***Western blot with specific antibody***

1. **Transfer to membrane**
   1. **Materials: Transfer Buffers**
      1. **Anode I (Blotting paper) Add enough deionized water to make 1 L**

|  |  |
| --- | --- |
| **300mM Tris base** | **36.34g Tris** |
| **0.05% SDS** | **2.5 mL 20% SDS** |
| **10% Methanol** | **100mL Methanol** |

* + 1. **Anode II (PVDF membrane) Add enough deionized water to make 1 L**

|  |  |
| --- | --- |
| **20mM Tris base** | **2.42g Tris** |
| **0.05% SDS** | **2.5 mL 20% SDS** |
| **10% Methanol** | **100mL Methanol** |

* + 1. **Cathode Buffer (Gel and 2nd blotting paper) Add enough deionized water to make 1 L**

|  |  |
| --- | --- |
| **25mM Tris base** | **3.03 Tris** |
| **0.05% SDS** | **2.5 mL 20% SDS** |
| **40 mM Caproic Acid** | **5.25g Caproic Acid** |
| **10% Methanol** | **100mL Methanol** |

* 1. **Methods**
     1. Set up 4 containers per gel
     2. Cut membrane to the size of filter and notch one corner being careful not to touch with fingers.
     3. Hydrate membrane in methanol.
     4. Remove the membrane from methanol and place into container marked Anode II buffer. Soak for 15 minutes on shaker
     5. Get out 2 blotting papers and place 1 in Anode 1 buffer and the 2nd in Cathode buffer.
     6. Remove gel cassette from electrophoresis chamber. Crack open both sides. Use spatula to shave off the extra portions of the gel and put a notch in the top left conrer (at about well #1)
     7. Carefully pick up gel and soak gel in Cathode buffer for atleast 5 minutes.
     8. Open the semi-dry transfer cell and stack the contents of the containers in the following order from the bottom to top: 1 blotting paper (Anode 1), membrane (Anode II), gel (cathode buffer), 2nd blotting paper (cathode buffer).
     9. Be sure to align the notches made in the membrane and gel. Use the small roller to roll out any bubbles/creases after putting on the membrane and gel layers to ensure good transfer.
     10. Close lid and set for a constant 0.28 A (0.56 A for 2 gels) for 45 minutes (time will vary according to protein size)
     11. Once complete, gently mark two bands of the ladder on the membrane and label its wavelength with a pencil.

