Protein analysis

Fluorescent western blotting—an introduction for new users

Western blotting is a fundamental technique in life science research. While many use chemiluminescent substrates for detection, more scientists are now taking advantage of fluorescence-based detection because of increased signal stability, reproducibility, and the potential to measure signals from multiple proteins of interest on a blot (multiplexing) without the need to strip and reprobe the blot. With advanced digital imaging instruments like the Invitrogen™ iBright™ FL1500 Imaging System, and improvements in the breadth of fluorescent dye–conjugated secondary antibodies available, scientists have the necessary tools to take advantage of the benefits fluorescence-based detection offers. Overall, the western blotting procedure is similar between chemiluminescent and fluorescent detection methods, with each method offering specific benefits (Table 1). Here we share best practices to enable your success when starting off with fluorescent western blotting.

Tips before getting started

Some reagents and steps will need to be optimized to help ensure background fluorescence does not interfere with detection of the protein of interest:

- Sample buffers containing bromophenol blue will fluoresce and can contribute to increased background. Consider using fluorescence-compatible sample buffers without bromophenol blue, such as Invitrogen™ Fluorescent Compatible Sample Buffer (Cat. No. LC2570).
- To eliminate a major source of background fluorescence, use transfer membranes with low autofluorescence, such as Thermo Scientific™ nitrocellulose membranes (Cat. No. 88024 or 88025) or low-fluorescence polyvinylidene difluoride (PVDF) membranes (Cat. No. 22860).

Tip: If using sample buffers with bromophenol blue, the dye front may be run off the gel prior to transfer, or cut from the membrane after transfer, to avoid background fluorescence.

Table 1. Comparison of detection techniques for western blotting.

	Chemiluminescent	Fluorescent
Signal source	Indirect signal from enzymatic reaction	Direct signal from fluorophore
Signal duration	Limited (minutes to hours)	Extended (days to weeks)
Sensitivity	Excellent, with a wide variety of substrates available	Good, but may require a higher concentration of secondary antibody
Consistency	Possible variation between blots	High reproducibility between blots
Detection	Film and imaging instrument	Requires imaging instrument with suitable light sources and filters
Quantitation	Single-channel detection makes normalization challenging	Multiplexing with an internal control makes normalization simpler
Other considerations	• Stripping and reprobing of blot is required for targets of similar molecular weight • Long exposure times possible, as no excitation light source required to capture signal	• Care is needed to avoid background fluorescence • Longer exposure times can produce high background because of the small amount of excitation light passing through the emission filters

- Decrease the amount of molecular weight markers loaded onto the gel. Standard prestained molecular weight markers can be used, but the loading amount will need to be optimized if the marker contains fluorescent bands, since overloading can increase background fluorescence and signal bleed-through to adjacent lanes. The Invitrogen™ iBright™ Prestained Protein Ladder (Cat. No. LC5615) contains twelve recombinant proteins, ten (11 to 250 kDa) that are stained blue for direct and near-IR fluorescent visualization and protein sizing, and two (30 kDa and 80 kDa) that are unstained and contain IgG binding sites to bind the primary and secondary antibodies to be used as a control for chemiluminescent and fluorescent detection of the target protein. Recommended loading volumes are provided in Table 2.

Table 2. Volumes of iBright Prestained Protein Ladder to load for different applications.

Gel type	Visual detection (1.0 mm gel thickness)	Fluorescent detection
Mini gel	1–3 µL (12-well and 20-well) 2 µL (26-well)	1–3 µL (12-well and 20-well) 2 µL (26-well)
Midi gel	2–4 µL (12-well and 20-well) 2 µL (26-well)	2–3 µL (12-well and 20-well) 2 µL (26-well)

Tip: Avoid using pens on membranes, as many inks fluoresce. Use a pencil instead.

- Use only high-quality filtered buffers, such as Thermo Scientific™ Blocker™ FL Fluorescent Blocking Buffer (Cat. No. 37565). Particles and contaminants in wash and blocking buffers can settle on membranes and create fluorescent artifacts.
- Only handle membranes with clean blunt forceps to limit contamination and scratches on the membranes, which can contribute to background fluorescence and artifacts.
- Leverage primary antibodies that are verified for western blotting. Our antibodies undergo advanced verification testing to help ensure that the antibody binds to the target of interest and with specificity. Specificity testing is combined with application verification to demonstrate that the antibody works in the intended application.

