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****Note: controlled versions of this document are subject to change without notice.***

Western Blot

I. Purpose

To probe with various primary antibodies and to detect a protein of interest in a cell line.

II. Scope

All procedures are applicable to the BCGSC Library Construction Core and Library Technology Development Group.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QA associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Assurance team to audit this procedure for compliance and maintain control of this procedure.

V. References

Document Title	Document Number
N/A	N/A

VI. Related Documents

Document Title	Document Number
N/A	N/A

VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the material safety data sheet (MSDS) for additional information.

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VIII. Materials and Equipment

Name	Supplier	Number: #	Model or Catalogue #	
Fisherbrand Textured Nitrile gloves	Fisher Scientific	270-058-53		✓
Ice Chest	Igloo	PM PAL BLUE		✓
Wet Ice	In house	N/A	N/A	N/A
DNA away	Molecular Bioproducts	7010		✓
Anhydrous Ethyl Alcohol (100% Ethanol)	Commercial Alcohol	People Soft ID: 23878		✓
Large Kimwipes	Fisher Scientific	06-666-117		✓
Black ink permanent marker pen	VWR	52877-310		✓
Small Autoclave waste bags 10”X15”	Fisher Scientific	01-826-4		✓
Gilson P10 pipetman	Mandel	GF-44802		✓
Gilson P20 pipetman	Mandel	GF23600		✓
Gilson P200 pipetman	Mandel	GF-23601		✓
Gilson P1000 pipetman	Mandel	GF-23602		✓
Diamond Filter Tips 10uL	Mandel	GF-F171203		✓
Diamond Filter Tips 30uL	Mandel	GF-F171303		✓
Diamond Filter Tips 200uL	Mandel	GF-F171503		✓
Diamond Filter Tips 1000uL	Mandel	GF-F171703		✓
RIPA buffer	Pierce	89900		✓
Halt Protease Inhibitor Cocktail (100X)	Pierce	78415		✓
Resolving Gel Buffer (1.5M Tris pH8.8)	Biorad	161-0798		✓
Stacking Gel Buffer (0.5M Tris pH6.8)	Biorad	161-0799		✓
40% 29:1 acrylamid/bis	Biorad	161-0146		✓
10% APS	In House	N/A	N/A	N/A
TEMED	Biorad	161-0801		✓
Ultrapure Water	GIBCO	10977		✓
Precision Plus Protein Kaleidoscope Standards	Biorad	161-0375		✓
1X Tris/Glycine/SDS Buffer (Gel running buffer)	Biorad	161-0772		✓
1X Tris/Glycine Buffer (Transfer Buffer mix)	Biorad	161-0771		✓
10X TBS	Biorad	170-6435		✓
10% Tween 20 Solution	Biorad	161-0781		✓
Biorad Blotting Grade Blocker (non-fat dry milk)	Biorad	170-6404		✓
Fisherbrand Filter Forceps	Fisher Scientific	09-753-50		✓
Mini-PROTEAN® Tetra Cell	Biorad	165-8000		✓
PowerPac 200	Biorad	1655050	✓	
Mini Trans-Blot Electrophoretic Transfer Cell	Biorad	170-3930		✓
Mini Trans-Blot Filter Paper	Biorad	1703932		✓
Trans-Blot Transfer Medium (Nitrocellulous Membrane)	Biorad	162-0115		✓
Glacial Acetic Acid	Fisher Scientific	CAS 64-19-7		✓
Ponceau Powder	In House	N/A	N/A	N/A
Disposable Polystyrene Weighing Dishes	Fisher Scientific	S40291		✓
Galaxy Mini Microcentrifuge	VWR	37000-700		✓
Rabbit IgG HRP	Cell Signaling	7074		✓
0.5mL Tube	Ambion	12350		✓

1.5 ml Eppendorf tube	Ambion	12400		✓
15ml Conical Tubes	VWR	CA21008-918		✓
50ml Conical Tubes	BD Falcon	352070		✓
50ml serological pipettes	Falcon	357550		✓
10mL serological pipettes	Costar	4488		✓
5ml serological pipettes	Costar	4487		✓
Goat Anti-Mouse HRP	Pierce	32430		✓
Supersignal West Femto Maximum Sensitivity Substrate	Thermo Scientific	34095		✓
Peltier Thermal Cycler	MJ Research	PTC-225		✓
Goat Anti-Rabbit HRP	Pierce	1858415		✓
Methanol	Fisher Scientific	A412P-4		✓
Mylar	In-house			
ChemiDOC XRS System with Image Lab Software	Biorad	170-8265		✓
Primary Antibody (variable)				

IX. Procedure

1. Equipment preparation in the ChIP Room

1.1. Put on a clean pair of gloves and lab coat.

1.2. Wipe down the work bench, small equipment, and ice bucket with DNA away and 80% Ethanol.

1.3. Open the Western Blot Worksheet Template at R:\Library Core\Epigenomics\Western Blots\Worksheets\Western_Blot_Worksheet_Template and fill in the Antibody name, Company, Catalog #, Lot #, LIMS ID, Aliquot Amt and dilution factor. See Appendix A for an example of the worksheet.

1.4. Retrieve the glass plates from the 6th floor ChIP room and scrub the glass gel plates with micro90 and rinse with H_2O. Rinse the plates with 80% Ethanol and wipe the plates dry with a kimwipe.

1.5. Assemble the BioRad gel casting apparatus as follows.

1.5.1. Place the short glass plate in front of the spacer glass plate. The spacer glass plate should read BIO-RAD with the arrows pointing up (see Figure 1). Ensure both plates are sitting level in the apparatus before locking them in place.

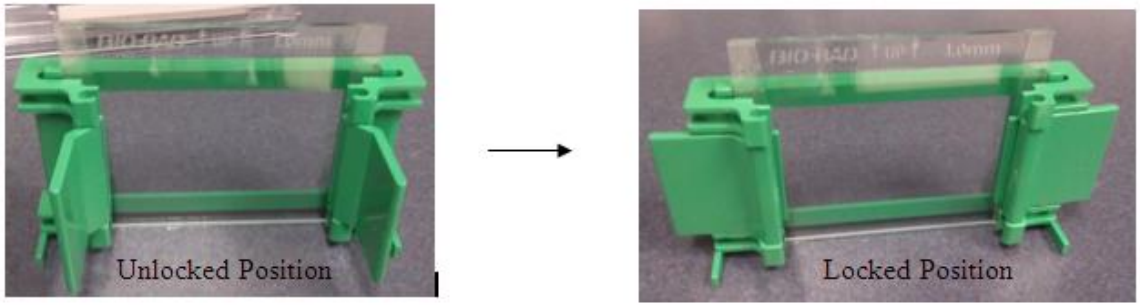


Figure 1

1.5.2. Place the plate on the base and clip the plates in place (Figure 2).

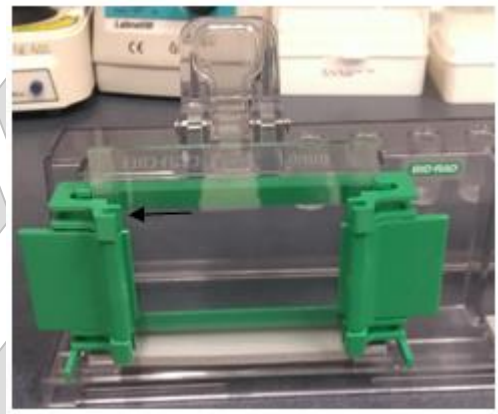


Figure 2

2. Making the Gel

2.1. Retrieve 40% 29:1 acrylamide/bis, 10% APS and TEMED from the 4ºC fridge. Retrieve Biorad Resolving Gel Buffer (1.5M Trip pH8.8) and dH_2O from room temperature.

2.2. In a fume hood, make a 10% or 15% Resolving Gel depending on protein size. See Table 1 for Resolving Gel preparation. In a 15mL tube, add the reagents in the order listed. Immediately after adding TEMED, invert the tube 5-6 times and pour the resolving gel up to line indicated by the arrow (see Figure 2).

Table 1. Resolving Gel

Protein Size	% Gel	Reagent	Volume
15-50kDa	15	Biorad Resolving Gel Buffer (1.5M Tris pH8.8)	2.5mL
		40% 29:1 acrylamide/bis	3.75mL
		dH_2O	3.75mL
		10% APS	50µL
		TEMED	30µL
>50kDa	10	Biorad Resolving Gel Buffer (1.5M Tris pH8.8)	2.5mL
		40% 29:1 acrylamide/bis	2.5mL
		dH_2O	5mL
		10% APS	50µL
		TEMED	30µL

2.3. With a 1mL pipette, pipette distilled water on top of the resolving gel and fill up to the top to level out the gel. Add the water carefully to avoid mixing the water with the resolving gel.

2.4. Allow the gel to polymerize and pour off the distilled water. Use a kimwipe to completely remove the distilled water from the resolving gel.

2.5. In a fume hood, make a 6% Stacking Gel. See Table 2 for Stacking Gel preparation. In a 15mL tube, add the reagents in the order listed. Immediately after adding TEMED, invert the tube 5-6 times and pour the stacking gel on top of the resolving gel.

