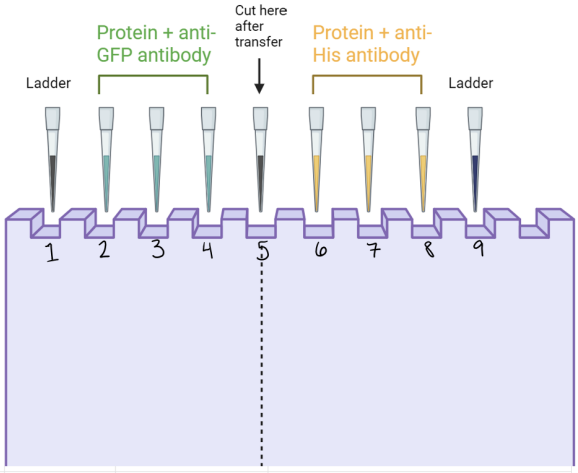
	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ** **PRODUCTON**
Western Blot **Module Hours: 5**	**Effective Date: 01.20.2024** **PRQs** **SDS PAGE**	**Revision 1.0** **Authors: C. Butcher** **August Kuca** **Checked by** **Editor: M. Stowell**

1. **Before completing this module:** Obtain an aliquot of purified GFP Protein and run the protein on an SDS-PAGE using the image below as a guide**



Lane in Gel	Contents	Antibody to use (after transfer)
1	Ladder	NA
2	Protein	anti-GFP (polyclonal)
3	Protein	anti-GFP (polyclonal)
4	Protein	anti-GFP (polyclonal)
5	Ladder	**NA (cut membrane here after transfer)**
6	Protein	anti-His (monoclonal)
7	Protein	anti-His (monoclonal)
8	Protein	anti-His (monoclonal)
9	Ladder	NA

** **Do not** add the antibody to the well with the protein. Proteins will be run on SDS-PAGE gels according to SDS-PAGE SOP. This diagram is meant to show you how to set up your SDS-PAGE gel so that you can compare Western blots using different antibodies in this SOP.

2. Background

Western blotting is a laboratory technique that allows for the detection of small molecules via antibodies with specificity to the target protein. Oftentimes during the analysis of biological samples, you will need to analyze samples that contain a

heterogeneous mixture of many different proteins and other molecules. Additionally, you might need to determine if a specific protein, which might be in small quantity, is present in your sample at all. For either of these scenarios, Western Blotting is a useful tool to help you detect specific proteins, even if they are in small quantities. This is accomplished by first resolving the molecular weight of your protein samples in solution via SDS-PAGE. The resulting gel from the gel electrophoresis can be placed on chemically treated blotting membrane paper, sandwiched with sponges and other materials to allow even conductance across the gel, and “blotted” from the gel to the membrane using a voltage gradient that passes from the front of the gel to the membrane placed on the back. After protein is blotted, the membrane can be treated with a variety of solutions to allow for blocking of non-specific binding positions on the membrane, and then incubated with a buffer solution containing the antibody specific for the target protein. After primary antibody has bound to target, and the remainder washed away, the membrane can be treated with a solution containing the secondary antibody which will bind the primary and allow for detection of your protein of interest via fluorescence or an enzyme-linked chemical reaction. With the proper control samples, western blotting can be a powerful technique for detection of small polypeptides with high specificity.

There are three types of Western blot transfers: wet transfers, semi-dry transfers, and dry transfers. Wet transfers, pictured in figure 2, are performed in a tank filled with transfer buffer and with an external power supply. The transfer apparatus is assembled as shown. Wet transfers are useful when you want to gain quantitative information from our blot, as the time, temperature, and voltage can be customized to best suit your protein of interest to ensure a successful transfer. A disadvantage of this method, however, is heat generation: as the apparatus runs, it heats up. This can result in inconsistent transfers or breakdown of the gel. Therefore, adding ice packs around the apparatus or running the transfer in a cold room is beneficial. Additionally, wet transfers may require a large volume of transfer buffer, depleting resources in the lab.

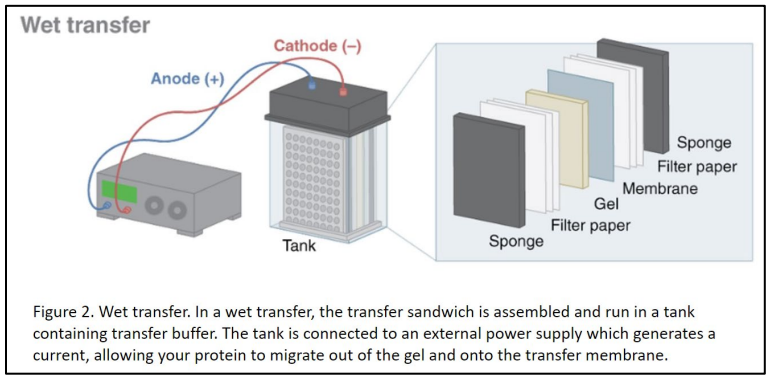


Figure 2. Wet transfer. In a wet transfer, the transfer sandwich is assembled and run in a tank containing transfer buffer. The tank is connected to an external power supply which generates a current, allowing your protein to migrate out of the gel and onto the transfer membrane.

