Immunoblotting

Immunoblotting identifies proteins using antibodies that recognize unique epitopes in a linear amino acid sequence or folded structure. Typically, proteins are first separated by electrophoresis SOP 0007, then immobilized ("blotted") onto a membrane SOP 0008. This facilitates antibody recognition and allows for thorough washing to remove nonspecific interactions.

Detection is either direct or indirect as dye- or enzyme-conjugated secondary antibodies bind to the primary antibody and generate a signal proportional to the amount of epitope on the membrane. Antibodies from different host species (mouse, rabbit, goat, chicken) or subclasses (IgG1, IgG2a, etc.) can be combined for multiplexed probing. To detect multiple targets with same-species antibodies, membranes can be cut or stripped and reprobed several times. Fluorescent detection (*Alternative A*) is particularly suited for multiplexed quantitative imaging across a wide linear range. Chemiluminescent detection (*Alternative B*) offers higher sensitivity for low-abundance targets and can be performed with inexpensive homemade reagents, but the signal decays over minutes and is less suited to quantification.

Risk assessment

- ▷ Wear gloves, safety glasses, lab coat
- ☐ Dispose milk in accordance with local regulations
- ☐ Some jurisdictions prohibit draining dairy products due to their biological oxygen demand

Reviewed: Jan 15, 2023

Procedures

›› *Immunoprobing*

- ☐ Incubation tray *or* Resealable plastic bag
- ☐ TBS with Tween™ 20, 1 L ⟨*R*⟩
- ☐ Bovine serum albumin *or* Nonfat dry milk powder ("Blotto")
- ☐ Antibodies, primary and secondary

(1.) Prepare blocking buffer by diluting Blotto to 5% or BSA to 1–5% in 20 mL TBST.

> ***Hint:*** Use Blotto for abundant targets or with high-affinity antibodies, as it is inexpensive and effective under most conditions. Use BSA otherwise or when low background is essential.

> ***Critical:*** Avoid milk-based blocking buffers such as Blotto for phospho-proteins or streptavidin-based detection. Milk contains phosphatases and biotin, respectively. Phosphatase activity can be quenched with 50 mM sodium fluoride.

(2.) Block the membrane face-up in 5 mL blocking buffer for 30 min at room temperature with agitation. 30 min

(3.) Dilute the primary antibody in blocking buffer (typically 1:1 000 to 1:10 000).

> ***Hint:*** Dilutions vary by antibody type. 1:10 to 1:100 for hybridoma supernatant, 1:100–1:1 000 for polyclonal, and up to 1:10 000 or higher for monoclonal antibodies.

> ***Hint:*** Primary and secondary antibodies can often be reused once or twice within two days to a week.

> ***Critical:*** For multiplexed experiments, avoid combining mouse and rat primary antibodies as they are difficult to cross-adsorb. Chicken antibodies tend to non-specifically bind to PVDF and nylon-based membranes, leading to high background.

(4.) Discard blocking buffer. Incubate with diluted primary antibody for 30–60 min at room temperature or overnight at 4 °C with agitation. 30–60 min

(5.) Transfer membrane to a clean tray.

(6.) Wash four times with 10–20 mL TBST for 3 min each with gentle shaking. 15 min

(7.) Dilute secondary antibody in TBST (typically 1:2 000 to 1:10 000) according to vendor instructions.

> ***Critical:*** Beware, secondary anti-mouse antibodies may be subclass-specific for IgG1, IgG2a, or IgG2b. Use highly cross-adsorbed secondaries when multiplexing with antibodies from different species.

(8.) Incubate with secondary antibody at room temperature for 30–60 min with agitation. 30–60 min

(9.) Wash again as before.

A › *Fluorescent detection*

(1.) Specifications for infrared dyes used, for example, with LI-COR Odyssey® Imagers:

- Use IRDye® 800CW or IRDye® 680RD secondary antibodies, at 1:20 000 dilution.
- Use IRDye® 800CW for the lowest-abundance target; it gives the lowest background.
- Rinse the membrane with 1 × TBS to remove residual Tween™ 20.

(2.) Clean the imager surface with distilled water, then 70% ethanol. Dry with a lint-free wipe.

(3.) Scan the membrane using a suitable fluorescence imager.

B › *Luminescent detection*

- ☐ 1 M Tris hydrochloride, 100 mL ⟨*R*⟩
- ☐ 100 × Luminol, 10 mL ⟨*R*⟩
- ☐ 200 × *p*-Coumaric acid, 10 mL ⟨*R*⟩
- ☐ ECL reagent, 10 mL ⟨*R*⟩

(1.) Prepare 10 mL ECL reagent per membrane just before use.

(2.) Drip-dry the membrane and distribute ECL reagent dropwise to cover the surface evenly. Incubate for 1 min at room temperature. 1 min

(3.) Briefly slip the membrane through deionized water and gently drag over the edge of a container to remove excess liquid.

(4.) Expose to film or camera. The signal will last for at least 10 min.

Stripping and reusing membranes

- ☐ Stripping buffer, 15 mL ⟨*R*⟩

(1.) Wash membrane in distilled water for 5 min.

