Western blotting

Western blotting transfers proteins from a gel onto a solid support such as a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Once transferred, individual proteins, protein–protein or protein–ligand interactions can be detected by probing for specific immunological epitopes or biochemical activity. The choice of the membrane and transfer conditions is largely dependent on the nature of the protein of interest and intended downstream applications.

Wet transfers (*Alternative A*) provide numerous opportunities for optimizing transfer conditions, including time, temperature, voltage, and buffer composition. When carefully tuned, they can achieve almost quantitative transfer for most proteins. Semi-dry transfers (*Alternative B*) are faster and use less buffer. However, they offer fewer opportunities for optimization. If transfer is incomplete, the blotting process cannot simply be extended since by that point, the thin buffer layer is typically exhausted.

The term 'Western blot' was coined by W. Neal Burnette (Burnette, 1981) in playful homage to the earlier 'Southern' and 'Northern' blots for DNA and RNA.

Risk assessment

- **Methanol is a REPRODUCTIVE TOXIN!**
- **Work with high voltage power sources**

▷ Wear gloves, safety glasses, lab coat

☐ Collect methanol containing solutions as HAZARDOUS WASTE

☐ DO NOT wash into sewer

Reviewed: Jan 13, 2023

Procedures

›› ***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	30 min
Histone	Denat.	Wet	NC, 0.20 µm	Towbin	20%	Omit	30 V	4 °C	70 min
Histone	Denat.	Wet	PVDF, 0.20 µm	CAPS	Omit	0.01%	100 V	4 °C	60 min
Membrane proteins	Denat.	Wet	PVDF, 0.45 µm	Towbin	20%	0.10%	35 V	4 °C	16 h
Transcription factors	Denat.	Semi-dry	PVDF, 0.20 µm	Bjerrum	10%	0.01%	15 V	25 °C	45 min
Acidic protein	Native	Wet	PVDF, 0.20 µm	Dunn	5%	0.01%	40 V	4 °C	60 min
Basic protein	Native	Wet	PVDF, 0.20 µm	CAPS	5%	0.01%	20 V	4 °C	60 min

Hint: For most proteins, the defaults will work. For especially large, small, or hydrophobic proteins that transfer poorly under default conditions, adjust membrane pore size, SDS and methanol concentrations, or buffer pH as indicated in the table.

(2.) Start by choosing the membrane material, considering the protein's abundance and hydrophobicity, the detection method, and whether you plan to reprobe the blot.

Membrane	Cost	Binding capacity	Hydrophobicity	Luminescence	Fluorescence	Stripping
PVDF	50 ¢/cm^2	150–200 µg/cm^2	High	✓	Limited	✓
NC, 0.45 µm	35 ¢/cm^2	80–100 µg/cm^2	Low	✓	✓	Limited

Hint: PVDF binds more protein, making it useful for low-abundance targets. However, as proteins penetrate deeper into PVDF membranes, diffusion may lead to fuzzy bands, especially if the transfer isn't tightly controlled. Soaking the gel in 30% PEG1000 (or higher) for 2 h can increase sensitivity 10- to 100-fold (Zeng et al., 1990).

Hint: While nitrocellulose is too brittle for repeated probing after stripping off the antibodies, it gives lower background in fluorescence applications, and often sharper bands.

(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%	0.01%
		20–80 kDa	10%	0.01%	Discouraged	Discouraged
		>80 kDa	10%	0.05%	Discouraged	Discouraged
	Wet	<20 kDa	20%	Omit	5–10%	0.01%
		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%

Hint: Methanol is thought to improve protein adsorption to nitrocellulose membranes and reduces swelling of polyacrylamide gels in low-ionic strength buffers. Too much methanol, however, may cause protein precipitation and impede transfer. Where possible, omit methanol to reduce hazardous waste disposal.

Hint: SDS is optional, but improves the transfer efficiency of proteins larger than 100 kDa or which have a tendency to precipitate. Small proteins or peptides, on the other hand, may completely penetrate through nitrocellulose membranes ("blow-through"). SDS should be omitted in this case.

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

Transfer			System		Application
Continuity	**Recipe**	**pH**	**Wet**	**Semi-dry**	
Continuous	Towbin	8.3	✓	Not suitable	Works for most proteins (wet transfer only)
	Bjerrum	9.2	✓	✓	Versatile; preferred for native proteins
	Dunn	9.9	✓	Not suitable	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	✓	

This is why: Buffer pH determines protein mobility during transfer. Buffers like Towbin and Dunn are not recommended for semi-dry transfer due to their low conductivity and limited buffering capacity in thin stacks. Discontinuous transfer uses separate anode and cathode buffer to generate an ion gradient, but these are rarely customized by users.

[KS09]

›› *Preparing the transfer system*

- ☐ Membrane, PVDF *or* nitrocellulose (NC)
- ☐ 1 × Transfer buffer, 100 mL
- ☐ Methanol

(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.

This is why: Removing excess SDS and salts minimizes band distortion and blow-through. The gel will settle to its final size.

