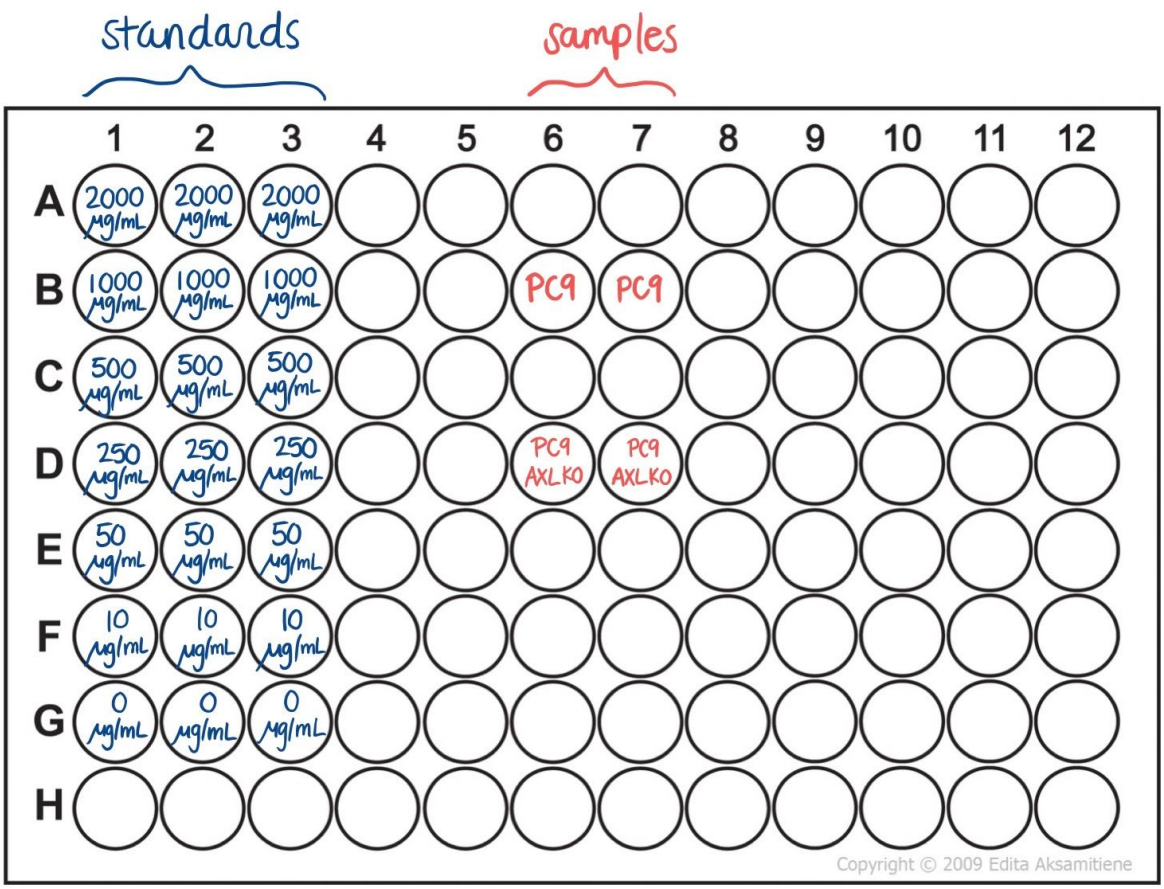
Recently, research labs (including **Meyer lab**) have been examining cancer therapies that inhibit the AXL receptor. UCLA researchers used CRISPR to remove the AXL gene from PC9 cells, creating AXL knockout cells (PC9 AXL KO). To determine if AXL inhibition is a viable form of cancer treatment, the morphology and metastatic behavior of these cells was then compared to the unedited PC9 cells.

As part of the procedure in this experiment, researchers had to confirm that the AXL knockout was successful. First, a **BCA Assay** was performed to confirm the presence of PC9 and PC9 AXL KO cells in the sample and determine their protein concentration. Next, a **Western Blot** was performed to confirm the presence of the AXL receptor in PC9 cells and the absence of the AXL receptor in PC9 AXL KO cells.