(2.) Strip antibodies using one of the following:

Stripping buffer	NC	PVDF
0.2 M NaOH	✓	✓
25 mM Glycine, pH 2.0	✓	✓
Stripping buffer, pH 6.8	Not recommended	✓

Critical: Do not use high concentration of SDS with nitrocellulose membranes as this can destroy membrane integrity.

(3.) Wash membrane twice in distilled water for 5 min.

(4.) Reblock and reprobe as needed.

Hint: Although it may not be strictly necessary to reblock the membrane after stripping with a mild stripping buffer, it is highly recommended to prevent high background noise. Use Blotto or casein to block when reprobing membranes. PVDF membranes can be reprobed up to five times with care.

Troubleshooting

Immunoprobing

In Step 1:

- No or little signal
  - Lower the percentages of Blotto or BSA as they might block antibody epitopes.

In Step 4:

- Weak or no signal
  - Ensure detergent concentrations are not too high; excess Tween or SDS may wash off loosely bound antibodies.

In Step 8:

- High background signal
  - Reduce antibody concentration.
  - Switch blocking buffer, or use more stringent washes.
  - Avoid milk-based blocking buffers such as Blotto for phospho-proteins or streptavidin-based detection.
- Non-specific bands appear in multiple lanes
  - Switch to highly cross-adsorbed secondary antibodies to eliminate cross-reactivity in multiplexed experiments.

Fluorescent detection

In Step 3:

- Fluorescent bleed-through between channels
  - Use spectrally distinct dyes and check for proper filter/channel selection.
  - IRDye® 800CW and 680RD are optimized for dual-channel detection with minimal overlap.
- Image noise or haze across the blot
  - Ensure platen glass is clean and dry before scanning. Dust can scatter excitation light, causing diffuse background.

Luminescent detection

In Step 4:

- Signal saturation or blown-out bands
  - Shorten exposure time or dilute antibody further.
  - Highly abundant proteins can overwhelm detection systems and obscure nearby bands.
- Blurry or smeared bands
  - Ensure even contact between blot and imaging surface.

Recipes

TBS with Tween™ 20 (TBST)

Amount	Ingredient	Stock	Final
100 mL	Tris-buffered saline (TBS), pH 7.4 R0053	10 ×	1 ×
5 mL	Tween™ R0058	20%	0.1%
To 1 L	Water, reagent-grade		

Add sodium azide to 0.05% to increase shelflife. Stable for 1 month at 4 °C. ***Hint:*** Compatible with PVDF or nitrocellulose (NC) membranes; do not use Tween™ 20 with nylon membranes.

TBS with Tween™ 20 (TBST)

1 × TBS, 0.1% Tween™

Expiry: Sign: R0169

Tris hydrochloride (Tris-Cl), pH 8.5, 1 M

Amount	Ingredient		Stock	Final
12.1 g	Tris base	[77-86-1]	121.14 g/mol	1 M
20 mL	HCl, 37%	[77-86-1]	11.65 M	
To 100 mL	Water, reagent-grade			

Adjust pH to 8.5–8.8 . Stable for 2 years at room temperature.

1 M Tris hydrochloride (Tris-Cl)

pH 8.5

Expiry: Sign: R0118

Luminol, 100 ×

Amount	Ingredient		Stock	Final
443 mg	Luminol	[521-31-3]	177.16 g/mol	250 mM
To 10 mL	Dimethyl sulfoxide (DMSO), reagent-grade			

Dispense into 50 µL aliquots. Store at −20 °C.

100 × Luminol

Date: Sign: R0119

p-*Coumaric acid, 200 ×*

Amount	Ingredient		Stock	Final
148 mg	*p*-Coumaric acid	[501-98-4]	164.16 g/mol	90 mM
To 10 mL	Dimethyl sulfoxide (DMSO), reagent-grade			

Dispense into 25 µL aliquots. Store at −20 °C.

200 × *p*-Coumaric acid

Date: Sign: R0120

ECL reagent

Amount	Ingredient		Stock	Final
1 mL	Tris-Cl, pH 8.5	R0118	1 M	100 mM
50 µL	Luminol	R0119	100 ×	1 ×
25 µL	*p*-Coumaric acid	R0120	200 ×	1 ×
3 µL	Hydrogen peroxide	[7722-84-1]	30%	5 mM
To 10 mL	Water, reagent-grade			

Prepare freshly; use immediately.

ECL reagent

100 mM Tris-Cl, 1 × Luminol, 1 × *p*-Coumaric acid, 5 mM Hydrogen peroxide

Date: Sign: R0121

Stripping buffer

Amount	Ingredient		Stock	Final
2 mL	Tris-Cl, pH 6.8	R0102	0.5 M	100 mM
98 µL	2-Mercaptoethanol	□ [60-24-2]	78.14 g/mol	140 mM
500 µL	SDS	R0047	20%	1%
To 10 mL	Water, reagent-grade			

Stripping buffer

100 mM Tris-Cl, □ 140 mM 2-Mercaptoethanol, 1% SDS

Date: Sign: R0122

Change log

2020-03-22	Michael Haugbro	Initial protocol.
2023-01-15	Benjamin C. Buchmuller	Adaptation as SOP. Added section on chemiluminescent detection and membrane stripping.

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