WESTERN BLOT USING BACTERIAL LIQUID CELL CULTURES

This experiment is a widely used technique to detect proteins from tissue samples or extracts. The first day consist of a gel electrophoresis to separate the native proteins according to their 3-D structures or denatured proteins according to their length.

For each experiment you can either order the components or make them yourself with the material listed in the tables bellow.

After the protein migration, they are transferred to a nitrocellulose membrane to be stained with specific antibodies. The membrane may be visualized depending on the immunostaining technique used.

DAY 1: SDS PAGE

MATERIALS:

Resolving Gel (for 2 gels 12%)	Stacking Gel (for 2 gels 4%)	Running Buffer (2L)
- 6.86ml H2O MQ	- 3.05ml H2O MQ	- Tris base (Tris hydroxyl methyl amino methane) = 6.06g
- 15ml TRIS HCl 1.5M (pH 8.8)	1.25ml TRIS HCl 1M (pH 6.8)	-Glycine = 28.8g
- 7.92ml AcrylBisacrylamide 30%	- 0.65ml AcrylBisacrylamide 30%	SDS = 20µl (10% SDS stock)
- 100µl SDS 20%	- 25µl SDS 20%	Mix in 2L DW and Add SDS separately
At the time of casting add:	At the time of casting add:	
- 100µl APS	- 25µl APS	
- 16µl TEMED	- 6µl TEMED	

PROCEDURE:

MAKING A STACKING AND RESOLVING GEL:

□ Assemble glass plates on a pad and make sure that they are tight by adding water to see if it leaks or not. The smaller plate should be facing the front.

□ Prepare the resolving and stacking gels. If you do it a day in advance, you can store them in 4°C by wrapping them in moist tissue paper.

- □ Pour the resolving gel between the glass plates. Put isopropanol on top to make sure your gel has a straight edge. Wait ~20min for the gel to solidify.
- □ Remove isopropanol and add the stacking gel. Place a 10 well 1.5mm combs (depends on your experiment) and wait ~20 min for the gel to solidify.

Preparing your samples

- □ Pellet 1ml of your overnight cells cultures (pellet can be stored at -20°C) in 1.5ml tubes.
- □ Resuspend pellets in 50-100µl laemmli lysis buffer.
- □ Sonicate samples for 10min with 0.5s ON and OFF cycle.
- □ Denaturate the proteins at 95°C for 5mins.
- □ Centrifuge at maximum speed for 2mins.

If the sample is too viscous it will be difficult to load on the gel, so you will have to repeat the heat denaturation step.

Loading your Samples, Running the Gel

- □ Place gels in running bath containing running buffer.
- □ Load 20-35µl of sample (avoid the lower part of the samples).

Don't forget to label your gels.

- □ Run the gel at 60V for ~20min or until all the bands are alinged.
- □ Run the gel at 80V for ~120min or until the bands resolve well.

Day 1: Western Blot

Materials:

Transfer buffer (500ml)	1X PBST (2L)	5% Non Fat Milk
- 1.52g Tris base (Tris hydroxyl methyl amino methane)	- 200ml 10X PBS	- 1.5g Non Fat Milk
- 7.2g Glycine	- 1800ml DW	- 30ml DW
- 100ml Ethanol (Absolute)	- 1ml 0.05% Tween 20	
- Make up volume in DW		

PROCEDURE:

□ Cut fiber pads, nitrocellulose membrane and whatman paper at the same size of your gels.

□ Arrange the transfer sandwich in the following order (bottom to top):

- Fiber pad
- Whatman Paper
- NC Membrane
- Gel
- Whatman Paper
- Fiber pad

The nitrocellulose membrane should always be below the Gel

□ Squeeze out extra Transfer buffer and place the gel in the above-mentioned order into the transfer maschine.

□ Run membrane sandwich at 17V for 60mins

You can do a ponceau staining to see if the transfer worked and wash the membrane to remove the reagent before blocking.

□ Block in 15-20ml 5% milk for 45mins in a rocker.

□ Dilute fluorescent antibody 1:1000 in 5% non-fat milk (i.e. 30µl in 30ml). This can be stored several months and used repeatedly.

□ Add 15ml of the antibody dilution to your membranes.

□ Keep away from light, overnight at 4°C, on rocker.

DAY 2: WESTERN BLOT AND VISUALISATION

MATERIALS:

- Stacking gel
- Resolving gel
- Running buffer
- Blocking buffer (milk)
- Antibody milk
- Samples

PROCEDURE:

□ Remove the antibody from your membrane into a falcon and store at -20°C (the antibody can be re used).

□ Wash the membrane with 15-20ml PBST, 3times, 5mins on a rocker.

□ If you use a primary antibody, you will need a secondary one, conjugated to HRP.

- □ You can prepare a 1:500 dilution of the secondary antibody in non-fat milk (i.e. 6µl in 30ml).
- □ Put 15ml of this dilution on you membrane and incubate 45-60mins on a rocker.
- □ Wash each membrane with 15-20ml PBST, 5 times, 5mins on a rocker.
- □ Incubate each membrane 2min with 1ml detection reagent peroxide solution + 1ml luminol enhancer solution. Then place your membranes in the cassette. Take a pair of scissors and film to the developing room.
- □ In the developing room, keep the red light on. Place one of the developing films in the cassette (note the orientation). Take several exposures and put it into the developer.

With a first antibody already conjugated to HRP; you can directly switch from the first 3 washes to the incubation with detection reagents. Then you can develop your gels.

With a first antibody being already fluorescently labeled; you can directly visualize your membrane with a typhoon scanner after the first 3 washes.