Note: On the following pages, images and information in italics were added this year to help make the protocol easier to understand in this online format.

Protocol for the PC9 BCA Assay:

1. Design layout for 96 well plate:



Note: Determine where triplicates of the standards for your BCA Assay standard curve and where duplicates of your two experimental samples will be in your 96 well plate. This will help you keep track of the well identities during your absorbance analysis.

2. Label seven eppendorf tubes to make standards:

Eppendorf Tube

BCA Assay Test Kit

In the Module 2 Worksheet, you calculated the volumes necessary for making the standards via serial dilution, as shown in the table below.

Each standard well contains 25 uL BSA Solution and 200 uL reaction buffer. Each sample well contains 25 uL of lysed protein sample and 200 uL reaction buffer.

BSA Concentration	**BSA Solution**	**Water (uL)**	**Total Volume after well (uL)**	**Total Volume at end (uL)**
2000 ug/mL	160 uL of 2000 ug/mL	0	160	80
1000 ug/mL	80 uL of 2000 ug/mL	80	160	80
500 ug/mL	80 uL of 1000 ug/mL	80	160	80
250 ug/mL	80 uL of 500 ug/mL	80	160	128
50 ug/mL	32 uL of 250 ug/mL	128	160	144
5 ug/mL	16 uL of 50 ug/mL	144	160	160
0 ug/mL	0 uL	80	80	80

3. Make reaction buffer:
    - Total number of wells = (7 standards x 3) + (2 samples x 2) = 25 wells
    - Total volume of buffer = 25 wells x 200 uL/well = 5000 uL total
    - Always make a little extra: 5500 uL
    - Combine BCA reagents in conical tube to obtain the BSA solution
        - Reagent A: Reagent B: Reagent C = 25:24:1
            1. Reagent A: (5500 uL)(25/50) = 2750 uL
            2. Reagent B: (5500 uL)(24/50) = 2640 uL
            3. Reagent C: (55500 uL)(1/50) = 110 uL
        - Vortex to mix, will turn green

Vortex Mixer

My (Miniature) Incubator

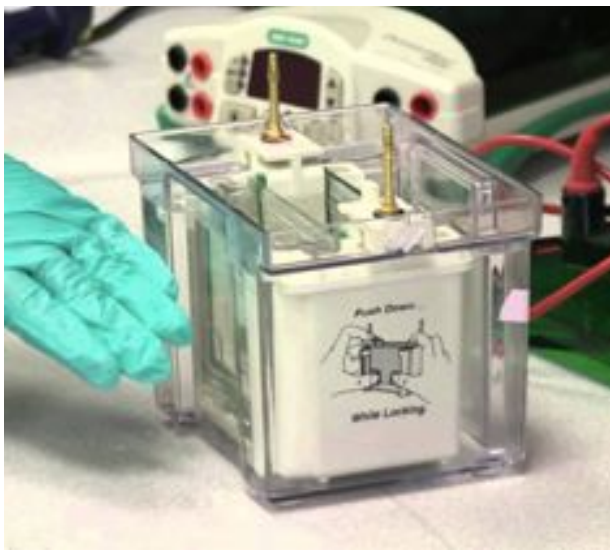
4. Load standards into 96 well plate
    - 25 uL of each standard per well
    - Make triples *(have three wells for each standard, see layout on Page 2)*
5. Load samples into 96 well plate
    - Keep samples on ice
    - Samples are protein lysates in PBS
        - *PC9 and PC9 AXL KO cells were lysed for this experiment and their protein contents were subsequently stored in PBS*
    - 25 uL of each sample per well
    - Make doubles *(have two wells for each sample - PC9 and PC9 AXL KO)*
6. Add 200 uL of BCA reagent to each well
7. Incubate at 37C for 30 minutes
    - Wells with a high protein content will turn purple!
8. Measure absorbances on plate reader *(we will perform this step in the Worksheet)*

Protocol for the AXL Western Blotting:

Day 1: SDS Gel Electrophoresis, Protein Transfer, and Primary Antibody Incubation