Table 2. Stacking Gel

Protein Size	% Gel	Reagent	Volume
15-50kDa, >50kDa	6	Biorad Stacking Gel Buffer (0.5M Tris pH6.8)	2.5mL
		40% 29:1 acrylamide/bis	1.5mL
		dH2O	6mL
		10% APS	50µL
		TEMED	30µL

2.6. Immediately insert a comb between the 2 glass plates and allow the stacking gel to polymerize (See Figure 3).

Figure 3

2.7. When the gel has successfully polymerized, unclip the plates from the base and unlock the casting apparatus.

2.8. Carefully slide out the two plates and set the polymerized gel aside until needed. Do NOT separate the plates and do NOT remove the comb.

2.9. The gels can be prepared up to 1 day in advance and left overnight. Wrap the gels with moist paper towels and saran wrap and store the gels in the 4°C fridge.

3. Preparing the Samples for Gel Running

*Note: A fresh batch of HL60 and HelaS3 cell lysate must be prepped as per LIBPR.0074 - Total Lysate Prep and BCA Protein Assay for each Western Blot.

3.1. Aliquot the HL60 and HelaS3 cell lysate in 25ug aliquots.

3.2. In a fume hood, add 10µL laemmli dye (1:1) to the cell lysate samples.

3.3. Boil the samples at 70°C in a thermocycler for 10 minutes. Do NOT boil the ladder.

3.4. Place the samples immediately on ice before loading on the gel.

4. Running the Gel

4.1. Prepare the 1X Gel Running Buffer (1X Tris/Glycine/SDS Buffer) and store at RT.

Table 3. 1X Gel Running Buffer

Reagent	Volume
10x Tris/Glycine/SDS Buffer	100mL
dH_2O	900mL

4.2. Load the polymerized gel plate into the gel running apparatus. Ensure that the short glass plate is facing inwards and that a gel buffer dam is in place if only 1 gel is being run. Clamp the frame closed to lock the plate in place (See Figure 4).

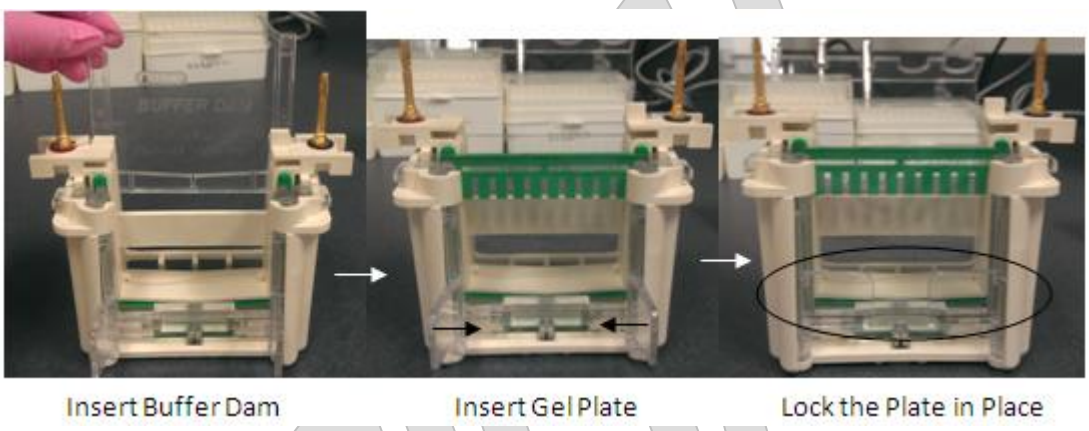


Figure 4

4.3. Pour the prepared gel running buffer in the inner chamber between the gel and the gel buffer dam. Fill the inner chamber until the buffer line is above the wells. Check for leaks. If leaks appear, re-seat the gel plate.

4.4. Carefully remove the comb. Using a 1mL pipette, gently rinse the wells.

4.5. Top up the inner chamber with gel running buffer and allow the buffer to overflow in to the outside chamber.

4.6. Fill the outside chamber with the gel running buffer. Approximately 500mL of gel running buffer is used to fill both the inner chamber and outer chamber of the apparatus.

4.7. Load the samples (20µL) and the Precision Plus Protein Kaleidoscope ladder (10µL) into the wells of the gel. Do not load the ladder or the sample into the wells at the ends of the gel. The sample can be loaded in the wells next to the ladder. Document the loading order in the worksheet (See Appendix B)

4.8. Place the lid on the gel box and connect the electrodes to the Biorad PowerPac 200.

4.9. Run the gel at 150V until the dye front just runs off (~65mins)

4.10. Remove the lid and remove the clamping frame from the gel box. Unload the gel plate from the gel running apparatus.

4.11. Carefully pry the plates apart with the plate separator and transfer the gel to the blot sandwich. See Step 5.3 for the blot sandwich assembly.

4.12. Rinse the gel running apparatus, gel comb and plate separator with micro90, rinse with H_2O and leave to dry overnight. Wash the glass plates with micro90, dH_2O and then with 80% Ethanol, allow the glass plates to dry overnight.

5. Transferring the proteins to a nitrocellulose membrane

dissassemble gel and assemble transfer set up in the fumehood

5.1. Prepare the 1X Transfer Buffer (1X Tris/Glycine Buffer) and store at 4ºC. Add the methanol in a fumehood.

Table 4. 1X Transfer Buffer

Reagent	Volume
10x Tris/Glycine Buffer	100mL
dH_2O	700mL
Methanol	200mL

5.2. Soak 2 pieces of sponges in 1X Transfer buffer for at least 10 minutes at room temperature in the FUMEHOOD. (See Figure 5)

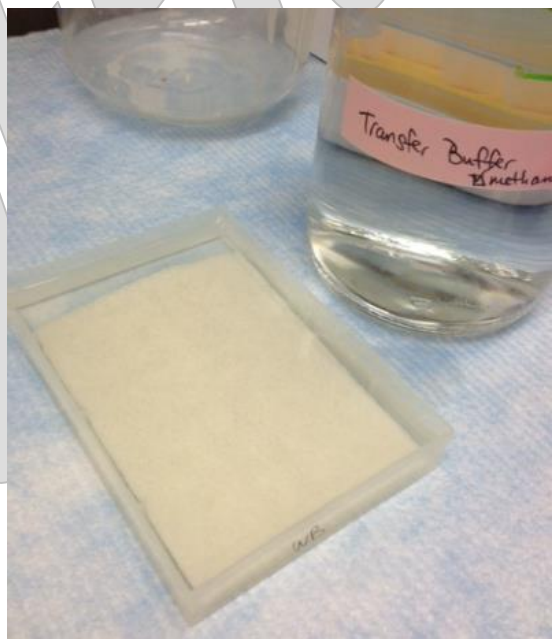


Figure 5

5.3. Once the gel is finished running, assemble the blot sandwich in a container filled with 1x Transfer Buffer. (See Figure 6).

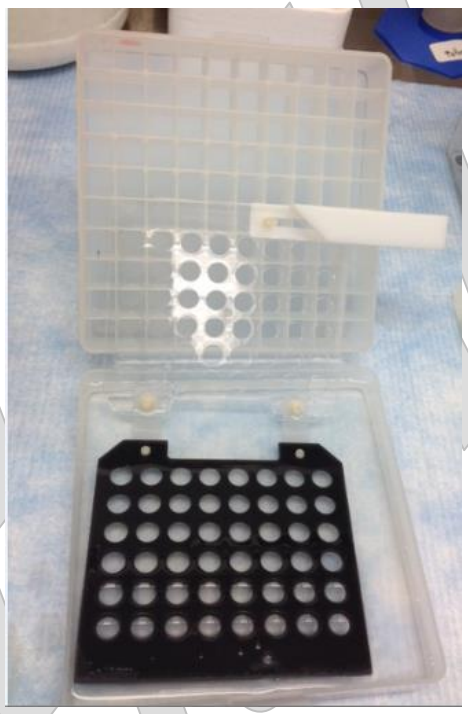


Figure 6

5.4. Set up the transfer in the following order: Ensure that all components of the sandwich are submerged in the transfer buffer to prevent the creation of bubbles during assembly.

