WESTERN BLOT PROTOCOLS

Western Blot (Always, ALWAYS, inform Aman when running a Western Blot, we use his apparatus and he will give you the membrane)

Sample preparation

Calculate amount of cells from OD_{600}.
Aliquote 10^8 cells in an eppendorf tube.
https://www.chem.agilent.com/store/biocalculators/calcODBacterial.jsp?_requestid=51001
Spin down cells for 1 minute at max speed.
Wash cells twice by discarding the supernatant, adding cold PBS, resuspending and spinning down at max speed for 1 minute.
After the second wash centrifugation discard the supernatant, add 1 ml of 1% lysis buffer in PBS to the samples and resuspend the pellet.
Lyse the cells 30 minutes on ice, with vortexing at 10 minute intervals.
Centrifuge the tubes at 13.000 rpm for 10 minutes at 4 degrees celcius.
Transfer the clear supernatant to a clean tube. The sample is now ready for assays.

Alternative WB lysis --> told by Max

same up to step 5.
Instead of step 5, aa double the amount of sample than we put of loading buffer (blue one, same as we use for loading the WB gel)
Heat up at 92 C degrees for 10 minutes
spin down maxium rpm for 2 minutes
KEEP supernatant (you can freeze it or load it to the gel)

Concentration

From floor 3 get the Vivaspin® 500 kit (assuming you are working with C protein or Cox).
Assemble the tubes (smaller, odd shaped tube inside the larger normal tube) and write the sample name on the lid of the smaller tube as the sample will end up there.
Max 500 µl sample to the assembly.
Centrifuge the sample for 30 min at 15 000 g's
Collect the sample from the smaller tube.

SDS-PAGE

Gel prep
Unpack each of the gels and place them against the electrode (text outward comb inward - the comb can be recognised by the small double ridge at the top of it) together. If you are running a single gel only, use the dummy gel for the second slot (facing the clamp). REMOVE THE WHITE STRIPE on the bottom of each gel so the current can flow through!
Use the clamp to fixate the gels and electrode in place.
Secure and test tightness by filling the center between the gels entirely with running buffer and check for leaking. Keep adding running buffer until the chamber overflows.
Add running buffer to the rest of the tray until it is filled half way.

Remove the combs of the gels by slowly pulling it straight out and prepare for loading.

Loading
After sample prep add the loading dye (sample dye) so that it is 1x, so if it's a 2.5x loading dye mix 15µl sample and 10µl dye
Load the gel by pipetting 20-30 µl sample into the wells. For each group of samples (read a. below) add 4 µl ladder. Pipet straight down and avoid the creation of bubbles by not pipetting the entire sample.
When loading think about the fact that C and Cox needs to be tested in different well and therefore be separated in a way so that the membrane is easily cut. So in a gel with 12 wells you need 2 wells for ladders, 1 that is empty so you can load 4 wells to test for C and 4 to test for Cox.
Cox and C are similar in size to in a westernblot it would be difficult to distinguish them, so a single sample is tested twice, once for C and once for cox.
In a cool room run the gel at the voltage of 190V (210V when running two gels) for 30 minutes. Repeat this step until the ladder is clearly separated. Our proteins are very large and needs time to travel to the bottom. Run until the blue line is 1-2cm from the end.
When finished, collect the running buffer. It can be re-used three times in total (mark on flask).

Protein transfer

Membrane handling
Before doing anything, inform Aman that you are ready to transfer from the gel to the membrane. He will give you a prepacked membrane so no membrane prep is needed. The room in which you work with the membrane is used by the Atlas Protein group, so Aman needs to be able to inform them that we are there and we need to abide by their rules.
Remove the gel from the chamber, rinse away the bubbles using dH_2O, and use the flat spatula to carefully break the gels plastic frame by inserting the spatula on the side (this will produce cracking sounds).
Seperate the frames whilst holding it vertically. Be gentle and run dH_2O continuously over the gel to help its separation from one on the plastic sides. Once one side is removed set the gel to the side plated on the plastic frame.
Open the membrane packaging without touching the membrane, the membrane is on the "bottom" side so be extra careful not to touch that.
The membrane's job is to capture proteins, so touching it risks contamination. Make sure that you wear fresh gloves that have not touched many sufaces and they are washed with ethanol.
Place the bottom membrane in the center of the Turbo Transfer cassette the same side up as it was packaged using tweezers, make sure to only touch the edges as to avoid contamination.
Always use the matching cassette for the machine/slot you are using
Return to the gel and gently remove it from the frame it is laying on. Be firm but gentle.
Place the gel on top of the bottom membrane.