Semi-dry transfers, pictured in figure 3, are beneficial when looking to conserve reagents and time. The transfer apparatus is assembled as shown. Semi-dry transfers, in addition to saving time (can be completed in 7 minutes to an hour, depending on the molecular weight of your protein, as opposed to the 1-2 hour wait time demanded by the wet transfer) and resources, can reliably be run at room temperature. However, this transfer method can require more optimization, and is not as reliable as a wet transfer for very small or very large proteins.

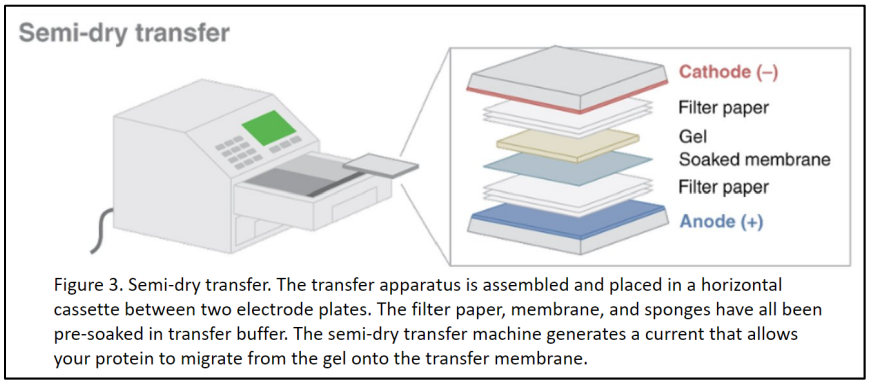


Figure 3. Semi-dry transfer. The transfer apparatus is assembled and placed in a horizontal cassette between two electrode plates. The filter paper, membrane, and sponges have all been pre-soaked in transfer buffer. The semi-dry transfer machine generates a current that allows your protein to migrate from the gel onto the transfer membrane.

The dry transfer, the method we will use in this protocol, does not use transfer buffer at all. Shown in figure 4, pre-assembled transfer stacks are used to transfer the protein from the gel to the membrane and are placed in a machine with a built-in power supply. Dry transfers can be completed in less than 10 minutes, are extremely user-

friendly, and have little need for optimization. Additionally, you do not need to worry about air bubbles, a problem in the other two methods which impacts successful protein transfer, as the anode does not release oxygen gas (a product of the current flowing through the transfer buffer used in the other methods.) However, dry transfers can be expensive because the transfer stacks are consumables not able to be created in our lab.

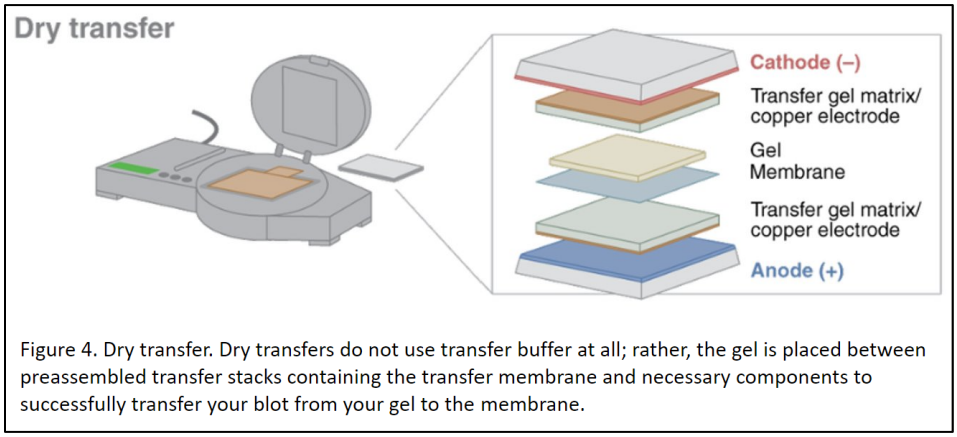


Figure 4. Dry transfer. Dry transfers do not use transfer buffer at all; rather, the gel is placed between preassembled transfer stacks containing the transfer membrane and necessary components to successfully transfer your blot from your gel to the membrane.