5.4.1. Place 1 piece of presoaked sponge on the black plate. (Figure 7a)

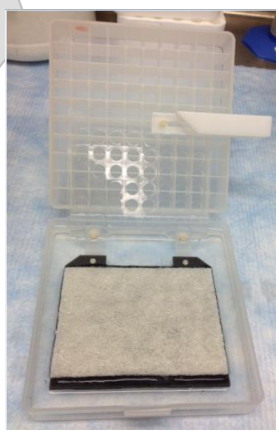


Figure 7a

5.4.2. Soak 1 piece of precut Whatman paper in transfer buffer and place on top of the sponge.

5.4.3. Dissassemble the gel running apparatus using the plate separator. Cut the stacking gel from the separating gel part and discard it. (Figure 7b)

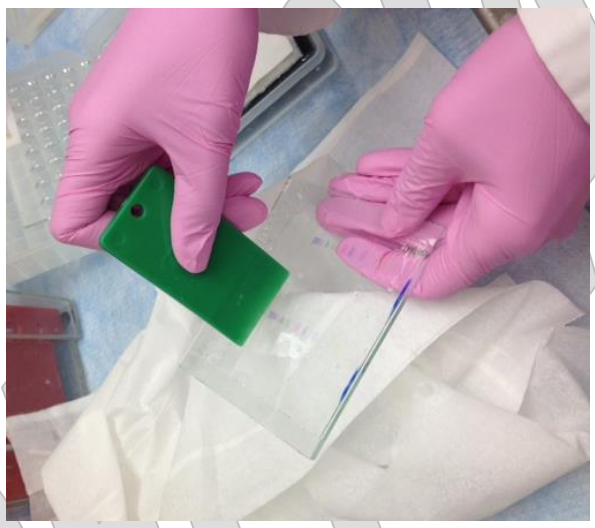


Figure 7b

5.4.4. Carefully transfer the gel onto the sandwich (ie on top of the whatman paper piece) and smooth out any air pockets by compressing gently with the plate separator. (Figure 7c and Figure 7d).

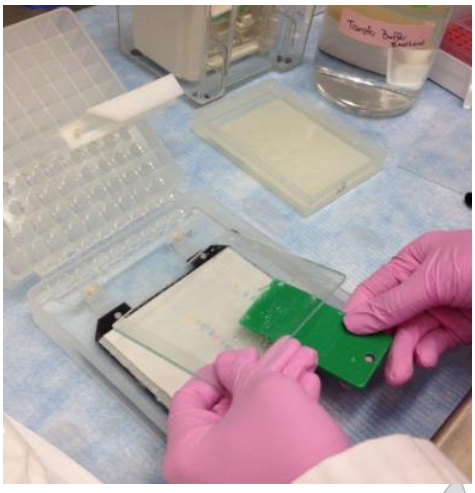


Figure 7c

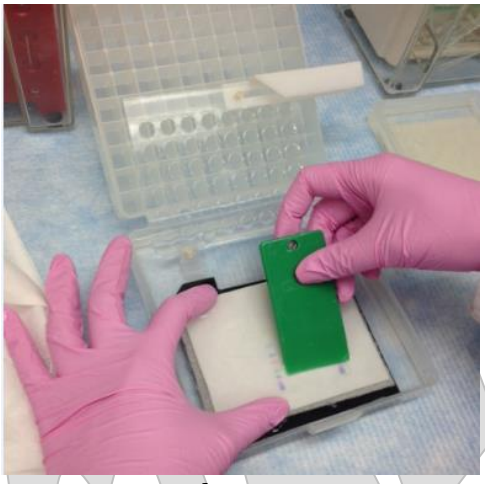


Figure 7d

5.4.5. Soak in transfer buffer 1 piece of nitrocellulose membrane cut to the size of the gel and place on top of the gel gently and release any air pockets by gently compressing with the plate separator. (Figure 7e and Figure 7f)

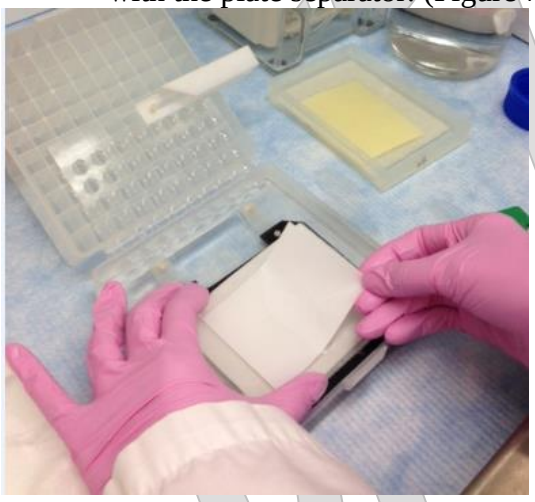


Figure 7e

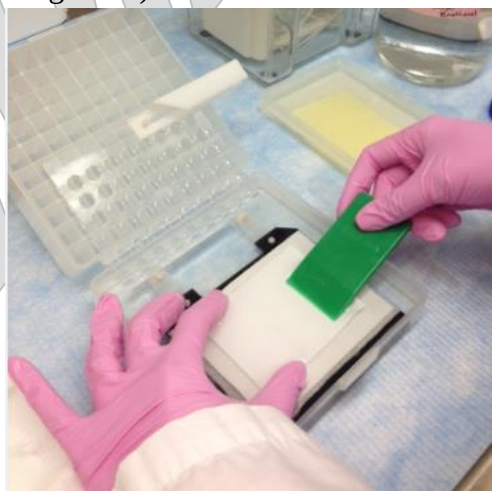


Figure 7f

5.4.6. Soak a piece of whatman paper in 1x transfer buffer and place gently on the sandwich. Again release any air pockets by gently compressing with the plate separator (Figure7g). Remove any air pockets by gently by compressing the sandwich with the plate separator, in a sweeping motion.

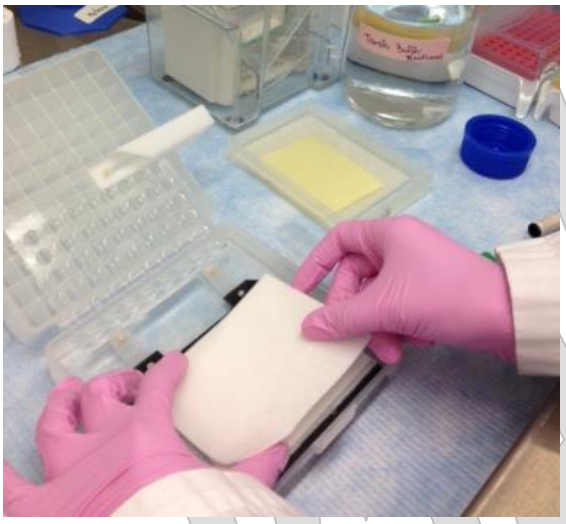


Figure 7g

5.4.7. Now place the presoaked sponge on top to finish the assembly of the sandwich. Roll out any air bubbles as described above. (Figure 7h)

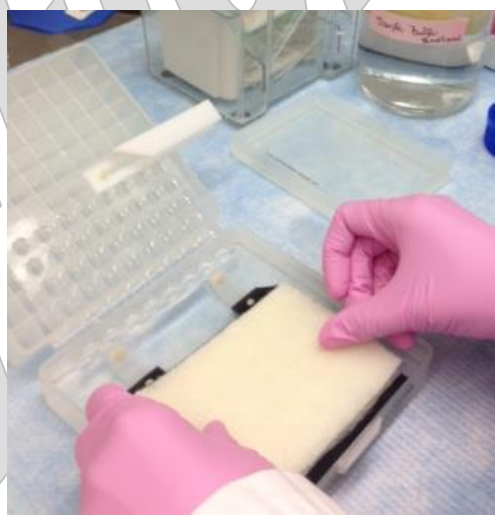


Figure 7h

5.4.8. Close the transfer holder by placing the white lever over the two plates (1) and slide the lever (2) to lock the plates together (See Figure 8a).

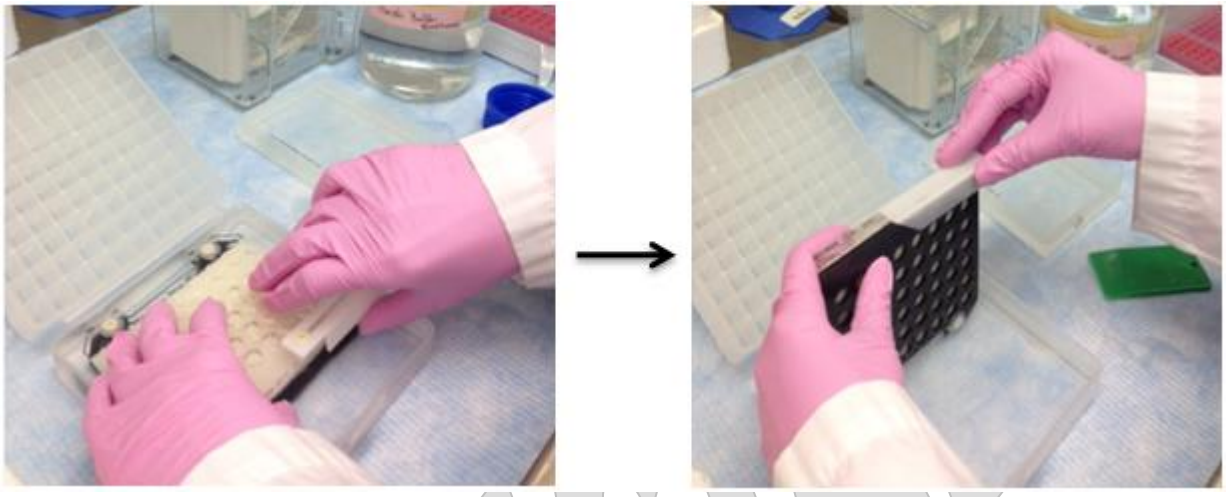


Figure 8a

5.5. Insert the cassette into the transfer apparatus with the black plate facing the black negative (-) electrode and the clear plate facing the red positive (+) electrode (See Figure 8b). **The membrane should be closest to the (+) electrode.**

