Western blotting

Risk assessment

- **Methanol damages the optic nerve and central nervous system**
- Methanol is HIGHLY FLAMMABLE
- Methanol is TOXIC if swallowed, inhaled, or in contact with skin
- Work with high voltage power sources

▷ Wear gloves, safety glasses, lab coat

□ Collect as HAZARDOUS WASTE
□ DO NOT wash into sewer



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This is a bench card. Full protocol available online.

›› *Choosing a Western transfer system*

(1.) Check the literature for transfer conditions of the protein of interest. Epitopes can be sensitive to the way proteins are transferred. Common starting points and optional adjustments:

Sample		Transfer		Transfer buffer			Transfer conditions		
Protein	State	System	Membrane	Recipe	MeOH	SDS	Voltage	Temp.	Time
General	Denat.	Wet	NC, 0.45 µm	Towbin	10%	Omit	100 V	4 °C	60 min
Histone	Denat.	Wet	NC, 0.20 µm	Towbin	20%	Omit	30 V	4 °C	70 min
Large or hydrophobic protein	Denat.	Wet	PVDF, 0.45 µm	Towbin	0–5%	0.10%	30 V	4 °C	16 h
Basic protein (pI > 9)	Denat.	Wet	PVDF, 0.20 µm	CAPS or Dunn	10%	0.01%	100 V	4 °C	60 min

(2.) Start by choosing the membrane material, considering the protein's abundance and hydrophobicity, the detection method, and whether the blot will be reprobed.

Membrane	Binding capacity	Hydrophobicity	Luminescence	Fluorescence	Stripping
PVDF	150–160 µg/cm^2	High		Limited	
NC, 0.45 µm	80–100 µg/cm^2	Low			Limited

(3.) Once the membrane and transfer system are chosen, fine-tune the methanol and SDS concentrations based on the protein's size and state.

Transfer			Denatured proteins		Native proteins	
Membrane	System	Protein size	MeOH	SDS	MeOH	SDS
NC	Semi-dry	< 20 kDa	20%	Omit	5–10%	Omit
		20–80 kDa	10%	0.01%	Discouraged	Discouraged
		> 80 kDa	10%	0.05%	Discouraged	Discouraged
	Wet	< 20 kDa	20%	Omit	5–10%	Omit
		20–80 kDa	10%	0.05%	Discouraged	Discouraged
		> 80 kDa	10%	0.10%	Discouraged	Discouraged
PVDF	Semi-dry	< 20 kDa	20%	0.01%	5–10%	0.01%
		20–80 kDa	10%	0.01%	5%	0.01%
		> 80 kDa	5%	0.05%	Omit	0.05%
	Wet	< 20 kDa	20%	0.01%	5–10%	0.01%
		20–80 kDa	10%	0.05%	5%	0.05%
		> 80 kDa	5%	0.10%	Omit	0.10%

(4.) Finally, choose a transfer buffer with a pH at least 2 units above the isoelectric point (pI) of the most basic protein of interest. Towbin buffer is often a good starting point.

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Transfer			System		Application
Continuity	Recipe	pH	Wet	Semi-dry	
Continuous	Towbin	8.3			Works for most proteins
	Bjerrum	9.2			Versatile; preferred for native proteins
	Dunn	9.9			Basic proteins
	CAPS	11.0			Basic proteins or prior to Edman sequencing
Discontinuous	Cathode buffer	9.4	Not applicable		
	Anode buffer	10.4	Not applicable		

›› *Preparing the transfer system*

(1.) Freshly prepare the 1 × transfer buffer (or cathode and anode buffer for discontinuous transfers), supplementing methanol or SDS as needed. Bring buffers down to 4 °C.

> ***Critical:*** Do not reuse transfer buffers! Gradient gels always require methanol in transfer buffers to prevent swelling into a trapezium shape during transfer.

(2.) Pre-equilibrate the gel in transfer buffer (or cathode buffer) for 10–20 min before assembly.

(3.) Cut a piece of membrane to the size of the gel. Avoid touching with bare hands.

(4.) *Critical:* Briefly activate PVDF membranes by soaking in methanol, even for methanol-free transfers.

(5.) Hydrate the nitrocellulose or PVDF membrane in transfer buffer (or anode buffer) for 5 min. The membrane will sink on its own once fully soaked.

A › *Wet transfer/Tank transfer*

(1.) Soak two sheets of blotting paper in transfer buffer until fully saturated.