All transfer methods use one of two main types of transfer membrane: PVDF or nitrocellulose. Transfer membranes are pieces of "paper," essentially, that have pores with a random, interconnected sponge-like structure (see figure 5.) This structure allows proteins to be immobilized for downstream analysis. When choosing the proper membrane, it is important to consider protein size, post-translational modifications, how you plan to detect the proteins after the transfer, and how likely it is you will have to strip and re-probe the membrane. For this SOP, we will be using nitrocellulose membranes, as they come with our pre-assembled

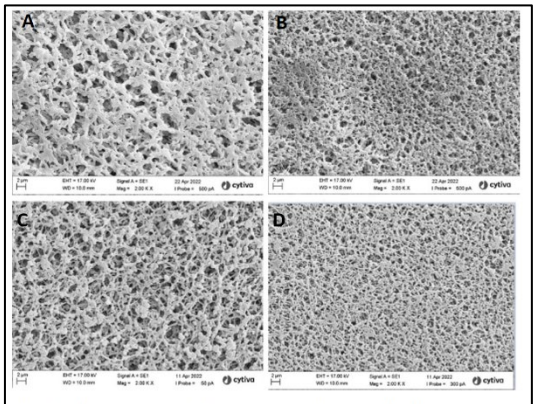


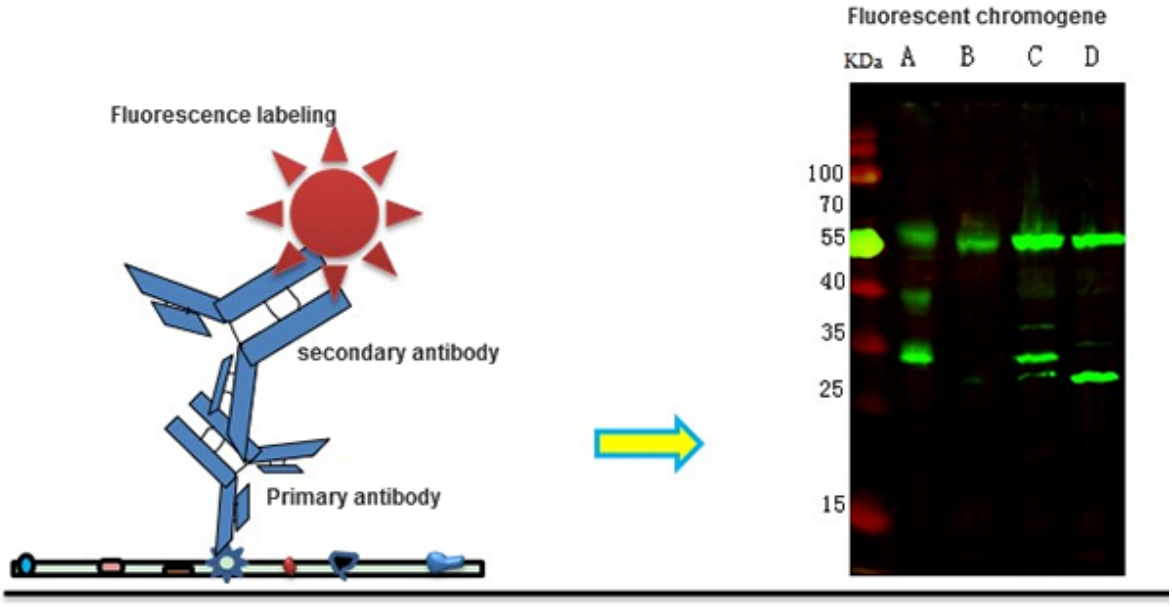
Figure 5. Electron micrograph of transfer membranes with different pore sizes. (A) and (B) show PVDF membranes, while (C) and (D) show nitrocellulose

transfer stacks. If you are curious, consult figure 6 from Cytiva Life Sciences, to learn more about the differences between the two types of membranes.

	Nitrocellulose	PVDF
Sample concentration	80-100 µg of protein/cm^2	150-200 µg of protein/cm^2
Protein size	Better for mid-to-low MW proteins	Better for high MW proteins
Protein binding interactions	Hydrophobic interactions	Hydrophobic and dipole interactions
Durability	Less durable	More durable
Saturation	Requires methanol or ethanol prior to transfer	Requires methanol in the transfer buffer
Chemical resistance	No, but improved by reinforced nitrocellulose membranes	Yes
Strip and re-probe	Suitable	Possible, but can lose sensitivity during rounds. Reinforced nitrocellulose membranes improve suitability
Autofluorescence	Low	High, but 'low-fluorescence' membranes are available
Detection	Well suited to chemiluminescence and fluorescence detection methods	Well suited to chemiluminescence detection but standard PVDF membranes can give high background. Dedicated low-fluorescence PVDF membranes can be used for fluorescence detection

Figure 6. From Cytvia Life Sciences, a comparison of nitrocellulose and PVDF membranes

Before you begin, it is important to identify the antibodies you are going to use. This brings us to a discussion of primary vs. secondary antibodies, and how one goes about choosing the correct antibody pairing. Primary antibodies recognize and bind directly to the target protein (the “antigen”). Secondary antibodies bind to the primary antibody and are typically conjugated with a fluorescent signaling molecule for easy detection. Here, our primary antibody will recognize and bind to GFP, and our secondary antibody will be conjugated with fluorescent dye. The intensity of this fluorescent signal (i.e. the “thickness” of the band on your Western blot) can be used to ascertain the abundance of the target protein on the transfer membrane. This is shown in figure 7 below.