1. Need 10 ug of lysate protein per well for gel
    - PC9: (10 ug *lysate protein*)/(1.3 ug *lysate protein* / uL *PC9 solution*) = 7.69 uL *PC9 solution*
    - PC9 AXL KO: (10 ug *lysate protein*)/(1.8 ug *lysate protein* / uL *PC9 AXL KO solution*) = 5.55 uL *PC9 AXL KO solution*
        - *Note: The lysate solutions were prepared with this protein concentrations in advance by last year's PMs*
2. Preheat heating block to 95C

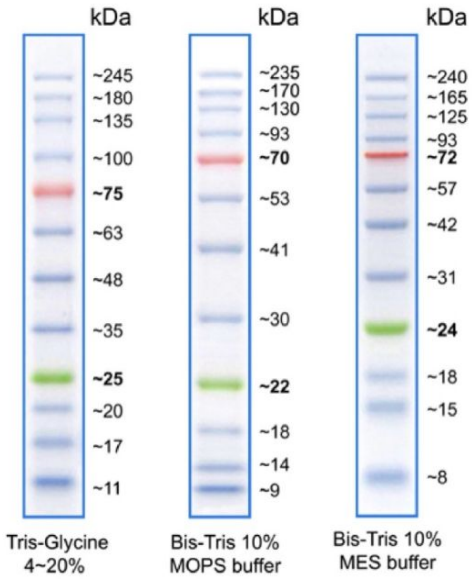
Heating Block



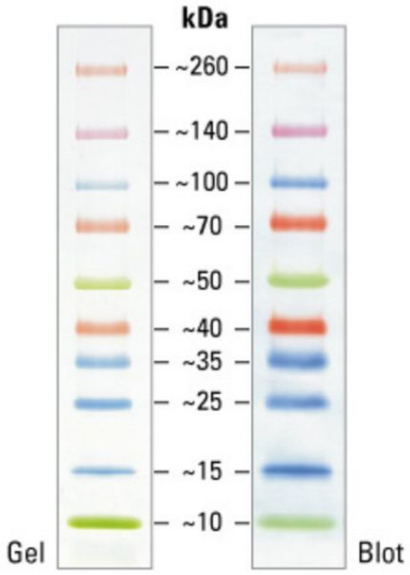
Gel Electrophoresis Chamber

3. In an eppendorf tube, combine:
    - 10 ug protein
    - 3 uL 6x SDS buffer
    - RIPA buffer to 18 uL
4. Thaw ladders on ice. *(Protein ladders to estimate size of sample protein after gel electrophoresis)*.
5. Heat samples on the heat block for 5 min.
6. Set up ***gel electrophoresis***
    - Obtain your gel and wash it with diH_2O
    - Place the gel in the gel electrophoresis chamber
    - Pour running buffer into the chamber to soak the gel
    - Remove comb from the gel

7. Gently load samples and standards into the wells of the gel
    - Samples: 18 uL
    - Dual and Blue ladders: 2.5 uL (*BLUEstain Protein Ladder)*
    - Broad Spectra ladder: 7 uL *(Spectra Multicolor Broad Range Protein Ladder)*
    - RECORD ORDER IN NOTEBOOK!

