

PROTOCOL		
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Protocol #24 – Western Blotting

Aim

The aim of Western blotting is to enable the detection of protein via binding with an antibody against a recombinant tag or a natural epitope determinant on the surface of the protein. This protocol utilizes the wet transfer protocol.

Materials & Equipment

- Pre-casted SDS-PA gel
- TGS buffer (See appendix A)
- PAGE apparatus
- Wet transfer apparatus
- Sandwich clasps
- Pair of sponges
- Transfer buffer¹ (See appendix A)
- Skim milk powder
- PBS-T (See appendix A)
- Antibody of choice
- Flat tweezers
- EZ-ECL kit (Beit Haemek)
- Nitrocellulose membrane (10x10cm rectangle)
- 2xWhatman papers (10.5 cm X 7.5 cm)
- Reserved ECL imager (Prof. Michal Shapira's lab)

Experiment procedure

1. Mount SDS-PA gel and fill the back-chamber with TGSx1 buffer till the top of the chamber. Fill the lower bath with TGS such that the bottom part of gel is submerged ~0.5cm.

¹ Can be recycled till color changes to dark yellow

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2. Load sample buffer-treated samples and marker (5-10 μ l).
 - a. **Recommended**: load several microliters of known positive control for the antibody of choice!
3. Close the apparatus with the dedicated cover while keeping alignment of electrode's color (Red $\Rightarrow$ Red, Black $\Rightarrow$ Black). Verify the electrode is correctly connected into the power supply.
4. Switch on the power supply and set the power to fixed voltage at the range of 160-180V (lower voltage than 50V will risk diffusion of samples across the gel).
5. Stop the power supply when marker separation suits the resolution needs (first-time runs should be stopped just before the buffer front reaches the bottom of the gel's cast).
6. Cut whatman's membranes and Nitrocellulose and submerge them in transfer buffer solution (Nitrocellulose membrane should be handled with gloves and tweezers).
7. Chill transfer apparatus by placing it onto an ice bucket filled with ice.
8. With caution, dismantle the gel's cast and submerge gel into the transfer buffer.
9. Spread a sandwich clasp and place on the white side the following items (keeping this order): Sponge, Whatman, membrane (press!), Gel (press!), whatman (press!), Sponge (press!).
10. Close sandwich clasp and insert into the transfer apparatus.
11. Fill the apparatus with transfer buffer and close the apparatus with the top cover keeping the **RED** electrode facing the **white** side.
12. Switch on the power supply and set fixed current at 150-200mA for 1.5-3hr, depending on the protein's molecular weight (the larger the protein, the longer the transfer should be applied).
13. At the end of the run dismantle the apparatus and mark with a ball pen the position of at least one band for each of identification.

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- a. Optional: Stain blot with Ponceau-S (see appendix A) for 5' at RT and then drain the dye back into dye reservoir.
 - b. Wash with MQ rotating till bands are visible.
 - c. Continue to wash till all dye is removed from blot.
14. Block membrane with 5% skim milk solution (usually 25ml; see appendix A) for 1hr at RT rotating (Cover plastic box to eliminate dust and acidification by atmospheric carbon dioxide).
 - a. Optional: Protocol can be stopped here by placing membrane at 4°C, covered.
15. Drain skim milk into a falcon tube and add appropriate amounts of antibody (see appendix A).
16. Incubate membrane with antibody solution at RT for 30'-60'.
17. Drain antibody solution and wash x3 with PBS-T, 5' each wash.
18. Optional – secondary antibody:
 - a. Prepare secondary antibody solution as by diluting it with 5% skim milk solution.
 - b. Incubate secondary antibody for 30'-60' at RT.
 - c. Drain antibody solution and wash x3 with PBS-T, 5' each wash.
19. While performing the washing step, turn on the cooling on the ECL imager (cooling takes a minimum of 20').
20. Prepare EZ-ECL mix by mixing 1ml of solution A with 1ml of solution B² in an aluminum foil-covered tube and incubate 5' at RT.
21. While incubating go to the ECL imager with the following items:
 - a. Membrane in PBS-T
 - b. 1000µl pipettor
 - c. 1000µl Tips
 - d. EZ-ECL mix
 - e. Tweezers

² Sufficient for 2 membranes

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- f. A4 Nylon sleeve
22. At the end of the incubation, spill the ECL mix over membrane and using the 1000µl pipettor splash the mix over the whole area of the membrane, specifically over the expected position of the bands.
23. Open the ECL imager door and place the Nylon sleeve over the tray.
24. Using the tweezers place the membrane and orient it such that it is centered across the tray and underneath the camera's lens.
25. Close the door and login into your user.
26. Initiate the software by double clicking on the icon titled " Las3000IR"
27. Choose "Tray/Method" that fits the size of the membrane/number and press "OK".
28. Check focus and brightness via the "Brightness and Focus" function.
29. Press "Method" and Choose "Digitize" to capture the current position of the membrane.
30. Choose "precise" methodology and set the exposure time to 1/60sec.
31. Press "OK".
32. Press "Start".???
33. Press "Save" and save the image at your folder (browse to Raz's lab H: hard drive within the server). See that you save as "Tiff" extension (16Bit is higher bit depth thus is recommended).
34. Click on "Method" and choose "Chemiluminescence" and press "OK".
35. Choose "Increment" methodology, 10sec each, and choose a sensitivity of "high".
36. Start capturing the images.
 - a. Optional: It is possible to increase sensitivity and contrast by playing with the Histrogram on the bottom side of the screen while in the capturing stage
37. When satisfied with the images, choose the picture and save each one as "Tiff" in the lab's folder.
38. Remove the membrane.

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39. In case there is no one listed afterwards, shutdown the software by exiting and choosing "Stop cooling..."

Appendix A

Buffers

Ingredient	Mass
<i>TGSx10 (1L)</i>	
Glycine	144gr
Tris Base	30gr
SDS 20%	50ml
MQ/DDW	Fill to 1L
<i>Transfer bufferx1 (3L)</i>	
Glycine	87 g
Tris Base	17.4gr
Methanol	600ml
MQ/DDW	Fill to 3L
<i>PBSx10 (1L)</i>	
NaCl	80gr
KCl	20gr
KH ₂ PO ₄	2.4gr
Na ₂ HPO ₄	14.4gr
MQ/DDW	Fill to 1L
<i>PBS-T (1L)</i>	
PBSx10	100ml
Tween-20	2ml
MQ	Fill to 1L