Choosing a primary-secondary antibody pair can be challenging, as there are several factors to consider. First, you must choose a primary antibody that is specific to your protein of interest while also trying to avoid cross-reactivity with other proteins.

This is where consideration of a polyclonal vs. Monoclonal antibody can be important. Polyclonal antibodies can target many epitopes (a part of the antigen that antibodies recognize and bind to) of a single antigen, while monoclonal antibodies target a single epitope of a single antigen. Thus, monoclonal antibodies are much more specific with a lower chance of cross-reactivity. However, they are expensive, take more time to produce, and are very prone to slight changes of an antigen. Polyclonal antibodies are more translational, have a high sensitivity and affinity to the target protein, and are cheaper, but have a higher risk of cross-reactivity and are prone to batch-to-batch variability. Here, we will use a polyclonal antibody. However, for personal experiments, this selection depends on how sensitive and specific you need your assay to be.

After you've chosen a primary antibody, choose a secondary antibody that will recognize your primary antibody. The secondary antibody must match the organism in which the primary antibody was raised. For example, a mouse primary antibody needs an anti-mouse secondary antibody produced in another species (a goat, perhaps, yielding a label "goat anti-mouse.") The secondary antibody must also match the class or subclass of the primary antibody. If the primary antibody is in the mouse IgG class (a common class of antibody), choose an anti-mouse IgG secondary antibody.

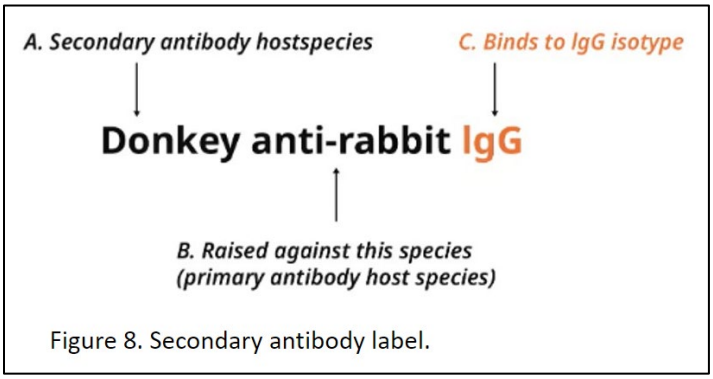


Figure 8. Secondary antibody label.

Choosing the proper antibody pair and using the most optimal transfer method and transfer membrane will ensure successful transfer and detection of your protein of interest.

2. Purpose

The purpose of this SOP is to understand the basic principles of the protein detection technique known as Western Blotting and to be able to conduct this process in the lab.

In this SOP, you will:

1. Transfer protein from an SDS-PAGE gel you have already run to a nitrocellulose membrane.

2. Compare Western blots using an anti-GFP antibody (polyclonal) and an anti-His antibody (monoclonal).

3. Scope

This procedure applies to qualified skills center users.

4. Responsibility

- It is the responsibility of the user to understand and perform the procedure described in this document.
- It is the responsibility of the user to fully document any deviations from the written procedure.
- It is the responsibility of the user to become trained on and display mastery of the procedure.

5. Definitions

- Transfer membrane: special paper composed of nitrocellulose or PVDF that has a sponge-like pattern of pores on the surface, allowing for protein immobilization during transfer.

- Primary antibody: antibody that directly recognizes and binds to your protein of interest (the antigen).

- Secondary antibody: antibody that recognizes and binds to your primary antibody. Usually conjugated with a fluorescent dye or enzyme (HRP) to allow detection.

- Horseradish peroxidase: enzyme that is conjugated to our secondary antibody if use the ECL reagent. Produces a chemiluminescent signal when the substrate is added to allow for protein detection.

- Enhanced chemiluminescence (ECL): substrate of HRP that helps produce the chemiluminescent signal for protein detection.

- Polyclonal antibody: antibody that recognizes a wide array of epitopes on a single antigen.

- Monoclonal antibody: antibody that recognizes only one epitope of a single antigen.

6. Materials/Equipment

6.1. iBlot2 transfer device
6.2. iBlot2 transfer stacks
6.3. Filter paper
6.4. Transfer stack roller
6.5. DI water in a suitably sized container
6.6. Pre-run, unstained gel run using SDS-PAGE
6.7. 1x TBST
6.8. Non-fat dry milk
6.9. Primary antibody
6.10. Secondary antibody
6.11. ECL kit
6.12. Micropipettes
6.13. Disposable pipette tips
6.14. Licor C-digit scanner and associated software

7. Recipes

7.1. 10x TBS, 1L

- 24 g Tris base
- 88 g NaCl
- Dissolve Tris base and NaCl in 950 mL DI water and adjust pH to 7.6 with HCl. Add DI water to bring the final volume to 1 liter.