1. **Western Blot-Blocking and Primary Antibody**
   1. **Materials**
      1. **Blocking Buffer**
         1. **PBS (make PBS solution with 5 tablets in 1 L of DI water)**
         2. **Odyssey blocking buffer**
         3. **Mix 1:1 PBS-Odyssey blocking buffer.** 
            1. **Eg:-to make 400 mL mix 200 mL PBS and 200 mL of blocking buffer**
      2. **Primary Antibody**
         1. **Mix 1:1 PBS-Odysssey blocking buffer with 0.2 % tween**
            1. **Eg to make 200mL 199.6 mL of PBS:Blocking buffer and 0.4 mL 100% tween**
      3. **Wash solution**
         1. **Mix PBS and 0.1% Tween**
            1. **Eg to make 1 L use 999 mL of PBS and 1 mL of 100% Tween**
   2. **Methods**
      1. Block membrane in a 1:1 Odyssey blocking buffer and PBS solution
      2. Incubate on shaker for 1 hour at room temperature or overnight at 4 C
      3. Choose the primary antibody and make a 1:1000 (15 uL) dilution in 15 mL (per membrane) of 1:1 Odyssey blocking buffer and PBS 0.2 % tween(solution B). For the most part we use 1:1000 dilutions but it can vary, always check
      4. Incubate on shaker for 2 hours at room temperature or overnight at 4 C. This can be cut down to 1 hour at room temperature if needed.
      5. After incubation, wash membrane 4 times for 5 minutes each time on shaker in PBS+ 0.1% tween
      6. \*\*\* All stages beyond this point should be done in the dark (wrapped in foil)
2. **Western Blot-Secondary Antibody**
   1. **Materials**
      1. **Secondary Antibody**
         1. **Mix 1:1 PBS-Odysssey blocking buffer with 0.2 % tween and 0.01% SDS**
            1. **Eg to make 200mL 199.4 mL of PBS:Blocking buffer and 0.4 mL 100% tween and 200 uL of 10% SDS**
         2. **Wash solution**
            1. **Mix PBS and 0.1% Tween**
   2. **Methods**
      1. Choose the appropriate secondary antibody for the primary antibody that was used. It should be anti-species used (ex. If primary was mouse, secondary shoul be anti-mouse) and the same class (ex.IgG,IgM,etc.)
      2. Prepare 50 mL of solution (BB:PBS + 0.2% Tween+0.01% SDS+antibody) per membrane. Using a 1:7500 dilution for the antibody, you'll need 6.7 uL for 50 mL solution.
      3. Incubate on shaker (wrapper in aluminum foil) for 60 minutes.
      4. After incubation, wash membrane 4 times for 7 minutes each time on shaker in PBS+ 0.1 % tween (solution C). Keep in dark
      5. Rinse the membrane once with PBS. Membrane can remain in PBS until ready to scan (keep in aluminum foil)
3. **Western Blot -Scan** 
   1. **Materials**
      1. Li-Cor odyssey in the core lab physiology room (MS-541) on the 6th floor
      2. Rolling tool
      3. Forcep
      4. Jump drive
   2. **Methods**
      1. Keep image from light
      2. Log on the computer as user physiology (password: physiology)
      3. Double-click the Odyssey V3.0 shortcut on the dsektop to launch the odyssey program.
      4. We will be keeping all our data in the 'Campbell Lab' Click File>Open> Projects, highlight the 'Campbell Lab' folder located at
      5. C:\Users\Physiology\Documents\Licor\Odyssey\Projects and click open
      6. To start a new scan, go to File>Scan>Scan…(or press Alt+F1)
      7. The scanner login dialog box will appear, log in using the username and password 'user'.
      8. Set scan parameters. First, scan membrane on the lowest quality with the desired channel and dimension (usually 10 X 15). Set intensity at 5. Press Start Scan.
      9. Once you obtain the desired results cancel the scan and rescan the membrane on medium quality with the previous set parameters.
      10. When scan is completed, press the save… button (on the same row as the start scan button you just pressed), name the scan with the following format: MMDDYY species muscle antibody gel # (if applicable) name, e.g. 052512 Hum Car cTNI Gel 1 Premi.
      11. Use the analysis name 'original analysis' and click ok.
      12. Before leaving, copy your scans folder (located at C:\Users\Physiology\Documents\Licor\Odyssey\Projects\Campbell Lab, Use shortcut to Projects located on desktop) on the jump drive (F:)
      13. Importing Scan into Odyssey. Open Odyssey and expand the Odyssey Projects folder.
      14. Identify which project you are working with and open it by double clicking on the folder.
      15. Click File>Scan>Import Scan. Select the file that you just saved on the jump drive and click OK.
4. **Western Blot- Ponceau Staining** 
   1. **Materials**
      1. **Ponceau Stain**
   2. **Methods**
      1. After scanning the membrane upstairs, download into Odyssey and make sure the scan worked. Only then begin staining with ponceau.
      2. Pour enough Ponceau S staining solution to cover the membrane and place on the shaker at medium speed for 45 minutes.
      3. Pour out the staining solution and spray with DI water to remove the stain. Continue until most of the red has disappeared on the surrounding area except for on the bands of protein.
      4. Place membrane on a blotting paper in a drawer in order to dry before scanning.
      5. Open Epson scan and verify the mode is set to Full Auto Mode.
      6. Click Customize>File Save Settings.
      7. Label the file exactly how it is labeled in Odyssey with “ponceau” at the end
      8. Select the file to be saved as a Tiff and click OK and then Scan.
      9. Edit the Tiff file in Photoshop. Under the image tab crop the image to only show the membrane and rotate if necessary. Click mode in the image tab and save it as a 16-bit, gray-scale image.
      10. Be sure to save original sane and altered scan (labeled the same with “16 bitat the end) in the correct project folder.
      11. Open Odyssey, go to File and click on Import image.
      12. Label the file exactly how it is labeled in Odyssey with “ponceau” at the end.
      13. Uses the Analysis name write Ponceau.
      14. Use the 800 channel and browse for the 16-bit, gray scale image in the project folder it was saved to, then click OK
      15. Go to alter image display under View. Click linear manual
      16. Go to adjust image curves under View. Select grayscale. Click the boc that says inverse gray scal and adjust the curve.
      17. Follow the Analyzing Scans in Odyssey (for westerns, ponceaus stains, and coomassie stains)protocol for the next step.