(2.) Assemble the gel sandwich in the blotting cassette, submerged in transfer buffer:

Top
Foam pad
Blotting paper
Gel
Membrane
Blotting paper
Foam pad
Bottom

(3.) Use a roller to gently remove air bubbles trapped between the layers of the blot assembly.

(4.) Close the cassette and insert it into the transfer tank with the membrane facing the anode (often red).

(5.) Add a stir bar and ice pack. Fill the tank with transfer buffer, place on a stir plate, and start stirring to ensure even temperature and ion distribution during transfer.

(6.) Connect the power leads. Run the transfer at one of the following regimes:

- Run at constant voltage: 100 V for 60 min at 4 °C.
- Run at constant current: about 2.5 mA/cm^2 (Mini Gel, 150 mA; Midi Gel, 300 mA) for 180 min at 4 °C.

60–180 min

(7.) Turn off the power supply and disconnect the electrical leads.

(8.) *Optional:* Stain the gel with Coomassie blue SOP 0010 to verify transfer efficiency.

B › ***Semi-dry transfer***

(1.) If using a discontinuous buffer system, soak one sheet of blotting paper in anode buffer and one in cathode buffer. For continuous systems, soak both sheets in transfer buffer.

(2.) Assemble the transfer stack on the anode plate (typically the bottom electrode):

Top	
Blotting paper	Cathode buffer
Gel	Cathode buffer
Membrane	Anode buffer
Blotting paper	Anode buffer
Bottom	

(3.) Use a roller to gently remove any air bubbles trapped between the layers.

> ***Quality assurance:*** Bubbles are common between the gel and membrane—press gently but thoroughly. Trapped air can lead to white spots (no transfer) on the final blot.

(4.) Close the semi-dry blotter and connect the electrical leads. Start the transfer according to the manufacturer's instructions.

- For discontinuous anode and cathode buffers, run at constant current: about 0.8 mA/cm^2 for 60 min at room temperature. 60 min
- For continuous buffers (Bjerrum, Towbin, Dunn, or CAPS), run Mini Gels at 10–15 V for 15–30 min with a current limit of 5.5 mA/cm^2 (3 mA/cm^2 for large gels). 15–30 min

(5.) Turn off the power supply and disconnect the electrical leads.

Reversible staining with Ponceau S for total protein normalization

(1.) Carefully remove the membrane from the gel sandwich and place it into a clean incubation tray or box filled with deionized water.

> ***Critical:*** Only handle membranes by the edges with clean forceps. Be careful not to touch the membrane with hands or gloves.

(2.) Decant the water. Add Ponceau S solution until the blot is submerged. Shake for 2–10 min on an orbital shaker (100 rpm) at room temperature.

(3.) Rinse with deionized water to remove the background stain.

(4.) Image the membrane. Quantify total transferred protein for normalization if needed.

(5.) Destain in aqueous buffers or deionized water. Proceed with blocking or detection.

Storing and reusing membranes

(1.) For short-term storage between probing rounds, keep the membrane in TBST or PBS-T at 4 °C. Wrap the container in plastic film to prevent evaporation.

> ***Quality assurance:*** Store membranes wet for short periods only, protected from light.

(2.) Rinse the membrane with deionized water and place it protein side up on a clean sheet of blotting paper. Do not cover the membrane during drying.

> ***Quality assurance:*** For prolonged storage, keep membranes dry at room temperature, protected from light.

> ***Critical:*** Drying can severely decrease the immunoreactivity of blotted proteins; keep membranes that will be reprobed wet [YS09].

(3.) Once dry, place the membrane in a resealable plastic bag or a clean document protector. Label with date and experiment details.

(4.) To reuse, rehydrate the membrane in water for 2 min, then transfer to TBST or another buffer.

> ***Critical:*** For PVDF membranes, pre-wet in 100% methanol for 10–30 s before water to ensure proper rehydration.

List of references

Bio-Rad Laboratories, *Protein blotting guide: a guide to transfer and detection* (Bio-Rad Laboratories, 2016). (Bulletin 2895 Ver C)

J. Kyhse-Andersen, *J. Biochem. Bioph. Methods* **10**(3-4), 203-209 (1984).

Y.-G. Yeung and E.R. Stanley, *Anal. Biochem.* **389**(1), 89-91 (2009).

Recipe (available online) Troubleshooting (available online) Notes (available online)

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