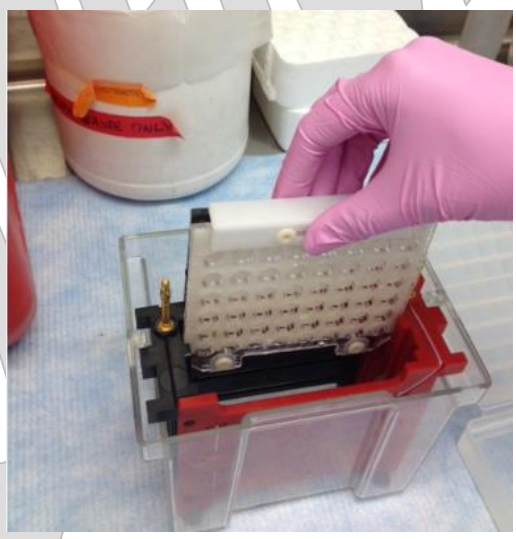


Figure 8b

5.6. Place the transfer apparatus on ice. The white container should be filled with water and prefrozen. Fill the chamber with cold 1X Transfer Buffer (See Figure 9).

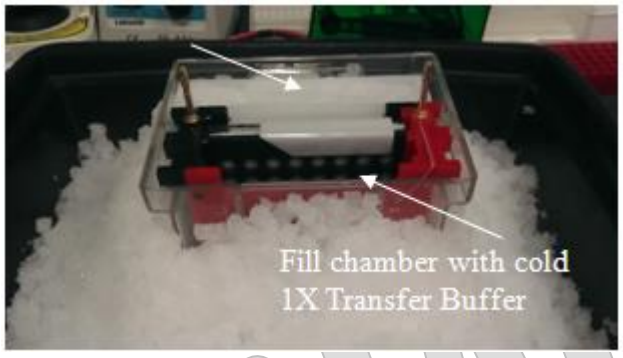


Figure 9

5.7. Place the lid on the gel box and connect the electrodes to the Biorad PowerPac 200.

5.8. Place the entire assembly including the ice bucket into the 4°C fridge. (Figure 10)

Figure 10

5.9. Transfer the proteins on the gel to the nitrocellulose membrane at 0.38A for 1 hour (See Figure 11). Ensure that the constant on the PowerPac is set to Amps (A). **LEAVE THE POWER PAC AT ROOM TEMP.**

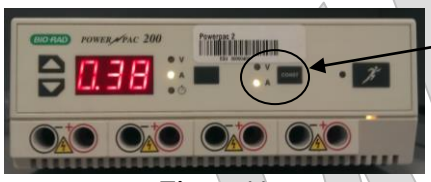


Figure 11

6. Blocking the nitrocellulose membrane

6.1. Prepare 1X TBST (1X TBS + 0.05% Tween) and store at room temperature.

Table 5. 1X TBST

Reagent	Volume
10X TBS	50mL
10% Tween	2.5mL
dH2O	447.5mL

6.2. In a 50mL tube, prepare fresh 5% milk in 1X TBST. **Confirm with the antibody data sheet that a 5% milk solution is the ideal blocking solution for it. If not, follow the data sheet and use the appropriate blocking solution.**

Table 6. 5% milk in 1X TBST

Reagent	Volume
Biorad Blotting Grade Blocker (non-fat dry milk)	1.25g
1X TBST	up to 25mL

6.3. Thoroughly shake the tube to resuspend the milk powder into solution. The prepared solution can be kept for up to a week. Keep at 4°C until ready to use.

6.4. After the transfer is complete, carefully remove the assembly from the 4°C and place the transfer unit on the bench. Dissassemble the sandwich, carefully. Leave the nitrocellulose membrane on a piece of whatman paper. (See Figure 12). The damp whatman paper helps prevent the membrane from drying out.

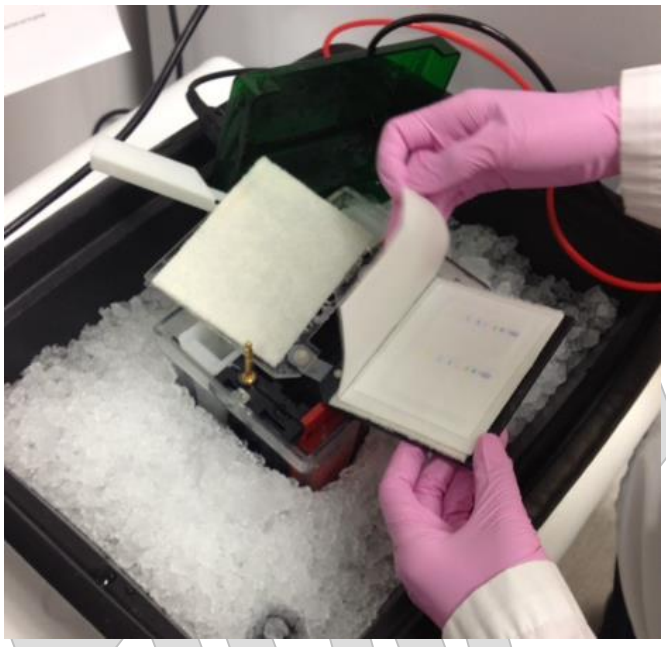


Figure 12

6.5. The actual gel should have no visible bands on it. If the transfer was successful, it should be clear. See Figure 13 for a successful transfer of the gel.

Figure 13

6.6. Cut the membrane to the shape of the gel, including the top part of gel, using the ladder as an indicator. **See Figure 14 below of the Protein ladder and where to cut the membrane, only if the size of band expected is less than 35kDa**. Save the top of the membrane for Step 6.10. Also see Figure 15 for an actual image of a gel being cut. **The steps following are for visualization of proteins less than 35kDa**

