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

– Sodium azide is FATAL if swallowed or in contact with skin
– Concentrated hydrogen peroxide causes serious eye damage
– 2-Mercaptoethanol is TOXIC if swallowed or inhaled

▷ Strip with warm 2-mercaptoethanol buffer in a certified chemical fume hood
▷ 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
☐ Collect azide-containing solutions as HAZARDOUS WASTE; azide forms explosive metal azides in drain pipes



Reviewed: Oct 26, 2026

Procedures

›› *Immunoprobing*

☐ Incubation tray
  • *or*: Resealable plastic bag
☐ 0.1% TBST R0169
☐ BSA
  • *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 phospho-epitopes and biotin, respectively.

(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:*** Without a vendor recommendation, start at 1:100 to 1:1 000 for serum or culture supernatant, 1:500 to 1:10 000 for purified antibodies, and 1:1 000 to 1:100 000 for ascites fluid.

> ***Hint:*** Primary antibodies diluted in TBST with 0.02% sodium azide keep their activity for months at 4 °C and can be reused; diluted in milk, they precipitate within weeks.

> ***Critical:*** 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.

> ***Hint:*** Mouse and rat immunoglobulins cross-react. Combining them in one multiplex needs secondaries cross-adsorbed against the other species, which costs signal; where the two primaries differ in subclass, subclass-specific secondaries help.

(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.

(2.) Rinse the membrane with 1 × TBS to remove residual Tween™ 20.

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

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

B › ***Luminescent detection***

- ☐ ECL reagent ◇ R0121

(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 lasts longer than 30 min.

Stripping and reusing membranes

- ☐ SDS stripping buffer, pH 6.8 ◇ R0122
  - *or*: Glycine stripping buffer, pH 2.2 ◇ R0211

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

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

Stripping buffer	NC	PVDF	Temp.	Time
0.2 M NaOH	✓	✓	25–37 °C	5–10 min
SDS stripping buffer, pH 6.8 ◇ R0122	✓	✓	37 °C	30–45 min
Glycine stripping buffer, pH 2.2 ◇ R0211	✓	✓	25 °C	2 × 10 min

Pre-warm the stripping buffer to the desired temperature in a water bath. Incubate the blot with stripping buffer with the protein-side up.

> ***Hint:*** If antibodies persist after SDS stripping, strip at up to 70 °C for 30 min.

> ***Critical:*** SDS stripping removes part of the transferred protein, and glycine at low pH may not remove all antibody [YS09].

> ***Hint:*** When probing for several targets in turn, start with the least abundant and work toward the most abundant, since each strip takes some of the transferred protein with it.

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

(4.) *Optional:* Test for complete removal of the primary antibody by probing with the secondary antibody alone; no signal should remain. ⊞

(5.) 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. Each round of stripping removes some protein from the blot, so detect the weakest signal first.

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 4:

- 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.

Materials

Immunoprobing

- ☐ **Incubation vessel**
  - **Incubation tray**
  - *or* **Resealable plastic bag**
- ☐ **TBS with Tween™ 20 (TBST)**, 0.1% ⌰ R0169
  Stable for 1 month at 4 °C.
- ☐ **Blocking agent**
  - **Bovine serum albumin (BSA)**
  - *or* **Nonfat dry milk powder ("Blotto")**
- ☐ **Antibodies**, primary and secondary

Luminescent detection

- ☐ **ECL reagent** ⌰ R0121

Stripping and reusing membranes

- ☐ **Stripping buffer**
  - **SDS stripping buffer**, pH 6.8 ⌰ R0122
  - *or* **Glycine stripping buffer**, pH 2.2 ⌰ R0211

Recipes

TBS with Tween™ 20 (TBST), 0.1%

Amount	Ingredient		Stock	Final
100 mL	Tris-buffered saline (TBS), pH 7.5	⌰ 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. ***Critical:*** Omit sodium azide for chemiluminescent detection, since azide inhibits horseradish peroxidase [Bio16]. Where a preservative is still wanted, use thimerosal at 0.01% instead.

0.1% TBS with Tween™ 20 (TBST)

1 × TBS, 0.1% Tween™



No GHS hazards at the indicated concentrations

☐ Hold for EHS review before disposal

Date: 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
2 mL	HCl, 37%	[7647-01-0]	12.0 M	
	Skin corrosion (H314)			
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

No GHS hazards at the indicated concentrations

☐ Dilute below the discharge limit
☐ Flush to drain with excess water

Date: Sign: R0118

Luminol, 200 ×

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.

