Western blot protocol

Original by Syeda Tayyiba Rahat. Updates and edits by Henna Kultalahti 2025

Table of contents

Table of contents
- Solutions needed

1. SDS gel preparation
- Recipe for 10 % SDS
- Separatig gel percentage
- Stacking gel percentage

2. Sample preparation
- Calculating sample & water & loading buffer
- Loading buffer preparation

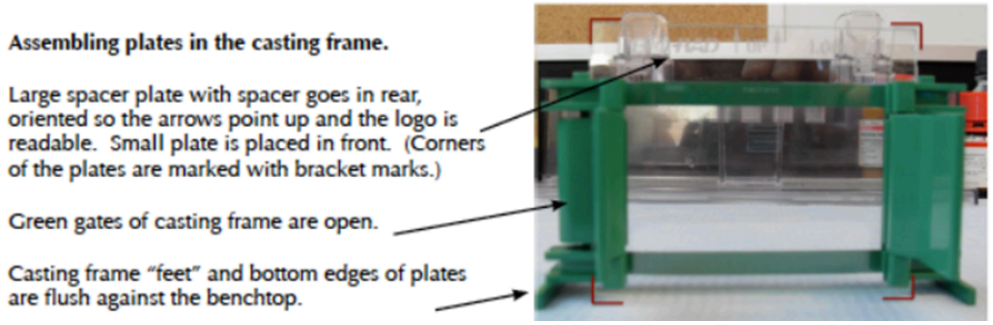
3. Running
4. Transfer
5. After transfer
6. Blocking
7. Primary antibody
8. Secondary antibody
9. Visualization
10. Stripping of membrane

Abbreviations=
(H) = HOOD, must be handled in a fume hood. Marked with red highlight also.
RT= room temperature
o/n = over night

Solutions needed

- **1xTBS:** 10X TBS diluted to 1x meaning: 25 ml 10x TBS diluted to 250 ml Milli-Q
- **1xTBSt:** 50 ml 10xTBS diluted to 500 ml Milli-Q + 50µl Tween-20 (thick, cut end of tip to obtain)
- **10% BSA**: 2.5g BSA powder dissolved in 1xTbst to make final volume of 25ml. +4 ºC
- **5% BSA:** 1:1 solution of 10% BSA and 1x Tbst. +4 ºC
- **Running buffer:** 100 ml of 10x Tris-Gly and 10ml of 10% SDS and water to make 1 L.
- **Transfer buffer:** 200ml of 100% ethanol, 100ml of 10x Tris-Gly and water to make 1 L. +4 ºC

1. SDS gel preparation



Prepare the stand, casting glass plates and assemble on top of a tray.

The casting glass plates have numbers (1 mm, 1.5 mm etc) according to width. Check that the same number is found in the comb.

Recipe for 10 % SDS

	Separating gel **10%**			Stacking gel **6%**		
No. of gels	1x	2x	4x	1x	2x	4x
ddH20	3.8 ml	7.6 ml	15.2 ml	2.9 ml	5.8 ml	11.6 ml
40% Acrylamide	2 ml	4 ml	8 ml	0.75 ml	1.5 ml	3 ml
1.5 M Tris pH 8.8	2 ml	4 ml	8 ml	-	-	-
0.5 M Tris 6.8	-	-	-	1.25 ml	2.5 ml	5 ml
10% SDS	80 µl	160 µl	320 µl	50 µl	100 µl	200 µl
10% APS	80 µl	160 µl	320 µl	50 µl	100 µl	200 µl
TEMED(H)	8 µl	16 µl	32 µl	5 µl	10 µl	20 µl
Final volume	8 ml	16 ml	32 ml	5 ml	10 ml	20 ml

APS and TEMED (H) start the solidifying process, that why they musts be added last, and casting done straight after that.

Separatig gel percentage

We used 10 % separating gel, since the molecular weight of our protein was 49 kDa.

PROTEIN SIZE	GEL PERCENTAGE
4-40 kDa	Up to 20%
12-45 kDa	15%
10-70 kDa	12.5%
15-100 kDa	10%
50-200 kDa	8%
>200 kDa	4-6%

💡 The lighter the molecular weight of your protein (kDa), the higher percentage the gel should have (especially separating gel). Do your own research.

