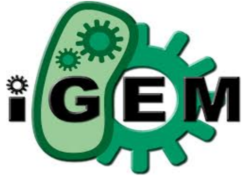
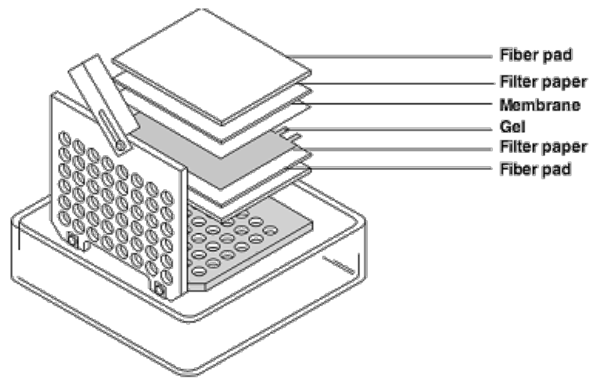


University of Melbourne iGEM 2014 Lab Procedure

Procedure	Name:	Western Blotting
	Version:	4
	Description:	How to run a Western Blot. See https://www.youtube.com/watch?v=VaXFwfkLdgw for a good instructional video. Visualising Proteins Video Transferring to PVDF Membrane Video
	Trigger:	Use this procedure on the same day as having run SDS-PAGE.
Last updated	Name:	Elizabeth Brookes, Michael Wei — Date: 28.07.14
You will need	Time:	6-7 hours
	PPE:	Gloves Lab coat
	Equipment:	Electroblotting Apparatus: Bio-Rad Trans-Blot Cell Power Supply: Bio-Rad Trans-Blot Cell Ice-block Magnetic Stirrer Rocking Platform
	Materials:	Transfer Buffer (10x Stock Solution): o 0.25M Tris o 100ml 2M Glycine o Add 200ml methanol when making 1L to 20% after dilution to 1x buffer and just before use add 1% SDS (1g for 1L). o *Note: Ensure that the transfer buffer is cooled in the fridge before use.* Blocking Buffer pH = 7.4: (make up 100mL and keep it in the fridge for up to 1 week). o 5% Non-Fat Dry Milk (0.5g dry milk) o 10 mL TBST PVDF Membrane Methanol Antibody Dilution Buffer: o 5% Non-Fat Dry Milk in 1% TBST Primary Antibody Secondary Antibody (Goat Anti-Rabbit IgG-HRP CAT#ASP00001)
Preparing the PVDF Membrane		
Step 1	Cut the PVDF membrane to the same size as the SDS-PAGE gel.	
Step 2	Soak the membrane sequentially in 100% methanol for 2 minutes, distilled water for 5 seconds and into transfer buffer for at least 10 minutes.	
Arranging the Gel Membrane Sandwich		
Step 1	Get a large plastic tub and fill it with transfer buffer so that you can immerse the sandwhich while you work. Arrange the Gel Membrane Sandwich in the following conformation:	

	 *Note: Set up the sandwich in a plastic tray filled with transfer buffer. Ensure that any bubbles on the membrane is removed using the roller brush.* Use a spatula or other device to CUT off the stacking part of gel, where the lanes are. Use same spatula to cut the sides of the gel so that it will come off. Place cut off gel in acrylamide waste bin. Don't touch the PVDF membrane with gloves. Use tweezers instead. Roll out the membrane just after you add it to the sandwich to visually ensure no bubbles are there.
Step 2	Place the transfer sandwich unit into a buffer tank and place a blank in the second unit space. Fill the tank with pre-cooled transfer buffer and attach the electrodes. Important: Ensure that there is transfer buffer almost overflowing between the gel area and the rest of the tank. No having enough buffer will cause the gel to run anomalously. *Note: Use a magnetic stirrer in the tank. And if possible, surround the tank with ice. The ice-block can be found in freezer 4 (near the small room at the entrance). Make sure the ice-block is remade and returned once finished.*
Step 3	Set the power supply to 100V, 400mA and transfer for 60 minutes. If transferring overnight, leave the apparatus in the cold room and use 10V with 50mA whilst setting the time limit to the maximum value possible. *Note: Ken says the power supply for the Bio-Rad Trans-Blot cell in the cold room is dodgy. Where possible, take the one on the outside into the cold room and use that one.*
Ponceau staining – Follow http://web.mnstate.edu/provost/PonseauSStainingProtocol.pdf, Ponceau Staining Protocol from Minnosota Uni on dropbox.	
Blocking	
Step 1	Disconnect the transfer apparatus, remove the transfer cassette and transfer the PVDF membrane to blocking buffer. Retain the used transfer buffer and note on the jar when it has been used. Transfer buffer can be used at least 5 times without loss of activity (see reference on the dropbox about this).
Step 2	Rinse off the transfer buffer with several washes of TBST, then rock in blocking buffer for 15-60 minutes (30 mins is good) at room temperature or keep it at 4°C overnight.
Immunoblotting	
Step 1	If we don't already have primary antibodies from last time, prepare the primary antibodies by making a dilution according the specific product details: e.g. 100ul thermisal (borrow from Cheng), X ul primary antibody, 10ml 5% milk in TBST. Currently we're using 4 uL of Genscript Anti-his.