7.2. 1x TBS+T, 100mL

- 10 mL 10x TBS
- 90 mL DI water
- 100 uL Tween 20 detergent
- Combine components in an appropriately sized receptacle. Mix well.

7.3. Blocking buffer (1x TBST + 5% dry non-fat milk)

- 1.5 g non-fat dry milk
- 30 mL 1x TBST
- Combine dry milk and TBST in an appropriately sized receptacle. Mix until milk is dissolved. Store in fridge until ready to use.

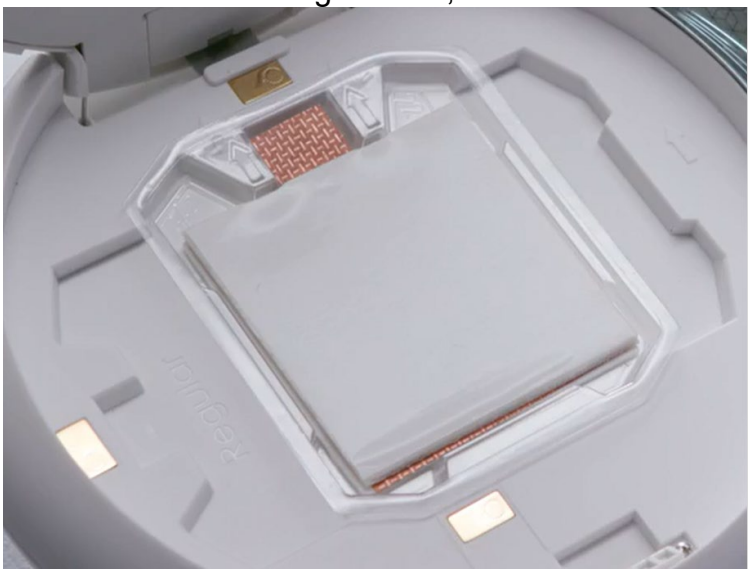
8. Procedures

The Western Blotting procedure begins with a pre-run, unstained gel from your SDS-PAGE module set up in the configuration detailed at the very beginning of this SOP. First, you will transfer your protein from the gel to a nitrocellulose membrane. Next, you will block non-specific binding positions on the membrane. Then, you will incubate the membrane first with a primary antibody solution, then with a secondary antibody solution to identify your protein of interest. Finally, you will image your gel on the imager.

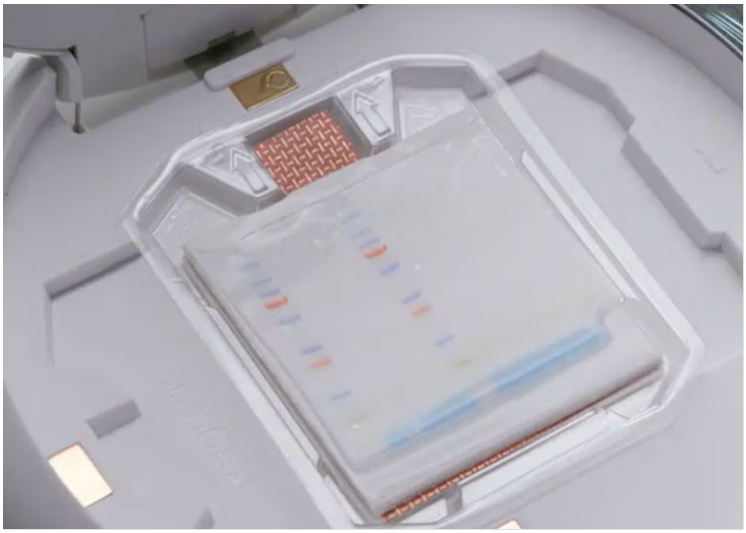
8.1. **Protein Transfer: using the iBlot2 dry blotting system**
https://www.youtube.com/watch?v=PN6ZMQWeMfI&t=1s
This 3-minute video is helpful if the instructions are confusing.

8.1.1 Turn on the iBlot2 dry transfer device and open the lid. The digital display will show icons for the available actions.
8.1.2. Unseal the transfer stack and separate the top stack from the bottom stack using tweezers. Place the top stack aside for now. **Keep the bottom stack in the plastic tray.** The bottom stack contains your nitrocellulose membrane.

8.1.3. Place the tray with the bottom stack on the blotting surface of the iBlot2. Be sure to align the electrical contacts on the tray with the corresponding contacts on the blotting surface, as shown.



8.1.4. Carefully pick up your pre-run SDS-PAGE gel and place it on the membrane



8.1.5. Briefly soak a piece of filter paper in DI water, then place the filter paper on top of your gel. Use the roller to remove air bubbles.