200 × Luminol

No GHS hazards at the indicated concentrations

- ☐ Collect as HAZARDOUS WASTE
- ☐ DO NOT wash into sewer

Date: Sign: R0119

p-Coumaric acid, 400 ×

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.

400 × *p*-Coumaric acid

No GHS hazards at the indicated concentrations

- ☐ Collect as HAZARDOUS WASTE
- ☐ DO NOT wash into sewer

Date: Sign: R0120

ECL reagent

Amount	Ingredient		Stock	Final
1 mL	Tris hydrochloride (Tris-Cl), pH 8.5 R0118		1 M	100 mM
50 µL	Luminol, in DMSO	R0119	200 ×	1 ×
25 µL	*p*-Coumaric acid, in DMSO	R0120	400 ×	1 ×
3 µL	Hydrogen peroxide Serious eye damage (H318)	[7722-84-1]	30%	2.9 mM
To 10 mL	Water, reagent-grade			

Prepare freshly; use immediately.

ECL reagent

100 mM Tris-Cl, 1 × Luminol, 1 × *p*-Coumaric acid, 2.9 mM Hydrogen peroxide

No GHS hazards at the indicated concentrations

- ☐ Collect as HAZARDOUS WASTE
- ☐ DO NOT wash into sewer

Date: Sign: R0121

SDS stripping buffer, pH 6.8

Amount	Ingredient		Stock	Final
1.25 mL	Tris hydrochloride (Tris-Cl), pH 6.8 R0102		0.5 M	62.5 mM
1.0 mL	SDS Skin irritation (H315); Serious eye damage (H318)	R0047	20%	2%
70 µL	2-Mercaptoethanol	☐ [60-24-2]	78.14 g/mol	100 mM
	Acute dermal toxicity (H310); Serious eye damage (H318); Reproductive toxicity (H361); Organ toxicity, repeated exposure (H373)			
To 10 mL	Water, reagent-grade			

Do not adjust the pH. Add 2-mercaptoethanol immediately before use. [KES87]

SDS stripping buffer

62.5 mM Tris-Cl, 2% SDS, ☐ 100 mM 2-Mercaptoethanol, pH 6.8



DANGER

Skin sensitization; Acute dermal toxicity; Organ damage through prolonged or repeated exposure; Reproductive toxicity

- ☐ Collect as HAZARDOUS WASTE
- ☐ DO NOT wash into sewer

Date: Sign: R0122

Glycine stripping buffer, pH 2.2

Amount	Ingredient		Stock	Final
750 mg	Glycine	[56-40-6]	75.07 g/mol	100 mM
430 mg	Magnesium acetate · 4 H_2O [16674-78-5]		214.45 g/mol	20 mM
370 mg	KCl	[7447-40-7]	74.55 g/mol	50 mM
To 100 mL	Water, reagent-grade			

Adjust pH to 2.2 with hydrochloric acid. [Bio16; LV81]

Glycine stripping buffer

100 mM Glycine, 20 mM Magnesium acetate, 50 mM KCl, pH 2.2



WARNING

Skin and serious eye irritation

- ☐ Neutralise to pH 6–9
- ☐ Flush to drain with excess water

Date: Sign: R0211

List of references

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Change log

2026-05-22 **Michael Haugbro, Benjamin C. Buchmuller** Version 1, 2020 to 2026. Initial protocol from the Tom Muir lab, adapted as an SOP in 2023 with fluorescent and chemiluminescent detection and membrane stripping; fluorescent detection after LI-COR Biosciences, "Good Westerns Gone Bad" (2008, rev. 2022), and the self-made ECL reagent from the Michael Knop lab.

2026-10-26 **Benjamin C. Buchmuller** Version 2. Credited the C. R. Hauck lab "Acrylamide gel reagents"; SDS stripping after Kaufmann et al. (1987), glycine stripping after Legocki and Verma (1981); corrected the Tris-Cl titrant, ECL and stripping-buffer concentrations, and antibody dilutions; added the azide and 2-mercaptoethanol hazards; dropped unsourced claims. Credited Abcam for the chicken-antibody note and Jackson ImmunoResearch for the mouse-and-rat multiplexing note, which is restored with the reason it matters.

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