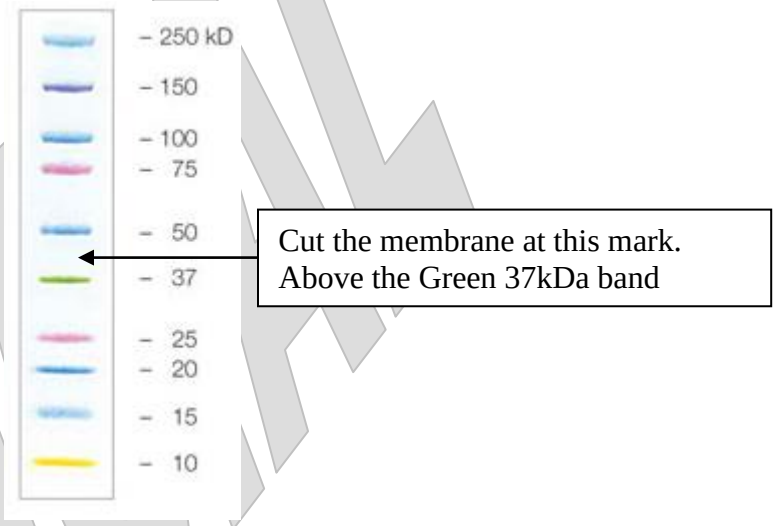


Figure 14. Kalidescope Protein Ladder

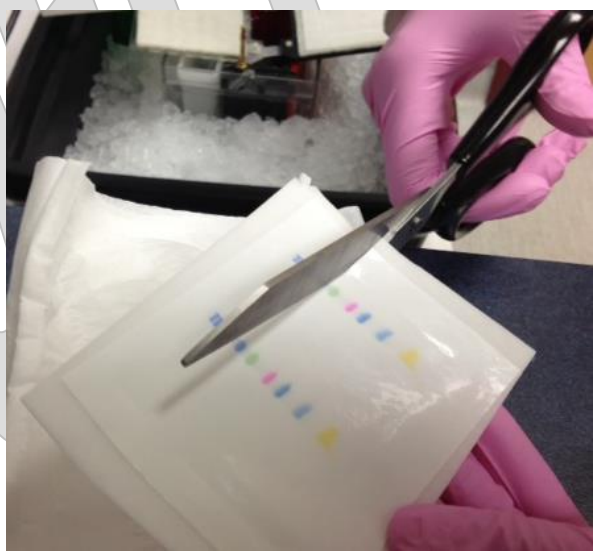


Figure 15

6.7. Trim the membrane along the sides and bottom. See Figure 16.

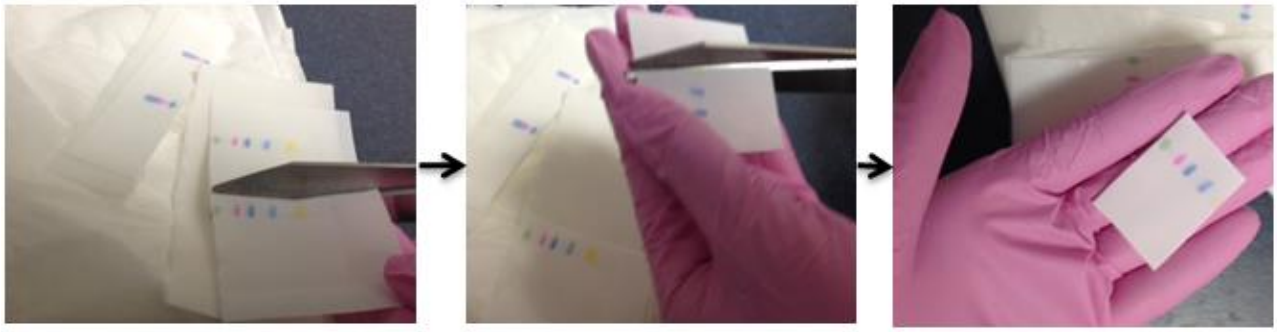


Figure 16

6.8. Place the membrane protein side up in the custom dish made by the Engineering Group (Figure 17).

Figure 17

6.9. Pipette 3mL of the prepared blocking solution (5% milk in 1X TBST or 5% BSA in 1X TBST) to cover the membrane.

6.10. Incubate and block the membrane for 1 hour at room temperature with shaking.

6.11. To confirm that the transfer of proteins was successful, stain the TOP part of the membrane (step 6.6) with Ponceau stain. **Refer to Appendix B for the Ponceau stain transfer confirmation process.**

6.12. Label each tray with the name of the antibody being tested. Include a positive control antibody if necessary.

7. Probe the nitrocellulose membrane.

7.1. Pour off the blocking solution.

7.2. Probe with the Primary Antibody.

7.2.1. Add 3mL of 5% milk in 1X TBST (or whichever blocking solution is suggested by the manufacturer noted on the data info sheet) to cover the membrane.

7.2.2. Add the appropriate amount of primary antibody directly to the blocking solution covering the membrane (see antibody data sheet for the appropriate dilution factor of antibody).

7.2.3. Incubate overnight at 4C with shaking.

7.3. Pour off the blocking solution.

7.4. Wash 3 times with 1X TBST, 10 minutes each with shaking. The Orbital Shaker is marked 'W" along the Speed dial indicating the intensity of the washing. Ensure the dial is set correctly.

7.5. Pour off the excess 1X TBST.

7.6. Probe with the Secondary Antibody.

7.6.1. Add 3mL of 5% milk in 1X TBST to cover the membrane.

7.6.2. Add1µL of secondary antibody Pierce Goat Anti-Rabbit (1 in 3000 dilution). Ensure the species origin of the primary antibody is rabbit. If the origin of the antibody is mouse, use Goat Anti-Mouse IgG.

7.6.3. Incubate the secondary antibody for 1 hour at room temperature with shaking. The Orbital Shaker is marked with an 'I' along the Speed dial indicating the intensity of the shaking speed. Ensure the Speed dial is set correctly.

7.7. Wash 3 times with 1X TBST, 10 minutes each with shaking.

7.8. Leave the last 1X TBST wash on the blot in the cassette. Wrap the cassette in plastic wrap carefully. This is to prevent spilling of the buffer while transporting it to the CRC.

8. Detection and Imaging at the CRC

8.1. Bring the following to the CRC for developing:
8.1.1. Pierce substrate: SuperSignal West Femto Maximum Sensitivity Substrate
8.1.2. 1mL pipettor and 1mL barrier tips

8.1.3. 15mL falcon tube
8.1.4. KimWipes
8.1.5. Timer
8.1.6. Black Marker
8.1.7. 2 Pair of Gloves
8.1.8. Disposable lab coat
8.1.9. Clean mylar sheets (2 per blot)
8.1.10. Blot in cassette

8.2. At the CRC

8.2.1. Put on disposable lab coat and gloves. Log into the computer connected to the ChemiDOC System (Figure 18). Double click on the Image Lab icon to initialize the software.

Figure 18

8.2.2. Mix together 1mL of the Enhancer Solution and 1mL of the Peroxide Buffer from the Pierce Substrate kit in a 15mL falcon tube. (ECL Stain)

8.2.3. Unwrap the cassette containing the blot and using gloved hands transfer the blot to a piece of mylar. Transfer the antibody label from the tray to the mylar sheet.

8.2.4. Add the ECL stain to the blot, enough to cover the blot but not soak it. After a few seconds gently pour off the stain from the blot and use a kimwipe to soak up the

excess stain. Cover the blot with another piece of mylar. Ensure no large bubbles are in the sandwich. Place the sandwich onto the middle of the imager deck. (See Figure 19) Close the lid.

Figure 19

8.2.5. Image the blot using the Image Lab Software.
8.2.6. Click on the 'New Single Channel' icon (Figure 20).

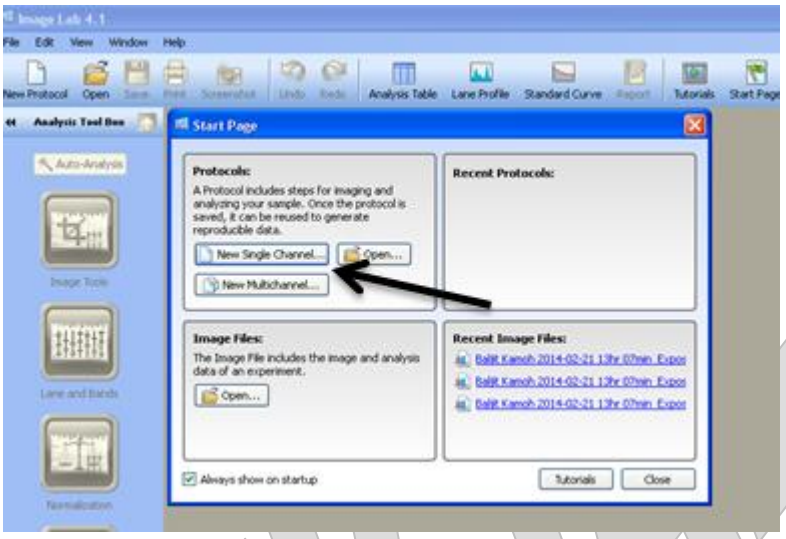


Figure 20

8.2.7. Set up the image capture with the following parameters (also see Figure 21):

a. Chemi Hi Sensitivity Blot under the Applications Icon
b. The software will automatically optimize the exposure time for Intense Bands
c. Deselect Highlight saturated pixels
d. Centre the blot on the tray by clicking on the 'Position Gel' icon

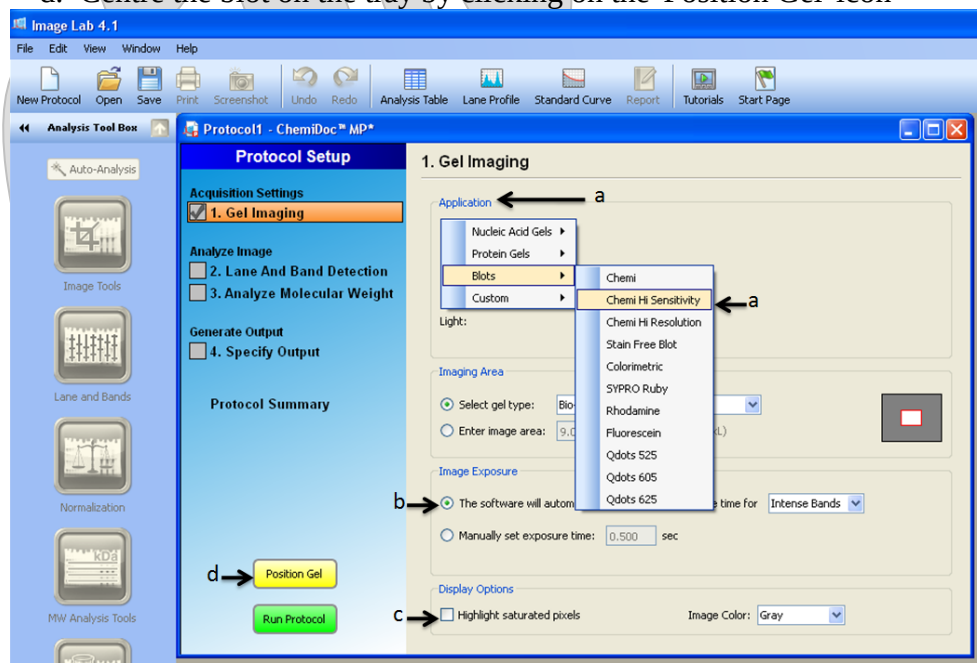


Figure 21

8.2.8. Centre the blot on the imager deck if needed to fit in the viewer of the software (Figure 22). If necessary, open the deck and physically move the blot to adjust. Once the blot is centered, click on the 'Run Protocol' icon.

