

General protocol for Western Blotting (AP and HRP conjugates)

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BUFFERS and Solutions:

Transfer buffer (1.5L, always prepare fresh before transfer):

- 50 mM Tris, 39 mM Glycine, 20% methanol (v/v), pH 8.0
- Methanol slows down the transfer, add SDS to larger protein to speed up the transfer

20X TBS stock (no Tween 20) (200 ml)

- 200 mM Tris, 3M NaCl, pH 8.0

1X TBS^T (prepare right before the analysis) (1L)

- 10 mM Tris, 150 mM NaCl, pH 8.0, 0.1% Tween

Blocking buffer:

- 1X TBST with 5% non-fat milk

For AP conjugates:

TSM

- 100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH 9.5

For development:

- BciP, NTB, TSM

For HRP conjugate (colorimetric only):

10X Tris-saline:

- 9% (w/v) NaCl in 1 M Tris-HCl, pH 8.0

Staining solution for HRP:

- 18 mg 4-chloro-1-naphthol (sigma cat#: C8890) in 6 ml methanol. Add 24 ml 1X Tris-saline followed by 600 ul 3% hydrogen peroxide (final 0.2% H₂O₂)

Procedures:

1. SDS-page Gel:

- Run a protein gel to separate the proteins, runs it until the bromophenol blue front reaches the end of gel.
- also load a protein marker to use as a control for protein transfer.

2. During gel running, prepare:

- PVDF membrane:

Cut appropriate size membrane, and activate it by:

- Soak in 100% methanol for 10 sec. (PVDF is hydrophobic)
- Soak in ddH₂O for 30 sec

- Soak in transfer buffer for >10 minutes

Whatman paper filter & sponges

- Soak cut filters (whatman paper) and sponges in transfer buffer for 10 minutes (push bubbles out)
- (don't soak the gel in transfer buffer if you are transferring small proteins); but soaking in transfer buffer for short time (eg. 3 minutes) can wash salt off gel to reduce current during transfer.

3. Assemble the transfer apparatus in the order of
Black(negative) –Sponge---Filter---Gel---PVDF memberane—filter---sponge—**RED end**
4. Ensure the packing is tight and the chamber does not leak. (if you press the sponges to the inner side, you should see the solution levels)
5. Transfer at RT (ice in apparatus) for 120 minutes at constant current (350 mA)
 - This setting may need to be optimized for diff. size of proteins. Small proteins (<20 kD) tends to transfer faster, larger protein might need longer transfer time
 - The setting that has worked for me (working with proteins with diff. sizes from 37kD to 180kD) is: total work (W) = I*V*time ~ 15 . For example, if using a constant voltage of 40 volts, current is 0.2 A, then a time setting of 2 hrs (40*0.2*2=16) usually is enough for complete transfer as judged by the marker.
6. Disassemble the chamber. To ensure your protein is transferred before all the following steps, commassie blue stain the SDS-gel to estimate the transfer efficiency
7. Block the membrane for 1 hr in blocking buffer: 5% (w/v) non-fat milk in TBST.
8. Wash membrane for 3* 5 min w/ TBST;
9. Incubate membrane in Primary antibodies diluted in blocking buffer (TBST) at 4 deg overnight and 1 hr at room temperature.
10. Wash for 3*5 min w/ TBST;
11. Incubate with 2nd Ab for 1 hr at RT in TBST
12. wash for 2*5 min w/ TBST
13. wash 1*5 min w/ TBS
14. wash 2*10 min/TSM (if using AP conjugated 2nd Antibody)
15. Development:
 - a) Colorimetric: Stain with AP or HRP staining solution until the signal is clearly visible
 - For AP-conjugated 2nd Ab, add 33 ul BciP , 42 ul NBT in 10 ml TSM, pH 9.5 (5-15 minutes)
 - For HRP: Staining solution for HRP: 18 mg 4-chloro-1-naphthol (sigma cat#: C8890) in 6 ml methanol. Add 24 ml 1X Tris-saline followed by 600 ul 3% hydrogen peroxide (final 0.2% H2O2) (1-5 minutes)
 - at RT for 5~15 minutes until the band shows up; (Don't shake blots during color development)
 - b) ECL solution followed by Film Exposure:

1. Prepare ECL reagents: mix the two reagents 1:1 (~1ml per blot) and pipette enough vol on a piece of Saran wrap taped to the bench.
2. Drag the blot along the edge of the tray, drain excess TBST, then place the blot with protein side down on the ECL solution.
3. Incubate for 1 min then drain excess reagent and transfer the blot to a plastic report cover.
4. Expose immediately (few seconds — 1 hr).

16. Stop solution: 20 mM Tris, pH 7.4, 5 mM EDTA OR rinse twice in water.

17. Dry the membrane and expose ASAP.

TIPs from the Millipore Protein Blotting Handbook (5th version):

To reduce persistent background:

- Use High salt wash after the 2nd Ab incubation (PBS or TBS with 0.5 M NaCl and 0.2% SDS)
 - ◆ Incubate the membrane in high salt buffer for 30 minutes w/ gentle shaking
 - ◆ Rinse blot with Milli-Q water and proceed as usual

To reduce overall high background:

Use higher dilution of the 2nd Ab

To reduce high non-specific signal:

Use higher dilution of the 1st Ab and lower protein yield.

To view the protein on the membrane:

Soak in 20% methanol, watch on the light box

Role of Tween:

0.05% -0.1% Tween can help renature the antigen, thus increasing improved recognition of specific antibodies; but too high % of Tween can wash away blotted proteins