Place the top half of the membrane package on the gel. Squeeze any air out using a small roller.
Place the top half of the cassette on the tray and close it. Place the cassette in the Turbo Transfer but do not slide it in entirely!
Run the turbo transfer using the following settings:
Press [List] > press [Bio-rad] > press [Minigel] > press [Low MW] > press [Run] > Push the cassettes into the machine > press [Run] for cassettes loaded.
Remove the cassette ASAP after running the Turbo Transfer.
After removing the top of the membrane sandwish, the gel should be clear.
[Optional] Cut the gel with the dedicated scisors.
Use tweezers to put the gel into a falcun tube with 10 ml 5% milk solution.
Clean the cassette with dH_2O and the dedicated sponge. Soft side of sponge for the tray, rough side for the lid.
Incubate the samples with the milk solution for 1 hour on a roller/shaker in the coldroom. This is called "blocking", we ensure there will be no unspecific binding of the antibody

Antibody treatment
After blocking, add a *certain amount* (see a.) of the primary antibody. Incubate for a *while* (see b.)
Add whatever amount is recomended for that specific primary antibody, usually it 1:10 000. Our FLAG antibody needs to be diluted 1:1000, so to a 10 ml 5% milk solution add 10µl.
Wait whatever amount of time that is recomended for that antibody. Waiting longer won't hurt but usually a good antibody needs only an hour. The FLAG antibody needs to incubate overnight.
Wash the membrane using TBST buffer for 5 min. Repeat this step 3 times.
After removing the last wash of TBST, add 10 ml of 5% milk solution and a *certain amount* of secondary antibody. Incubate for 1-2 hour.
Like in step 2, wash the membrane with TBST for 5 min three times.
After removing the third wash, store the membrane in TBST.

Visualization

Staining - This depends on the substrate you are using. This is the guide for the Immobilon Western: Chemiluminescent HRP Substrate
In a falcon tube mix the 2 sub-substrates 1:1, mix 3 ml of each.
This is a commercial kit, so they don't divulge what the contents of the kit is, so we will call them sub-subsrates. Together the sub-substrates form a substrate which reacts to the peroxide.
The substrate is light-sensitive and needs to be stored in room temperature, so until use put it in our pocket.

Imaging - Inform Aman that you are using the computer
In a dark box (like a pipette-tip box with an aluminumfoil covered lid) pour the subsstrate.
On the computer, open the software "Image lab"

Click on *New protocol --> single channel*. Repeat this twice and 2 windows should pop up.
Under the slide "Application"
In window 1 select *custom --> High sensitivity*
In window 2 select *blot --> Chemi Hi Sensitivity*
Under the portion "imaging area"
Click on *select gel --> Bio-Rad criterion gel*
Take out the membrane and place it in the box and cover it with the substrate. Put on the lid and let rest for 30 sec.
During these 30 sec, cut out a film of plastic 2x the size of the membrane and place it in the visualizer tray.
Take the membrane out and place it on half of the plastic film
Fold the other half over the membrane and remove air bibbles with a roller. Close the vizualizer tray.
Return to the computer. Check if the membrane is visible by clicking on *Position gel* in window 1. Look and correct if there is any issue.
In window 1 click *Run protocol*. Wait, and observe.
In window 2 click on *Run protocol.* Wait and observe.
Then we merge the positioning image with the gel imaging using image tools --> *Merge.*
Discard the plastic film and membrane and clean the visualiser.

Congratulations! You have successfully run a Western Blot!

YFP: excitation at 450-550 / emission 500-580