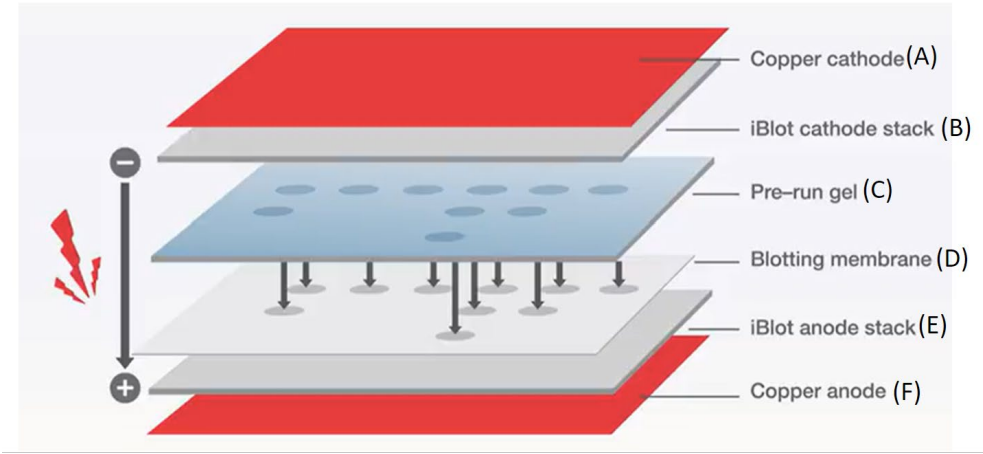
Note: it is important to ensure that you remove all air bubbles between the stack and the gel. Otherwise, protein transfer will be less effective.

8.1.6. Place the top stack over the pre-soaked filter paper and use the roller to remove air bubbles.

Note: there is a piece of white paper that separates the bottom stack from the top stack. Remove it before placing the top stack on the sandwich, otherwise the machine will display an error.

8.1.7. Place the absorbent pad on top of the top stack. Be sure the electrical contacts are aligned with the corresponding contacts on the blotting surface.

Your final “transfer sandwich” should appear as follows:



Important note about the sandwich: The current will flow from the negatively charged cathode (black) to the positively charged anode (red). The proteins within your gel are negatively charged because you added SDS to help denature

them. Therefore, the proteins will move towards the anode, making it imperative that you correctly assemble the sandwich.

8.1.8. Close the lid. Select “templates” → “P0” on the touchscreen. Wait approximately 7 minutes for your transfer to be completed. When the transfer is complete, select the “done” icon to stop the beeping.
Note: the transfer usually takes much longer. But this device is fancy and saves you about an hour of waiting around. Pretty awesome.

8.2. **Disassembling the transfer stack**

8.2.1. Open the lid of the transfer device and discard the absorbent pad and top stack.
8.2.2. Carefully remove and discard the gel (C) and filter paper (between B and C) BUT NOT THE TRANSFER MEMBRANE! (D)
8.2.3. Remove the transfer membrane (D) from the stack and place into a container of DI water. Proceed to the blocking procedure.

8.3. **Blocking non-specific binding sites and antibody incubations**

8.3.0. Before incubating with antibodies, use scissors to cut your transfer membrane down the middle ladder (see image on first page.)
8.3.1. In a 50mL conical tube, make 30mL of blocking buffer: 5% non-fat dry milk in 30mL, 1x TBST

Weight of dry milk to add:
(0.05)*(volume of TBST) = _____ g non-fat dry milk powder

8.3.2. Dump out DI water from the container with the transfer membrane making sure not to touch the top of the membrane with your fingers. Add about 8mL of blocking buffer to the side of the container (not directly onto the membrane) and leave on a rocker at room temperature for 1 hour, or in the fridge overnight.
8.3.3. Make your primary antibody solutions: in a 15mL tube, add your primary antibody to proper dilution to 8mL of blocking buffer.

Anti-GFP antibody: want a 1:3000 dilution

Calculation: (mL blocking buffer) / 3000 = _____ mL anti-GFP antibody to add to blocking buffer.

Anti-His antibody: want 1 ug/mL

Calculation: (x mL antibody stock) * (1000 ug/mL) = (8 mL buffer solution) * (1 ug/mL). Solve for x. This is the volume, in mL, that you will pull from the "stock" antibody solution

8.3.4. Once membrane blocking is complete, dump out blocking buffer and add your primary antibody solution to the side of the container. Place on rocker at room temperature for 2 hours or in the fridge overnight.

8.3.5. After primary antibody incubation, remove primary antibody solution and add it back into the 15mL tube. You can save it to use again if you need to re-attempt the antibody stain.

8.3.6. Wash your membranes for 5 minutes, 3 times with 8mL of TBST. Place on rocker at room temperature for washes.

8.3.7. Make your secondary antibody solution: in a 50 mL tube, add your secondary antibody (fluorescently labelled anti-rabbit IgG) at a dilution of 1:4000 to 16 mL of blocking buffer.