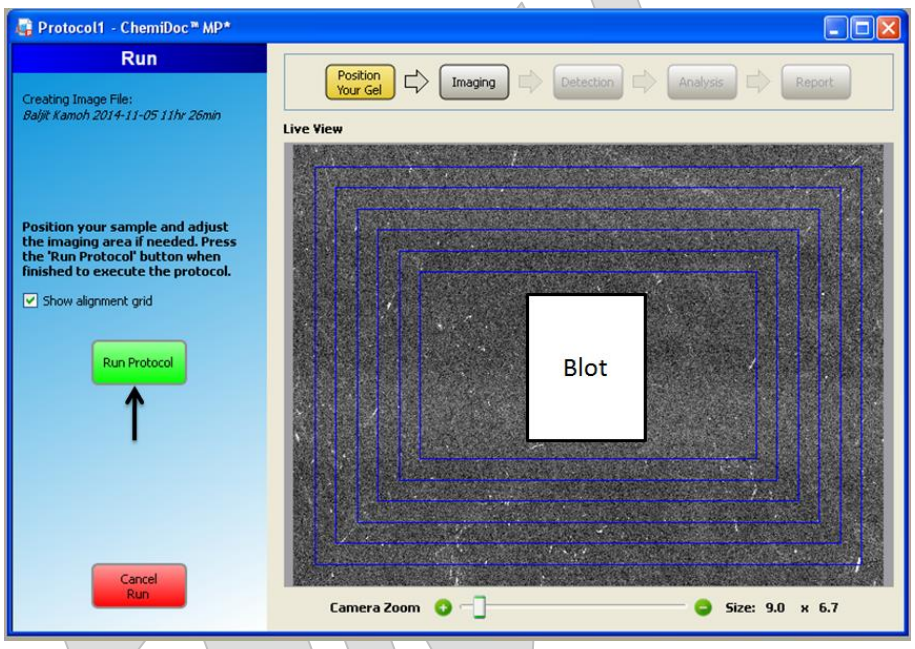


Figure 22

8.2.9. Auto exposure of the blot. See Figure 23.

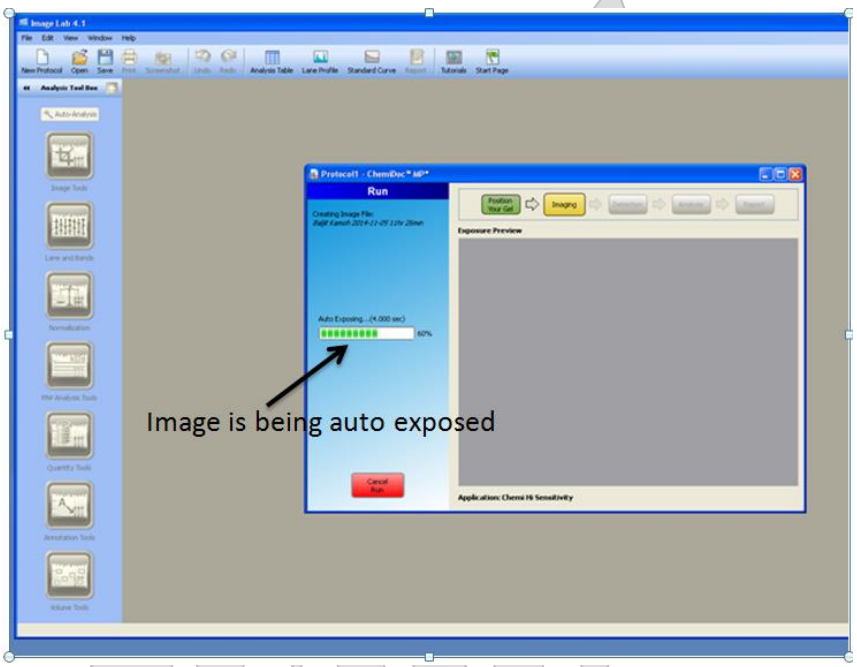
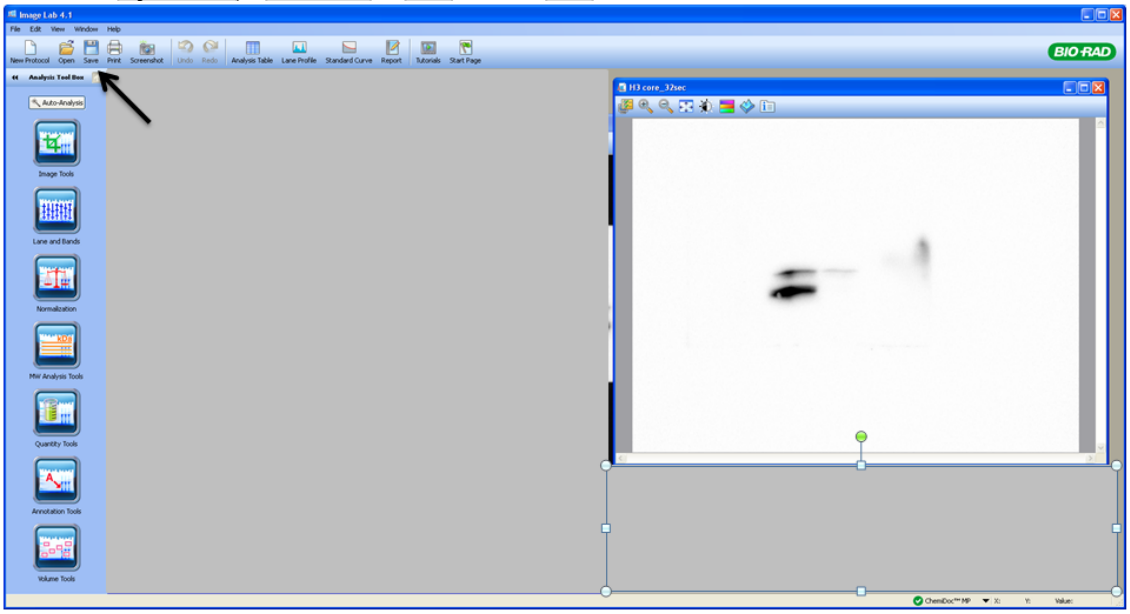


Figure 23

8.2.10. Save the blot image with the name of the primary antibody and date in a pre-labeled folder in R:\\filer05b\geneexplab\Library Core\Epigenomics\Western Blot\(1° Antibody Name)



8.2.11. Take an image of the ladder by following the lettered steps in Figure 25. Note the red arrows for auto exposure set up, and unhighlighted saturated pixels. Since the blot has not been moved, it does not need to be positioned. Simply run the protocol.

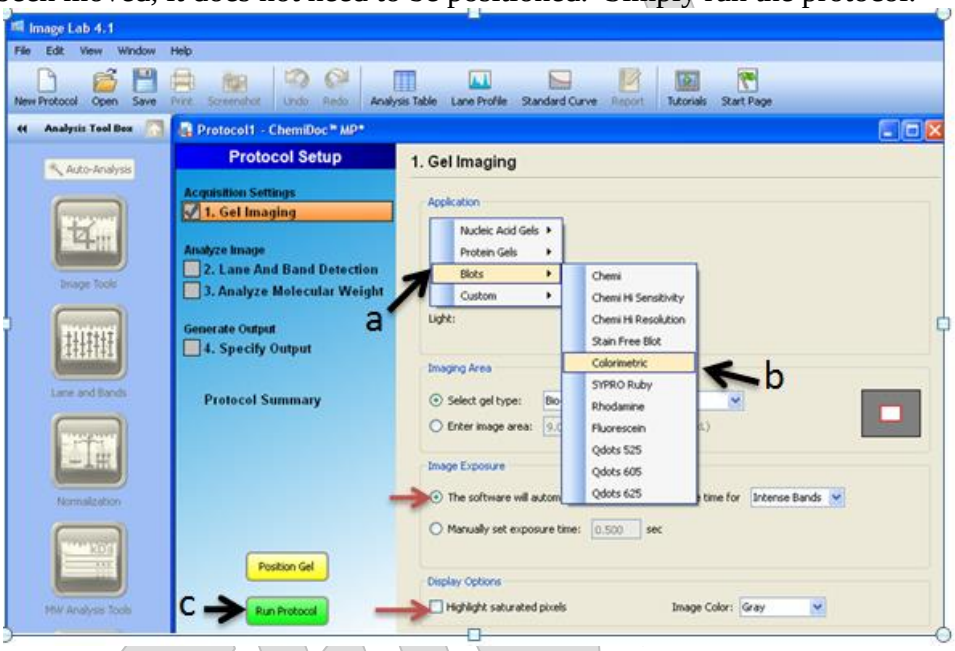


Figure 25

8.2.12. Save the ladder image in the directory location noted in Step 8.2.10. and naming the image file 'ladder' and include the date. See Figure 26