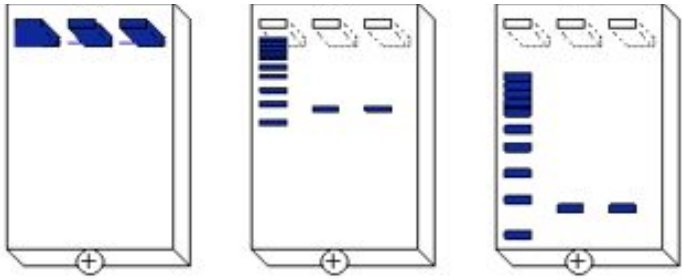


BLUEstain Protein Ladder used for 11-245 kDa proteins



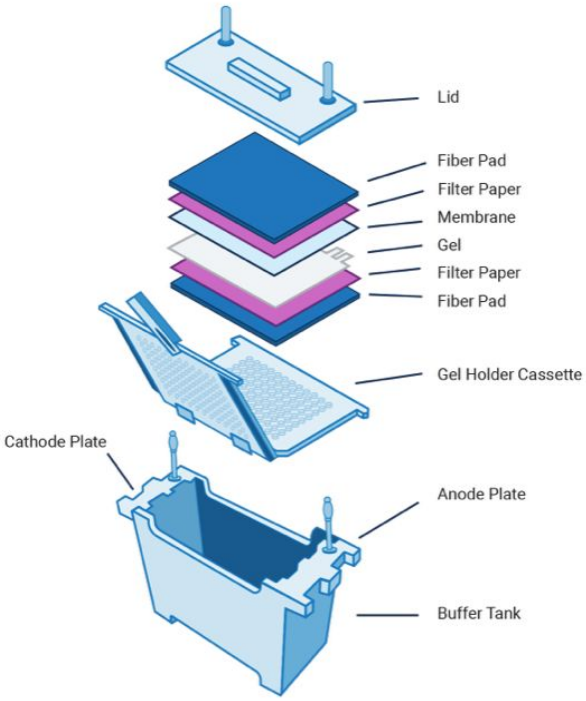
Spectra Multicolor Broad Range Protein Ladder
used for 10-260 kDa proteins

8. Run samples at 60V for 15 mins then 165V for 1 hour.

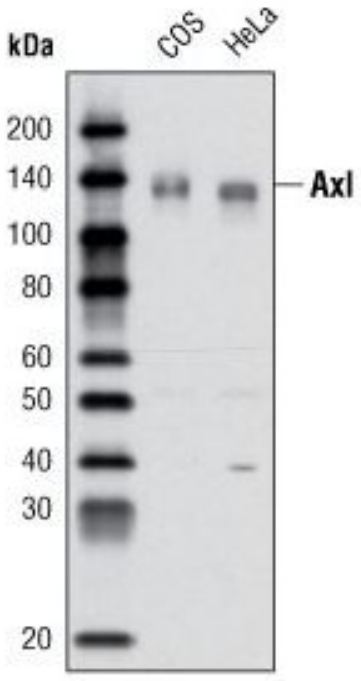


Gel Development over the course of the hour looked something like this

9. Once gel electrophoresis run is complete, ***prepare for transfer***
    - Remove gel from container and soak in transfer buffer
    - Soak 2 sponge pads and 2 filter papers in transfer buffer
    - Soak blot membrane in methanol for 2 min to activate
    - Move blot to soak in transfer buffer

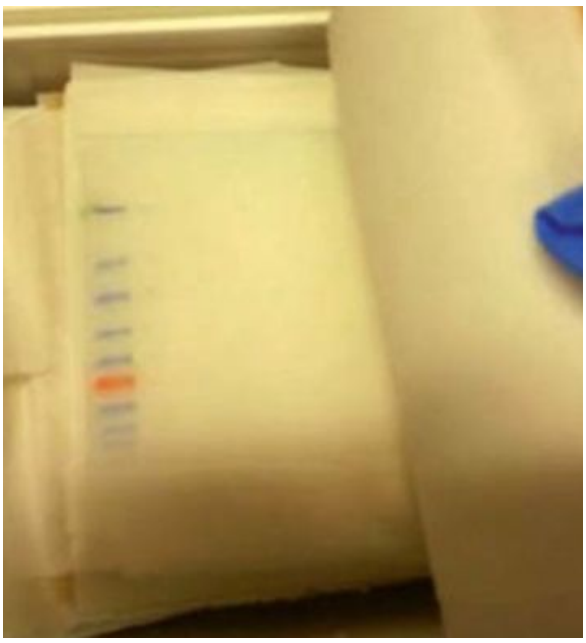


Overview of Sandwich Assembly



AXL and Actin Bands in HeLa Western
(HeLa is another cancer cell line)

10. Prepare sandwich assembly of gel and blot
    - Put the following on top of each other in order: filter paper, blot, gel, and filter paper.
    - DO THIS IN TRANSFER BUFFER TO AVOID BUBBLES
    - Place sandwich set in electrode container between the two sponges with the BLOT MEMBRANE closer to the POSITIVE side
11. Place electrode container in gel electrophoresis chamber
12. Pour all transfer buffer into the chamber
13. Run transfer at 20V for 1.5 hours.
14. Cut the transferred blot between expected bands
    - Actin bands: 42 kDa
    - AXL band: 135 kDa