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

> ***Hint:*** Use a paper trimmer to pre-cut membrane sheets that fit Mini (7.2 cm × 8.6 cm) or Midi Gels (13.3 cm × 8.7 cm).

(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***

- ☐ Blotting cassette
- ☐ Blotting paper, 2 mm thick
- ☐ Foam pads
- ☐ Stir bar
- ☐ Transfer tank
- ☐ Ice pack
- ☐ Power supply
- ☐ 1 × Transfer buffer, 1 L ⟨*R*⟩

(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

> ***This is why:*** Placing the gel on top of the membrane allows you to visually check the alignment and remove air bubbles. The blotting paper protects the gel and membrane from concentrated ions near the electrodes. Too little paper results in lower transfer efficiency and leads to damaged electrode surfaces in the long run.

> ***Hint:*** When transferring multiple gels in one sandwich, interleave them with pieces of water-soaked dialysis membrane.

(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 and perform the transfer according to your preferred settings. 30–180 min

Transfer	**For 1 h**		**For 3 h**		**For 16 h**	
Recipe	**Voltage**	**Current**	**Voltage**	**Current**	**Voltage**	**Current**
Towbin	50–100 V	200–400 mA	25–50 V	100–200 mA	25–40 V	40–80 mA
Bjerrum	50–100 V	200–400 mA	25–50 V	100–200 mA	25–40 V	40–80 mA
Dunn	40–80 V	200–500 mA	20–40 V	100–250 mA	10 V	40–80 mA

- Run at constant voltage: 100 V for 30 min at 4 °C, *or*
- Run at constant current: 3 mA/cm^2 (Mini Gel, 150 mA; Midi Gel, 300 mA) for 180 min at 4 °C

> ***Hint:*** For proteins larger than 150 kDa, increase transfer time to 60 min; for proteins smaller than 30 kDa, decrease to 20 min. Transfer efficiency and quality generally increase with reduced power and increased transfer time.

> ***This is why:*** Constant voltage ensures that field strength remains constant, providing the most efficient transfer possible for tank blotting, but generates more heat. Use cooling systems or run at lower voltages to minimize band distortion.

> ***Quality assurance:*** Stain gel after transfer with Coomassie blue to verify transfer efficiency.

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

B › *Semi-dry transfer*

- ☐ Blotting paper, 2 mm thick
- ☐ Semi-dry blotter unit
- ☐ Power supply
- ☐ 5 × Anode buffer, 1 L ⟨*R*⟩
- ☐ 5 × Cathode buffer, 1 L ⟨*R*⟩
- ☐ 1 × Anode/Cathode buffer, 100 mL ⟨*R*⟩

(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.

> ***Hint:*** In most cases, a continuous buffer system like Bjerrum or CAPS is preferred. Discontinuous systems are used for specific commercial protocols and require exact matching of buffer formulations.

(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	

> ***Hint:*** Make sure the membrane is between the gel and the anode side so proteins migrate toward it. The positive electrode (anode) attracts the negatively charged proteins.

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

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

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

- Run at constant current: 1.5 mA/cm^2 (Mini Gel, 75 mA) for 15–60 min at room temperature. ⏲ 15–60 min

> ***This is why:*** Running at constant current minimizes heat generation. Field strength decreases over time, slowing the transfer.

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

Reversible staining with Ponceau S for total protein normalization

- ☐ Incubation tray *or* Incubation box
- ☐ 0.01% Ponceau S solution, 100 mL ⟨*R*⟩

- Ponceau S is the standard for reversible membrane staining
- Amido black is sometimes reversible under harsh destaining conditions

(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.

> ***This is why:*** Total protein staining with Ponceau S provides a complementary loading and transfer control compared to single housekeeping gene antibodies, which can vary with experimental conditions. Quantify lane intensities to normalize target protein signals across samples.

(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:*** Membranes stored wet should be used within one to two weeks. Extended wet storage increases the risk of microbial contamination and signal degradation.

(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:*** Drying preserves protein–membrane interactions and reduces risk of microbial growth. Membranes can be stored dry at 4 °C for months or even years.

(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.

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

Troubleshooting

Wet transfer/Tank transfer

In Step 2:

– Blurry or wavy bands or lateral smear
  - Ensure gel and membrane are evenly aligned and firmly pressed.
  - Use intact, uncompressed foam pads. Avoid reusing compressed blotting paper.

In Step 3:

– White spots or voids on Ponceau S stain
  - Remove trapped air bubbles by gently pressing the sandwich with a roller or conical tube.
  - Pre-wet blotting paper and pads thoroughly. Degas buffer before use if needed.

In Step 5:

– Uneven transfer efficiency across blot
  - Stir transfer buffer more vigorously during transfer.
  - Use fresh transfer buffer. Cool if appropriate.

In Step 6:

– Faint or absent bands
  - Check transfer time, voltage, and buffer composition. Verify methanol and SDS levels for the protein size.

– No visible transfer on membrane
  - Some membranes, especially PVDF, may have a designated active or coated side; ensure this side faces the gel during assembly.
  - Ensure membrane is between gel and anode.
  - Confirm black lead (cathode) is behind the gel and red lead (anode) is behind the membrane.
  - Verify electrode cables and apparatus function.

