NuPAGE® Bis-Tris Mini Gels Western blot protocol

Reagent preparation

PBS/T (Wash buffer 0.1%)
1ml Tween-20 per 1L PBS (1L dH20 +10 tablets of PBS)

5% milk solution

2.5g milk solution
50ml PBS-T

MOPS Running buffer (1X SDS Running buffer)
50ml 20x MOPS SDS Running Buffer to 950ml deionized water

Transfer buffer
100ml Methanol
1ml Antioxidant
50ml 20 X Transfer buffer
Made up to 1L with deionized water

Western blot

If lysate used for 1st time, spin at 13,000 rpm for 20 mins in cold room. Remove and save supernatant, discard the pellet.

1. Prepare samples (for reduced samples):

Sample	x µl (e.g. 5µl)
NuPAGE®LDS Sample buffer (4X)	2.5 µl
NuPAGE® Reducing agent (10X)	1 µl
Deionized water	To 6.5µl
Total volume….	15µl

   Heat at 100°C for 5min, centrifuge at 13000rpm for 2 min

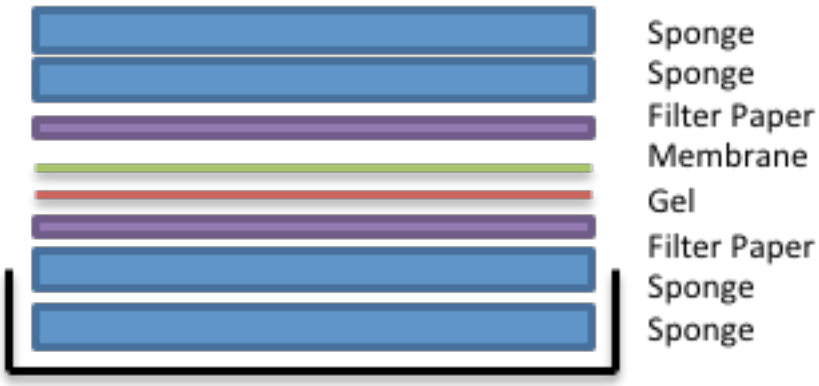
   Markers: 3µl/gel

2. Set gel in tank (remove white strip and comb), shorter plates inwards.
   Fill tank with 1X SDS Running Buffer. *Add 500µl NuPAGE® Antioxidant to upper Buffer Chamber.*
   Load samples e.g. 15µl each well – **remember the loading order**! Use 3µl of marker (Precision protein plus marker/Ladder).
   Run gel at 125V for 100min

3. Remove gel from casing and cut off wells and bottom of gel. Use the gel knife to crack open the cassette by separating the two plates and carefuly remove the gel from one of the plates. Store gel in transfer buffer.

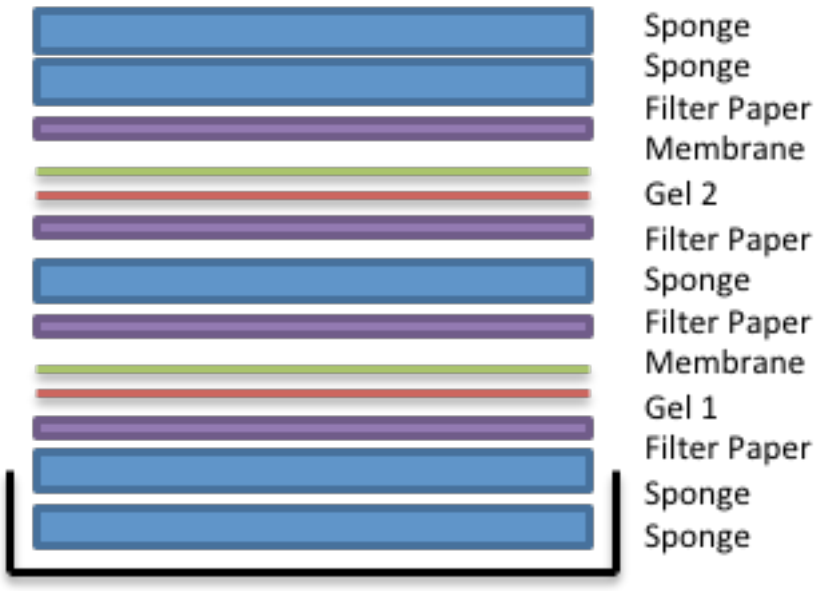
Cut filter paper & membranes (Don't touch membrane itself) , soak with sponges in transfer buffer . Place 2 sponges in deep side of cassette, filter paper, gel, membrane, filter paper, sponge and repeat (or fill with sponges). Assemble cassette and place in tank.

Sandwich for the transfer of one gel:



Cathode Chamber of Blot module

Sandwich for the transfer of two gels:



Cathode Chamber of Blot module

4. Fill cassette with transfer buffer
   Fill outer tank with dH_2O (Two third)
   Run at 25V for 90min

5. Place membrane in **5% milk solution (2.5g in 50ml PBS-T)** for **60min at RT** (or O/N in cold room)

6. Roll membrane in 50 ml tube and add 1:1000 – 1:500 primary antibody (2 - 4µl 1^{o} in 2ml 5% milk), incubate overnight on rocker in cold room (or 1 hour at RT)

   Calculation of Antibody:
   If using 1:500 dilution, then 6 **µL** antibody in 3ml of 5% milk (3000 **µL/500=6 µl)**

   Wash in weight boat x 3 in PBS-T (5 mins each)

7. Incubate in 1:10,000 β-actin (0.5µl β-actin in 5ml 5% milk) and incubate at RT for 1 hour (use different species as compared to primary antibody e.g if primary antibody is produced in Rabit, use Anti **β** actin antibody produced in mouse.

   Wash in weight boat x 3 in PBS-T (5 mins each)

8. Incubate in 1:10000 secondary antibody solution (0.5µl anti-mouse + 0.5µl anti-rabbit in 5ml 5% milk) in covered tray for 1 hour at RT – antibodies are light sensitive. So cover tube with foil paper and incubate for 1 Hour on roller at RT.

   (Use secondary antibody which is produced in different species as compared to primary antibody and anti **β actin** antibody. e.g if primary produced in rabbit and anti **β actin** in mouse then use Donkey anti rabbit and Donkey anti mouse Secondary antibody but with different wavelenths.)

   Wash x 2 in PBS-T (5 mins each)
   Wash x 1 in PBS (5 mins each)

9. Examine using Licor Odyssey machine (Lab 2)