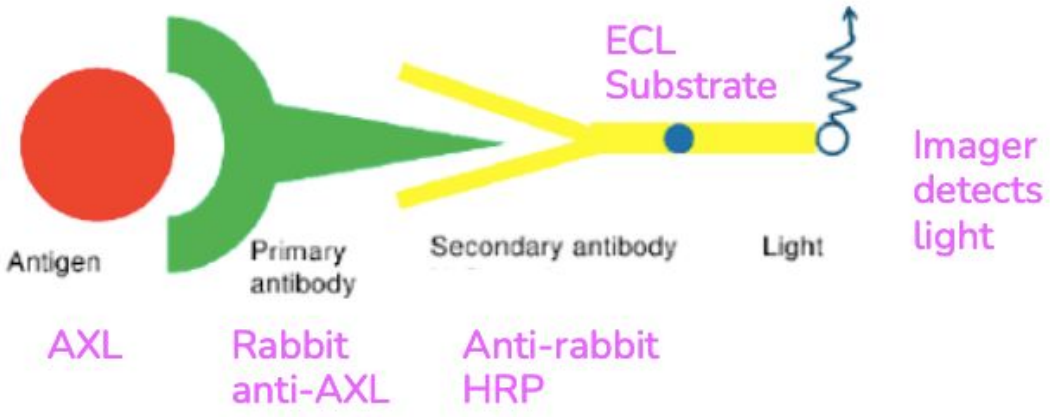
Membrane After Protein Transfer

Primary Antibody Incubation (on a shaker)

15. Incubate the transferred blot to block nonspecific binding
    - Making blocking buffer: 5% BSA in TBST
    - Incubate at room temperature for 1 hour
    - *Note: Blocking is usually done with BSA or nonfat dried milk. Nonfat milk is a cheaper, more accessible option, but is not compatible with the secondary antibody in this experiment.*
16. Prepare primary antibodies
    - Dilute 5% BSA-TBST to 2.5% BSA-TBST
    - Place primary antibody on cooling rack
        - Actin: anti-B actin (8H10D10) HRP conjugated
        - AXL: rabbit anti-AXL (C44G1)
    - Dilute antibodies in 2.5% BSA-TBST 1:1000 → make 3 mL total of each
17. Once blocking is complete, move membranes into clear box
18. Primary antibody incubation
    - Add actin antibody to actin blot half
    - Add AXL antibody to AXL blot half
    - Incubate overnight on shaker at 4 °C

Day 2: Secondary Antibody Incubation and Imaging

19. Wash blots with TBST 3 times
    a. Remove existing liquid
    b. Add 5 mL of TBST to each blot half
    c. Place on shaker at RT for 5 minutes
    d. Repeat
20. Prepare secondary antibody
    a. Dilute 5% BSA-TBST to 2.5% BSA-TBST
    b. Place secondary antibody on cooling rack
        i. Anti-rabbit IgG HRP-linked -- binds to rabbit anti-AXL
        ii. Actin primary antibody already has HRP so does not need secondary antibody!
    c. Dilute antibody in 2.5% BSA-TBST 1:2000 → make 3 mL total
19. Incubate with secondary antibody on shaker for 1.5 hours at RT
21. Wash blots with TBST 3 times
    a. Remove existing liquid
    b. Add 5 mL of TBST to each blot half
    c. Place on shaker at RT for 5 minutes
    d. Repeat
22. Mix Clarity Western ECL substrates A and B in a 1:1 ratio
    a. Make 3 mL for each blot
    b. Cover this mixture in aluminum foil to prevent photobleaching
23. Pour ECL substrates into two compartments of an aluminum foil-covered box
24. Place blots in substrate mixtures for 5 min to develop
25. Image!!



In the final imaging step, chemiluminescence from the chemical reaction between the enhanced chemiluminescence (ECL) substrate and the horseradish peroxidase (HRP) enzyme conjugated to the secondary antibody is detected.