Learn more about the Invitrogen™ antibody verification process for western blotting

- Secondary antibody concentrations are typically higher in fluorescence applications. Optimization is required to achieve the best signal-to-noise ratio, but the recommended concentration range, regardless of fluorescent conjugate, is typically between 0.4 and 0.1 µg/mL (1:5,000–1:20,000) for imaging on the iBright FL1500 Imaging System (Figure 1). Invitrogen™ Alexa Fluor™ Plus secondary antibodies were designed to provide high signal-to-noise ratios and lower cross-reactivity, reducing the time needed for optimization.

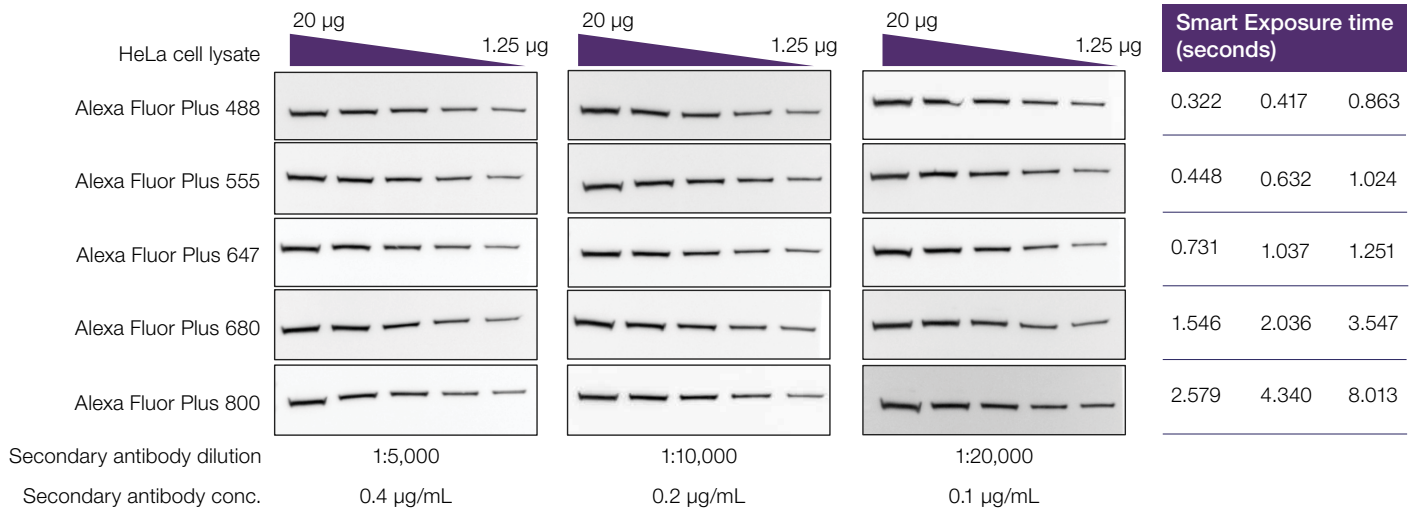


Figure 1. Excellent detection and sensitivity can be achieved with Alexa Fluor Plus fluorescent conjugates. The images are shown in grayscale to facilitate comparison. Using secondary antibody dilutions between 1:5,000 and 1:20,000 (0.4–0.1 µg/mL), exposure times are less than 10 seconds. HeLa whole cell lysates were serially diluted (to load 20 to 1.25 µg per lane) and electrophoresed on 4–12% Invitrogen™ Bolt™ Bis-Tris Plus Mini WedgeWell™ gels (Cat. No. NW04120BOX). Proteins were transferred to PVDF membranes using Invitrogen™ iBlot™ 3 Transfer Stacks (Cat. No. IB34002X3) on the iBlot™ 3 Western Blot Transfer Device (Cat. No. IB31001). The blots were processed overnight using the Invitrogen™ Bandmate™ Automated Western Blot Processor (Cat. No. BW1000). Membranes were blocked in 1X Blocker FL Fluorescent Blocking Buffer (Cat. No. 37565), then probed with a 1:20,000 dilution of Invitrogen™ HSP90 alpha Polyclonal Antibody (Cat. No. PA3-013) followed by either a 1:5,000, 1:10,000, or 1:20,000 dilution of Invitrogen™ Goat Anti–Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, conjugated to Alexa Fluor™ Plus 488 (Cat. No. A32731), Alexa Fluor™ Plus 555 (Cat. No. A32732), Alexa Fluor™ Plus 647 (Cat. No. A32733), Alexa Fluor™ Plus 680 (Cat. No. A32734), and Alexa Fluor™ Plus 800 (Cat. No. A32735). Images were captured on the iBright FL1500 Imaging System using the Smart Exposure tool; exposure times for each membrane are shown on the right. All the images were adjusted separately to the same black, white, and gamma levels.