<table>
<tr><td>Step 2</td><td>Pour on the primary antibodies. Leave the gel to rock in the solution for 2-4 hours at room temperature or at 4°C overnight.</td></tr>
<tr><td>Step 3</td><td>Thoroughly rinse off the primary antibodies. Save the primary antibody solution first by pouring into the Wash the membrane by pouring off the primary antibody solution and rinsing the membrane in TBST six times. Then, immerse the membrane in TBST and rock for half an hour. Pour off the TBST. (Source: Dave)</td></tr>
<tr><td>Step 4</td><td>Dilute 2 μL of the Antibodies Australia GAM 2.2 antibodies (the secondary antibodies) in 10 mL of 5% milk in a small centrifuge tube (source: Dais). For this step, you will need to use the Coles brand dry skim milk. To make the solution, fill up one of the 15 mL centrifuge tubes with distilled water, and add 5% weight/volume milk (e.g. add 0.5 g to 10 mL of water)

Cover the membrane in the secondary antibody solution and leave it to rock for 1 hour.

(try not to leave it on for more than one hour. If you leave it on for a day, for example, this will lead to nonspecific binding of the antibodies; also secondary antibodies will go off).

You can store the secondary antibody solution in -20 and reuse it 2-3 times (source: Daisy)</td></tr>
<tr><td>Step 5</td><td>Wash the membrane by pouring off the secondary antibody solution and rinsing the membrane 3-6 times with TBST.</td></tr>
<tr><td colspan="2">Developing the Gel</td></tr>
<tr><td>Step 1</td><td>Prepare the lightbox by using the computer to start it up. It needs to cool down to -30°C and this will take approximately 10-15 minutes.</td></tr>
<tr><td>Step 2</td><td>Lay a transparency sheet (piece of clear plastic) on top of the black plate to stop the membrane form touching the plate. Place the membrane on top of the sheet.</td></tr>
<tr><td>Step 3</td><td>Load both the left and right filter on the lightbox. Use the blue filters for proteins and the green filters for DNA.</td></tr>
<tr><td>Step 4</td><td>Because the molecular weight marker is usually quite faint, use a ballpoint pen to draw lines over the various marker protein bands.</td></tr>
<tr><td>Step 5</td><td>Load the plate into the lightbox with the circles pointed towards you.</td></tr>
<tr><td>Step 6</td><td>Click the 'Pro' button and ensure that the machine is ready. Then click chemiluminescne > tray 3 > focusing > brightness adjustment and ensure that you can see the membrane clearly.
Note: use some text written on masking tape to aid you in focusing the membrane (i.e. try to bring the text into focus).</td></tr>
<tr><td>Step 7</td><td>Get the ECL solution from the bar fridge. It is covered in aluminium foil and is usually on the side. Add ~2 mL ECL solution to the 15 mL tube attached to ECL bottle. Add trace amount of HRP (HRP in a microcentrifuge tube taped to side of ECL solution). Can take a yellow pipette tip, dab the HRP, then dab the 2mL ECL solution (source: Dave). Add the ECL solution to the PVDF membrane, spreading it around with a plastic hand pipetter to cover it completely.</td></tr>
<tr><td>Step 8</td><td>Select the exposure time:
• Auto
    o With the auto setting, it will automatically find the optimal exposure time. However, you may wish to have a longer exposure time. It is possible to set the machine to incrementally increase the exposure time</td></tr>
</table>

	by 10 seconds each round, taking photos after each round. You can then pick the best one. • High sensitivity
Step 9	Save the files in tif format (this is the format which is most compatible). To get a normal image, go to Method/Tray and switch it to DPI→Digitize Take a screenshot of both the marker and the exposed gel and paste both files to Microsoft PowerPoint. Then, on the screenshot of the marker, label the molecular weight bands. Then, send a screenshot of the marker to the back. Now you have an image of the gel with the molecular weight bands labeled
Version history	*V2 Original protocol was a generic one from the net. Changing it to be specific to Cheng lab* *V3: Added link to video and altered dilutions in step 5 of immunoblotting* *V4: Development of membrane section added.* *V5: Transfer buffer recipe altered, step 2 methanol soaking time change, primary antibody recipe added, wash buffer step removed.* *V6 Sean made lots of fixes*