Stacking gel percentage

We did 6%. Normally it's 4% or 6%. (8+ would be separating percent). 4% gel is thin, good for heavier proteins > 150-200 kDA

Prepare separating gel, mix quickly and properly, and load it immediately between glass plates.

- With 1mm glass plate load 4.6 ml
- With 1.5 mm glass plate, load 7.2 ml.

Add 0.25-0.75 ml isopropanol on top of gel to make it smooth.

Let solidify 10-15 min. Save the falcon where you made the gel, once that solidifies, you know your gel is solidified.

Remove isopropanol with blot paper.

Prepare stacking gel and layer it on top of separating gel.

Insert comb(s).

Let solidify. Glass plates can be stored in a wrap of wet paper towel and stored +4 ºC overnight.

2. Sample preparation

Proteins used are probably frozen. Let thaw and centrifuge in high speed for 5 min. (To eliminate degraded proteins).

Proteins must be kept on ice.

Concentration is measured by BCA assay, Victor (protein) or nanodrop (DNA, RNA).

Calculate what amount of sample, water and loading buffer you need (that already has BME)

Calculating sample & water & loading buffer

For example:

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
concentration of sample	c= unit µg/ml, convert to µg/µl				
volume of sample (has 20 µg protein)	calculate				
water (to make 12 µl)	=12µl-the amount of sample				
loading buffer	3 cause 1:4 and sample + water= 12	3	3	3	3

Protein expression determines the amount of protein we want in the sample that is going to be loaded. 5-60 µl depending on protein expression.

The higher the protein expression, the lower amount (in µg) is used in WB.

💡 The amount of protein wanted can be optimised. For example. If you want to load all together 15 µl,
c= concentration from assay
m= the mass of protein we want in our sample
V= amount of sample taken
After figuring out V you make it up to the amount you want to load, in this case 15 µl.

Loading buffer preparation

Loading buffer comes in a 4x stock. Add 4x loading buffer equivalent to 25% of the protein sample, meaning volume of protein/4= is the amount of buffer added.

Beta mercaptoethanol (H) 4% meaning 40: 1000 is added to loading buffer. Loading buffer+BME can be stored by freezing it.

The well size determines the max volume of sample + buffer(+BME).

- With a 1 mm glass the max well size is 30 µl.
- With a 1.5 mm glass the max well size is 40 µl.

Heat sample at 95ºC for 5 min. Put back on ice immediately after that back on ice. That prevents proteins from refolding.

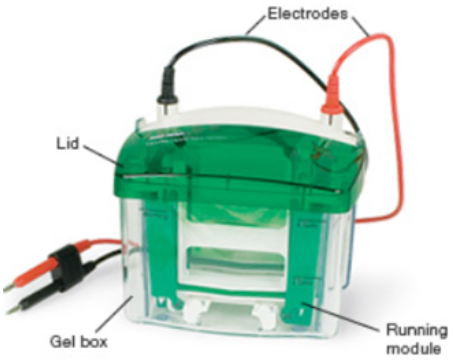
3. Running

Fix glass plates to green stand, the lower glass away from you. If running only one gel, fix plastic plate on the other side.

Place gasket inside running container and pour running buffer between plates to check for leakage. Remove comb carefully.

Load ladder/page ruler, then load samples to wells.

Pour running buffer carefully between plates up till “blotting” line.



Start running at ~50 V for 5 min. Then increase to ~100-120 V for 80-100 min until samples are 1mm away from the bottom of the glass.

Running buffer can be reused about 3-4 times.

4. Transfer

Cut nitrocellulose membrane 9 cm x 7.5 cm. Put in milli-Q 5 min and then in transfer buffer.