Calculation: (mL of blocking buffer) / 4000 = volume, in mL, of secondary antibody to pull from "stock" and add to buffer solution.

8.3.8. Add 8mL of secondary antibody solution to each half of the membrane after removing TBST and let react for 1 hour at room temperature on the rocker.

8.3.9. Remove your secondary antibody solution and add it back into the 15mL tube in case you need to use it again.

8.3.10. Wash your membranes for 5 minutes, 3 times with 8mL of TBST. Place on rocker at room temperature for washes.

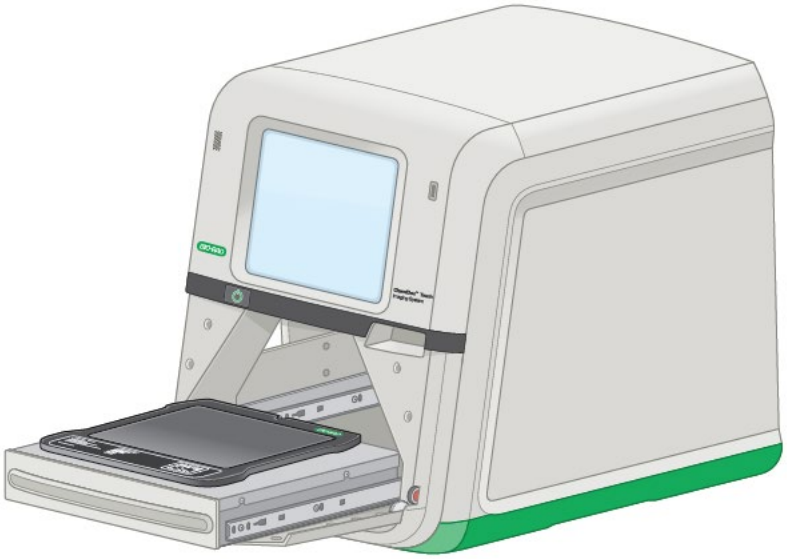
8.3.11. If you need to store your membranes to image another day, store covered in 10mL of TBST in the fridge. Otherwise, proceed to the next section.

Note: do not leave your membrane for more than 3 days in the fridge.

8.4. **Fluorescent imaging – using the ChemiDoc MP Imaging System and associated software**

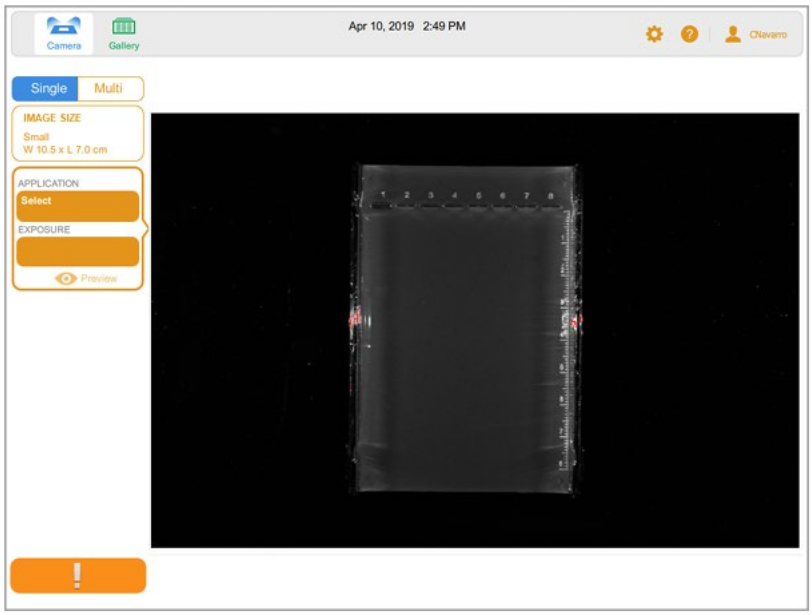
** Perform the following with one half of your membrane, then repeat for the other half.

8.4.1 once ready to scan, dump out TBST solution from the containers holding the membranes
8.4.2. Log into imager by using the drop-down suggestions for usernames and click the option that says ‘Stowell’