Semi-dry transfer

In Step 4:

– Arcing or excessive heat during transfer
  - Check that all components are properly wetted and assembled with no gaps.

Recipes

Tris-Gly transfer buffer (Towbin), pH 8.3, 10 ×

Amount	Ingredient		Stock	Final
30.3 g	Tris base	[77-86-1]	121.14 g/mol	0.25 M
144.1 g	Glycine	[56-40-6]	75.07 g/mol	1.92 M

Do not adjust pH.

10 × Tris-Gly transfer buffer (Towbin)

25 mM Tris base, 192 mM Glycine, pH 8.3

[At 1 × dilution]

Date: Sign: R0112

Tris-Gly transfer buffer (Bjerrum), pH 9.2, 10 ×

Amount	Ingredient		Stock	Final
58.2 g	Tris base	[77-86-1]	121.14 g/mol	0.48 M
29.3 g	Glycine	[56-40-6]	75.07 g/mol	0.39 M
To 1 L	Water, reagent-grade			

Do not adjust pH. ***Hint:*** Use for native proteins.

10 × Tris-Gly transfer buffer (Bjerrum)

48 mM Tris base, 39 mM Glycine, pH 9.2

[At 1 × dilution]

Date: Sign: R0113

CAPS buffer, pH 11.0, 100 ×

Amount	Ingredient		Stock	Final
22.1 g	3-Cyclohexylamino-1-propanesulfonic acid (CAPS)	[1135-40-6]	221.32 g/mol	1.0 M
To 1 L	Water, reagent-grade			

Adjust pH with sodium hydroxide. ***Hint:*** Use for basic proteins. ***This is why:*** Some protocols use 100 mM CAPS for protein crystallography or general biochemistry, but this is not typical for Westerns.

100 × CAPS buffer

10 mM CAPS, pH 11.0 *[At 1 × dilution]*

Date: Sign: R0114

Transfer buffer, 1 ×

Amount	Ingredient		Stock	Final
100 mL	Transfer buffer		10 ×	1 ×
100–200 mL	Methanol, analytical grade [67-56-1]	☐	32.04 g/mol	10–20%
2.5 mL	SDS	☐ R0047	20%	0.05%
To 1 L	Water, reagent-grade			

Prepare freshly. Methanol and SDS are optional.

1 × Transfer buffer

1 × Transfer buffer, ☐ 10–20% Methanol, ☐ 0.05% SDS

WARNING

Reproductive toxicology

Date: Sign:

Anode buffer, pH 10.4, 5 ×

Amount	Ingredient		Stock	Final
15.1 g	Tris base	[77-86-1]	121.14 g/mol	125 mM
To 1 L	Water, reagent-grade			

Adjust pH with sodium hydroxide.

5 × Anode buffer

25 mM Tris base, pH 10.4 *[At 1 × dilution]*

Date: Sign: R0115

Cathode buffer, pH 9.4, 5 ×

Amount	Ingredient		Stock	Final
15.1 g	Tris base	[77-86-1]	121.14 g/mol	125 mM
26.2 g	6-Amino-*N*-caproic acid	[60-32-2]	131.17 g/mol	
To 1 L	Water, reagent-grade			

Adjust pH with sodium hydroxide.

5 × Cathode buffer

25 mM Tris base, pH 9.4 *[At 1 × dilution]*

Date: Sign: R0116

Anode/Cathode buffer, 1 ×

Amount	Ingredient		Stock	Final
20 mL	Anode/Cathode buffer		5 ×	1 ×
10–20 mL	Methanol, analytical grade [67-56-1]	☐	32.04 g/mol	10–20%
0.25 mL	SDS	☐ ⟲ R0047	20%	0.01%
To 100 mL	Water, reagent-grade			

Prepare freshly. Methanol and SDS are optional.

1 × Anode/Cathode buffer

1 × Anode/Cathode buffer, ☐ 10–20% Methanol, ☐ 0.01% SDS

Date: Sign:

Ponceau S solution, 0.01%

Amount	Ingredient		Stock	Final
1 mg	Ponceau S	[6226-7-5]	760.6 g/mol	0.01%
1 mL	Acetic acid, glacial	[64-19-7]	1.74 M	1.0%
To 100 mL	Water, reagent-grade			

This is why: The 0.01% solution is as sensitive as a 0.1% solution.

0.01% Ponceau S solution

0.01% Ponceau S, 1.0% Acetic acid

Date: Sign: R0117

List of references

W. Burnette, *Anal. Biochem.* **112**(2), 195—203 (1981).

B.T. Kurien and R.H. Scofield, *Methods Mol. Biol.* **536** 9—22 (2009).

C. Zeng, Y. Suzuki, and E. Alpert, *Anal. Biochem.* **189**(2), 197—201 (1990).

Change log

2020-03-22	Michael Haugbro	Initial protocol.
2023-01-13	Benjamin C. Buchmuller	Adaptation as SOP. Added selection guide from Carl Roth GmbH + Co. KG.