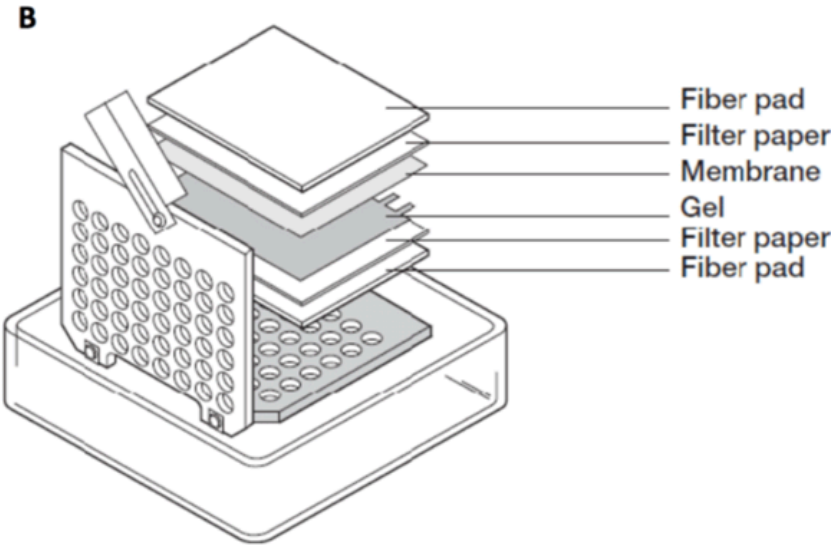
Cut filter paper and put filter paper (whatman's paper) and filter pads in transfer buffer.

Take gel out of casket and separate glass plates gently.

Preparing the sandwich:

Black side > Filter pad > Filter paper > Gel > Membrane > Filter paper > Fiber pad >White side

Ladder should be on the left side of the membrane, meaning mirror image and ladder on the right when putting gel and membrane on sandwich.



Add magnetic stirrer, ice box and sandwich to transfer tank. Black side of cassette towards black side.

Pour transfer buffer up till blotting.

Run transfer 20 V overnight in +4 ºC or 80-110 V for 150-90 min.

Transfer buffer can be reused 2-3 times.

5. After transfer

Mark membrane ladder corner with non-smudge pen. Put membrane in Tbst.

OPTIONAL: Staining of the gel

Move gel to water and microwave 30 secs, remove water and add commasie dye. Let incubate in room temperature for 1 h and was with water. Visualise.

6. Blocking

Prepare blocking solution: 5% or 10% BSA in 1xTbst in a falcon.

Shift rolled (proteins inside) membrane to blocking solution. Incubate 1-2 h or overnight at + 4 ºC.

7. Primary antibody

Prepare primary antibody solution: 5% BSA + antibody in a falcon. Dilution information can be found on the primary antibody product description.

For example: 3ml 5% BSA and 1:500 antibody meaning:

$$\frac{x}{3000\mu l} = \frac{1}{500} => x = \frac{3000\mu l}{500} = 6\mu l \text{ of antibody}$$

Incubate 1-1.5 h at RT or o/n at +4 ºC

Wash 3 times for 5-10min in Tbst in a shaker.

8. Secondary antibody

Check the host in which primary antibody is prepared then take the antibody against that hosts cells. For example if primary antibody host is mouse, secondary antibody should be anti-mouse.

Prepare secondary antibody in 5% BSA. Same calculations as with primary antibody. Check secondary antibody product information for dilution.

Incubate 0.5-1.5 h in RT.

Wash 3 times in Tbst for 5-10 min in a shaker.

Do last wash with Tbs for 5-10 min in a shaker.

9. Visualization

Move membrane to a folded biofilm.

Prepare chemiluminescent substrate kit (light sensitive, keep in foil). Kit has instructions for the preparation.

Add substrate to membrane that’s on film and let be for 5 min, keep covered.

Visualize using Image reader LAS 3000 with settings:

- Exposure time: Precision
- Exposure time: Auto
- Sensitivity: High
- Tray position: 2

Take a print and save on computer.

Membrane can be stored in +4 ºC in film for longer.

Membrane can be stored in Tbst in + 4ºC for some days.

10. Stripping of membrane

Wash membrane once with Tbst in a shaker for 5 min.

Always prepare fresh stripping buffer:

- 10 ml milli-Q
- 400 µl of 5M NaOH (corrosive!)

Incubate membrane in stripping buffer for 15 min in a shaker at RT.

Wash 3 times 10 min in Tbst in a shaker.

Membrane can be stored in Tbst (last wash) overnight + 4 ºC o/n.

Membrane can be stored in blocking solution in + 4 ºC o/n.

After blocking, primary, and secondary antibody steps can be repeated.