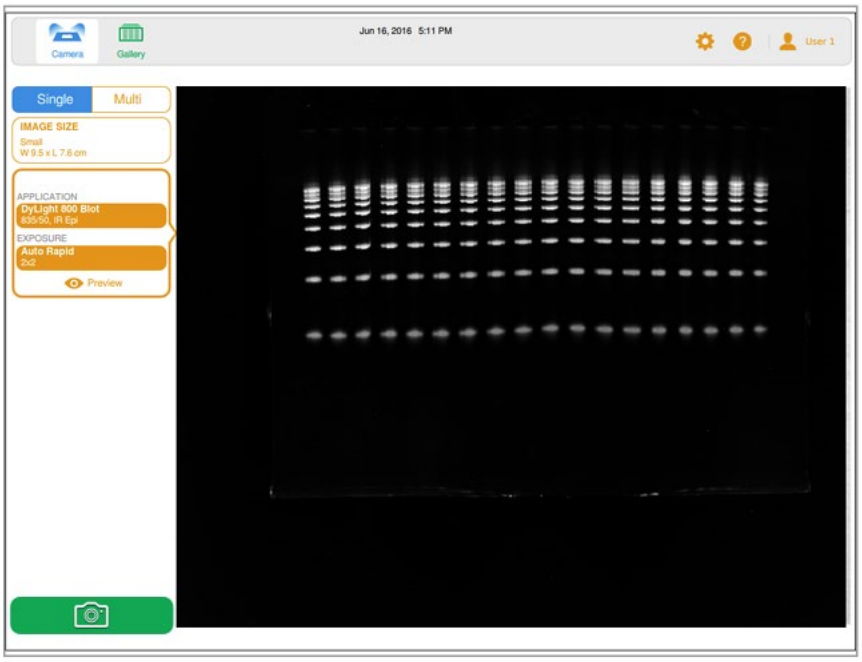


8.4.3. Place your membrane on the **Blot/UV/Stain-free tray.** If the try is not in the rack it may be in the instrument. Gently open the imaging system and pull out the tray; place your membranes so that the side with visible bands is facing upwards at you

8.4.3.2 The view the imager should be set to is camera view which will allow you to determine if you need to shift your membrane/s to the left or right



8.4.5.1 From here, click the ‘Application’ setting, and open up the application options for ‘blots’. From here, choose the fluorescein application.
8.4.5.2 Click the ‘exposure’ setting and select either optimal or rapid auto-exposure. Optimal exposure will take longer to generate but produce more visually appealing bands whereas Rapid exposure takes less time but may generate under/overexposed bands.
8.4.6 After adjusting the exposure and application settings it will be possible for you to preview your membrane. The result should be a lower resolution image of your bands



8.4.7 Once any necessary adjustments have been made, click the camera icon in order to acquire an image of your membrane

8.4.8. When acquisition is complete, select the best image and click “done.” Save your image by emailing it to yourself or using a thumb drive. Exit out of the software, remove your membrane from the scanner, **clean the scanner plate with a little bit of ethanol,** and close the lid.

9. Troubleshooting

9.1. No current

9.1.1. Incorrect placement of the plastic tray leading to interference with contacts

9.1.2. Plastic tray was removed when assembling the stack

9.1.3. The metal safety contacts in the lid hinge may have been dirty, precluding full contact

9.1.4. Metal contacts were bent

9.2. No protein transfer or distorted bands

9.2.1. No current or incorrect method used

9.2.2. Non-uniform electric field created around wells due to a gel that was not completely flat in the apparatus.

9.3. Empty spots on the membrane

9.3.1. Presence of air bubbles between the gel and membrane

9.3.2. Transfer time may have been too long

10. References

1. *1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent (TBST) for Western Blotting*. (n.d.). Retrieved November 1, 2023, from https://www.sigmaaldrich.com/US/en/support/calculators-and-apps/1x-tris-buffered-saline-tween-20
2. *1X Tris-Buffered Saline (TBS) Working Solution for Western Blotting*. (n.d.). Retrieved November 1, 2023, from https://www.sigmaaldrich.com/US/en/support/calculators-and-apps/1x-tris-buffered-saline
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11. *Western Blotting Protocol (Primary Ab Incubation In Milk) Cell Signaling Technology*. (n.d.). Cell Signaling Technology. Retrieved November 1, 2023, from https://www.cellsignal.com/learn-and-support/protocols/protocol-western-milk
12. *ChemiDoc and ChemiDoc MP Imaging Systems With Image Lab Touch Software from* 10000062126.pdf (bio-rad.com)

13. Module Methods Task (MMT)

11.1. Take a picture of your Western blot transfer stack and attach it to your document. Describe the order of materials in the stack you constructed and why you constructed it that way.

11.2. Show your calculations for how you made your primary and secondary antibody solutions.

11.3. After your transfer and blotting, use the ChemiDoc system to image your blot as described above. Attach the image from the imager to this document, labeling the ladder and lanes. Based on your results, does your sample contain your protein of interest? How do you know?

11.4. Describe the utility of Western blotting. What makes it different from SDS-PAGE? Why would you use Western blotting instead of SDS-PAGE?

11.5. Describe how proteins are transferred from the SDS-PAGE gel to the blotting membrane. Why is it imperative that the transfer sandwich is assembled correctly?

11.6. Explain why we use both a primary and a secondary antibody to identify our protein. Why can't we just use a primary antibody?

11.7. Describe how to choose an appropriate primary/secondary antibody pair.

11.8. Describe the difference between a monoclonal and a polyclonal antibody? What are the benefits and drawbacks of each? Based on the images you acquired, compare the half membrane stained with a monoclonal antibody vs. the polyclonal antibody. Describe what you see and any differences you observe.