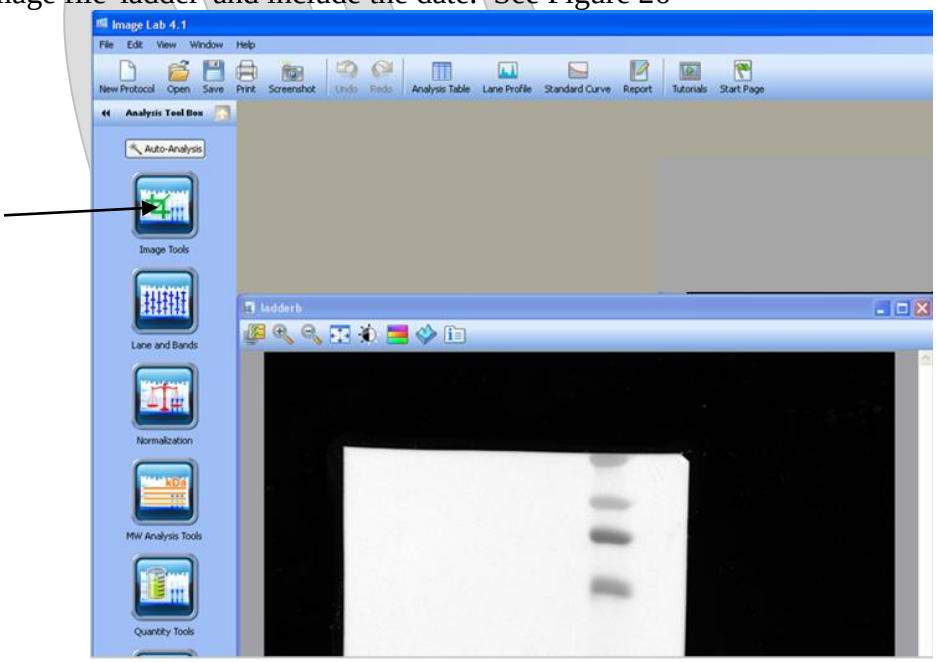


Figure 26

8.2.13. Merge the 2 images together. Click on the 'Image Tools' icon on the left side of the screen (Figure 26). Next, click on the 'Merge' icon. See Figure 27. Select the images to be merged (a). Then click OK (b).

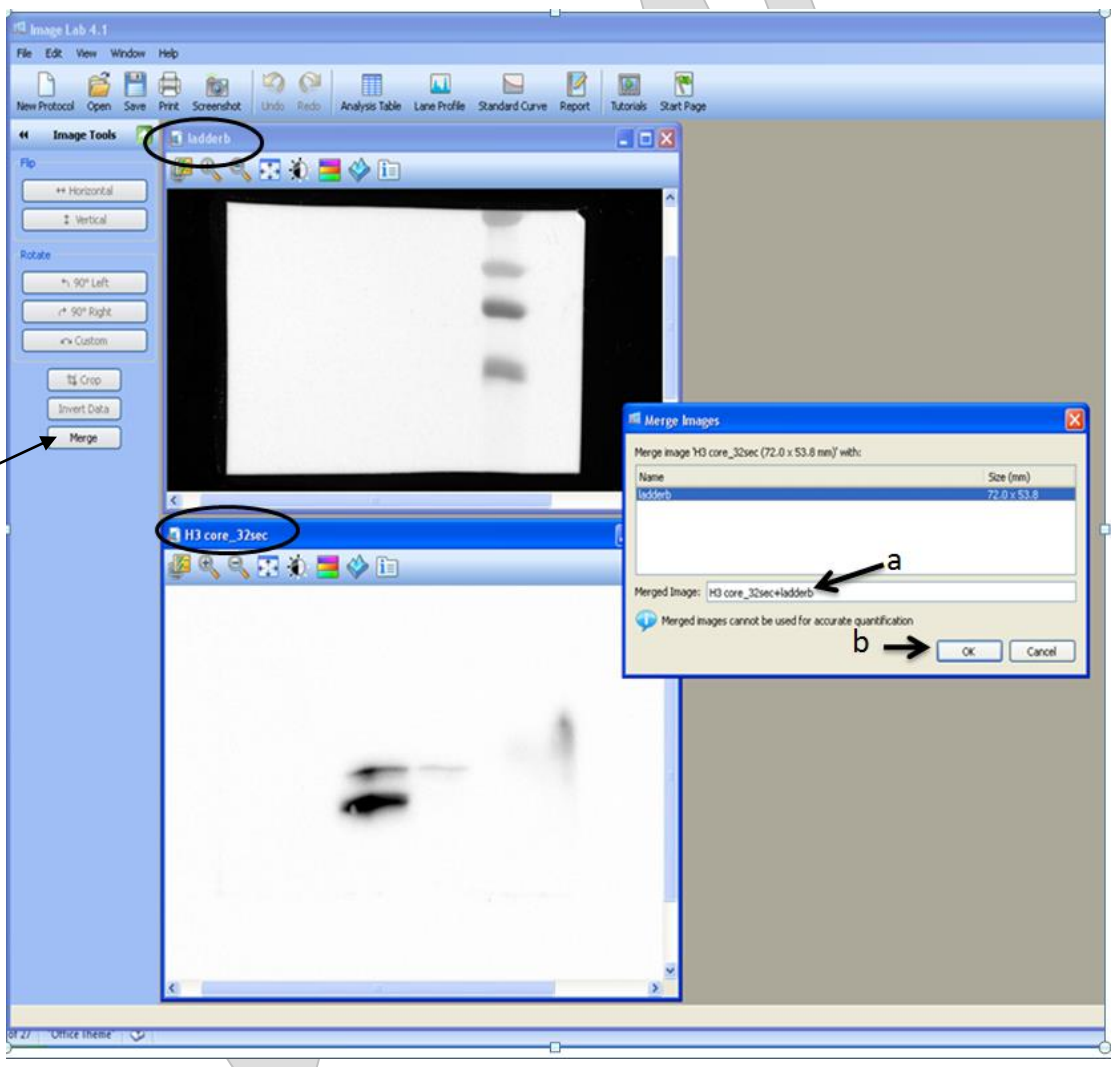


Figure 27

8.2.14. Save the merged image as a screenshot (Figure 28). Click on the 'Screenshot' icon. A window will pop up, prompting the user to which image to save. Select 'Current Image View' (b). Ensure the merged image is the image in the current view window of the software. Click on ' To File' to save the image to a file (c)

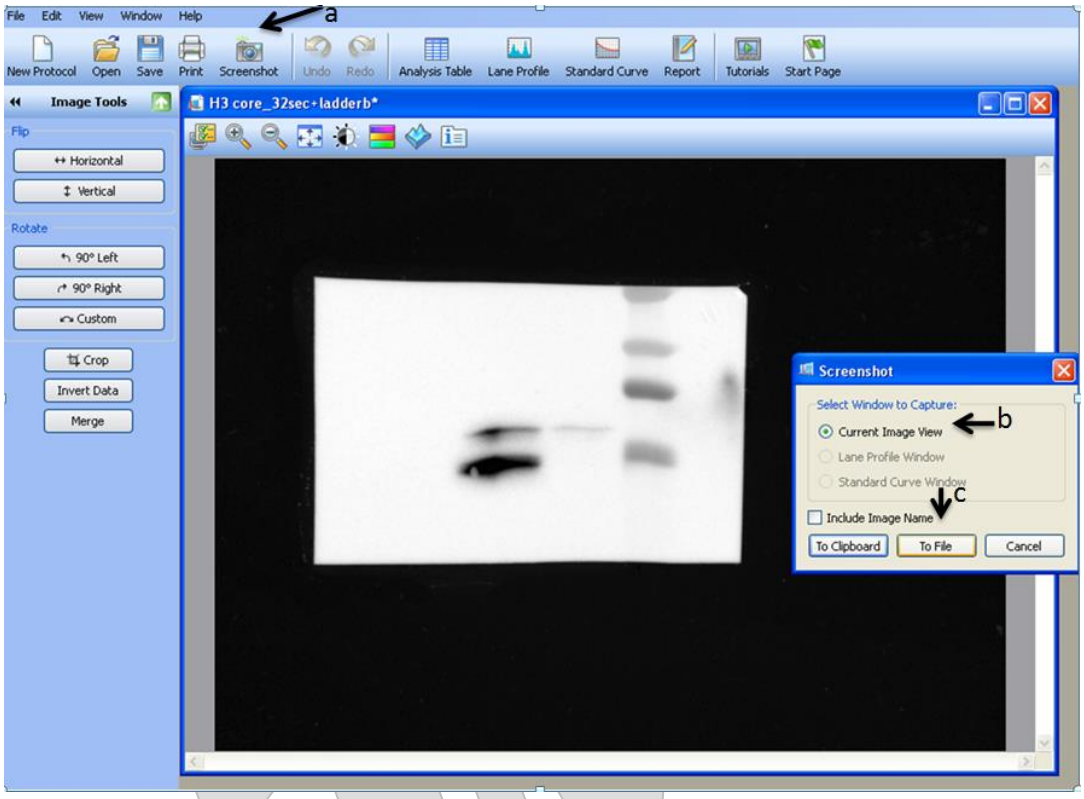


Figure 28

8.2.15. Change the file type to a jpeg image, and save the merged image in the same folder in the directory noted in Step 8.2.10 (Figure 29). Change the name of the file to reflect that it is a merged image.

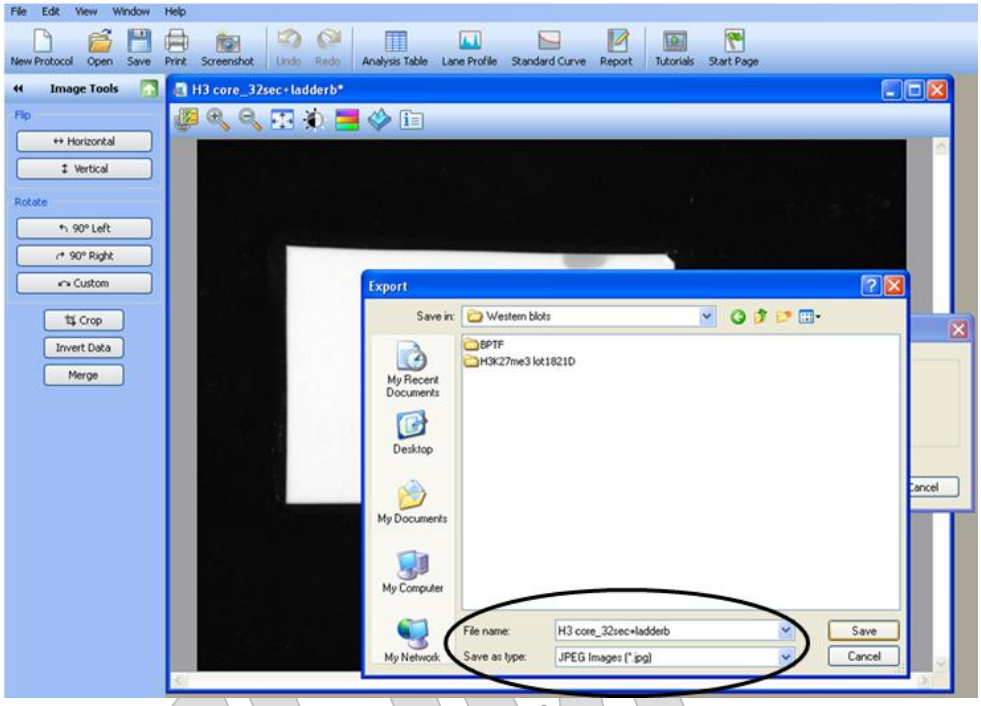


Figure 29

8.2.16. Once the image is saved, exit out of the software and log off of the computer.

8.3. Remove the blot from the imager deck and wipe down the deck with dH_20. Tidy up area and place all supplies and reagents back into transporting bag. Discard gloves and disposable lab coat.

8.4. At Echelon, send the results of the western blot to supervisors in a PowerPoint document with details of the experiment. Update the Antibody Database with the results as well.

APPENDIX A – Western Blot Worksheets

Western Blot - Gel Running

Date: _____

Gel Preparation

15% Resolving Gel		Added
dH2O	3.75mL	
Biorad Resolving Gel Buffer (1.5M Tris pH8.8)	2.5mL	
40% 29:1 acrylamide/bis	3.75mL	
10% APS	50µL	
TEMED	30µL	

☐ Pour resolving gel and immediately pipet distilled water on top

6% Stacking Gel		Added
dH2O	6mL	
Biorad Stacking Gel Buffer (0.5M Tris pH6.8)	2.5mL	
40% 29:1 acrylamide/bis	1.5mL	
10% APS	50µL	
TEMED	30µL	

☐ Pour off water

☐ Pour stacking gel and add comb

Prepare 1X Gel Running Buffer

Reagent	Volume	Added
10X Tris/Glycine/SDS Buffer	100mL	
dH20	900mL	

Prepare Total Lysate Samples

Lysate concentration 9.25 ug/ul (HL60)

Lysate Amount	Dilution
25ug	2.7ul HL60 + 7.3ul RIPA + PIC

Lysate concentration 14.2 ug/ul (Hela)

Lysate Amount	Dilution
25ug	1.8ul HL60 + 8.2ul RIPA + PIC

Gel Layout

1	2	3	4	5	6	7	8
	10ul ladder	HL60 (25ug)	Hela (25ug)	10ul ladder	HL60 (25ug)	Hela (25ug)	

☐ Add 10ul of loading dye to lysate samples

☐ Boil samples at 90°C in a thermocycler for 10 minutes and then place on ice

☐ Load Ladder and Lysate according to Gel Layout

☐ Run gel at 150V for 65 minutes

Western Blot - Transfer

Date: _____ *prepare in fume hood

Prepare 1X Transfer Buffer*

Reagent	Volume	Added
10X Tris/Glycine Buffer	100mL	
methanol*	200mL	
dH20	700mL	

☐ Store Transfer buffer at 4°C

Assemble Blot Sandwich*

☐ Pre-soak sponges and whatman paper in transfer buffer

Clear Plate (+)
1 Piece of sponge
1 Piece of Whatman Paper
1 Piece of nitrocellulose membrane
Gel
1 Piece of Whatman Paper
1 Piece of sponge
Black Plate (-)

☐ Assemble sandwich in transfer buffer
☐ Remove bubbles with serological pipette
☐ Insert sandwich into transfer apparatus (black plate closest to black side of chamber)
☐ Transfer proteins at 380mA for 60mins

Preparing Blocking Solution

1X TBST

Reagent	Volume	Added
10X TBS	50mL	
10% Tween	2.5mL	
dH20	447.5mL	

☐ Store 1X TBST at room temperature

5% milk in 1X TBST (blocking solution)

Reagent	Volume	Added
Biorad Blotting Grade Blocker (non-fat dry milk)	2.5g	
1X TBST	up to 50mL	

☐ Shake vigorously to resuspend milk powder into solution
☐ Store 5% milk in 1X TBST at 4°C

Blocking the nitrocellulose membrane

☐ Pipette 3mL of blocking solution in a petri dish
☐ Cut the membrane and place into the blocking solution
☐ Incubate and block membrane at room temp for 1hr, with shaking

Western Blot - Probing

Date:

Prepare 1° Antibody Probing Solution

Blot # 1

Antibody	H3K4me1
Company	Diagenode
Catalog #	C15410037 (pAb-037-050)
Lot #	A1657D
LIMS ID	
Aliquot Amt	
Dilution Factor	1: 500
	6ul H3K4me1 + 3ml of 5% milk in 1X TBST

Blot # 2

Antibody	H3 Core
Company	Abcam
Catalog #	ab1791
Lot #	GR65697-1
LIMS ID	sol137289
Aliquot Amt	3ul
Dilution Factor	1: 1000
	3ul H3 Core + 3ml of 5% milk in 1X TBST

Blot # 3

Antibody	H3K4me3
Company	Cell Signaling
Catalog #	9751S
Lot #	Lot#7
LIMS ID	sol145197
Aliquot Amt	5ul
Dilution Factor	1: 500
	6ul H3K4me3 + 3ml of 5% milk in 1X TBST

Blot # 4

Antibody	H3K9me3
Company	Diagnode
Catalog #	pAb-056-050
Lot #	A1675-001P
LIMS ID	sol135235
Aliquot Amt	3.29ul
Dilution Factor	1: 500
	6ul H3K9me3 + 3ml of 5% milk in 1X TBST

- ☐ pour off blocking solution
- ☐ pour diluted 1° Antibody on membrane and incubate overnight @ 4°C with shaking
- ☐ wash 3x with 1X TBST, 10min each with shaking

Prepare 2° Antibody Probing Solution

Antibody	Goat Anti-Rabbit HRP
Company	Pierce
Catalog #	1858415
Lot #	HA102347
Dilution Factor	1: 3000
	1ul Goat Anti-Rabbit HRP + 3ml of 5% milk
	in 1X TBST

- ☐ pour diluted 2° Antibody on membrane
- ☐ incubate for 1hr at room temperature, with shaking
- ☐ wash 3x with 1X TBST, 10min each with shaking

ECL detection

- ☐ mix together 500ul of Enhancer Solution and 500ul of Peroxide Buffer per Blot
- ☐ cover membrane with ECL detection buffer and incubate for 2mins
- ☐ while incubating, cover blots with a mylar sheet
- ☐ after incubation, use kimwipe to absorb excess ECL and cover with fresh mylar sheet
- ☐ expose to film and devlop in dark room

APPENDIX B

Confirmation of Protein Transfer Using Ponceau Stain

Follow the steps below if it is necessary to confirm the transfer of the cell lysate from the gel to the nitrocellulose membrane:

1. To prepare Ponceau stain, see Table 7. Prepare the stain in a fume hood.

Table 7. Ponceau Stain

Reagent	Volume
Ponceau Powder	50mg
Glacial Acetic Acid	2.5mL
Ultrapure H_2O	up to 50mL

2. Pour enough Ponceau stain on to the membrane and to cover the membrane completely.

3. Pour off the Ponceau stain immediately and rinse the membrane with dH_2O. Continue to rinse until the pinkness in the membrane disappears and the bands of interest are visible. If the protein bands are not visible, consult with an APC.