General fluorescent western blotting procedure

Materials

- Nitrocellulose or low-fluorescence PVDF membrane (Cat. No. 88024, 88025, 22860, or equivalent)
- Filtered blocking buffer (e.g., Blocker FL Fluorescent Blocking Buffer, Cat. No. 37565)
- Wash buffer (e.g., Tris-buffered or phosphate-buffered saline with 0.05% Tween™-20 detergent, Cat. No. 28360 or 28352)
- Primary antibody and fluorescently labeled secondary antibody

Explore primary antibodies for western blotting

Explore fluorescent secondary antibodies for western blotting

- Incubation trays or containers (e.g., Invitrogen™ Incubation Trays, Cat. No. LC2102)
- iBright FL1500 Imaging System or equivalent

Tip: The same quantity of protein sample can be used for fluorescence and chemiluminescence. Typically, 10–50 µg of lysate protein is loaded, depending on the abundance of the target and concentration of the sample.

Protocol

1. After protein transfer, wash the membrane in deionized water 4 times for 5 minutes each with agitation.
2. Dilute Blocker FL Fluorescent Blocking Buffer (10X) to 1X with deionized water.
3. Incubate the membrane with a sufficient volume of blocking buffer for 15–30 minutes at room temperature with agitation.

Tip: For typical incubation trays, use at least 15 mL for mini blots and 30 mL for midi blots to fully cover the membrane. Avoid low volumes, as differences in agitation and coverage can produce high or uneven background.

4. Dilute the primary antibody per supplier recommendations in the blocking buffer (typically 1:1,000, assuming a 1 mg/mL stock).
5. Incubate the membrane in the primary antibody with gentle agitation for 1 hour to overnight. When incubating overnight, place at 4°C.
6. Wash the membrane 6 times for 5 minutes each in wash buffer with agitation.

Tip: The wash time may be reduced by filling and decanting the tray with distilled water 4 times, then moving forward with three 5-minute washes in wash buffer.

7. Prepare dilutions of the conjugated secondary antibody, at 0.4 to 0.1 µg/mL, in appropriate volumes of wash buffer. Alternatively, the secondary antibody can be diluted in blocking buffer. From a 2 mg/mL antibody stock, dilute 1:5,000 to 1:20,000:
   - **1:5,000:** 3 µL of secondary antibody in 15 mL wash buffer
   - **1:10,000:** 1.5 µL of secondary antibody in 15 mL wash buffer
   - **1:20,000:** 0.75 µL of secondary antibody in 15 mL wash buffer
8. Incubate the membrane in diluted secondary antibody for 1 hour at room temperature with agitation. Protect from light.
9. Wash 6 times for 5 minutes each in wash buffer with agitation. Protect from light.
10. Blots can be imaged immediately while still wet, or alternatively may be dried prior to imaging (Figure 2). The signal from some fluorescent dyes can decrease with drying, while others may be improved. Place each blot in a sheet protector or on a clean surface prior to imaging, to prevent contamination. Image on the iBright FL1500 system using fluorescence detection for the appropriate conjugate and selecting the Smart Exposure tool.
11. To dry the membrane, place it between two sheets of western blot filter paper to protect it from light exposure while drying. Drying the membrane allows for extended storage of the blot and can reduce exposure times. Store blots in the dark to prevent photobleaching.

Tip: When using Alexa Fluor Plus secondary antibodies, the membrane may be dried to improve the signal-to-noise ratio; signals will remain stable for days to weeks with proper storage of the membrane.

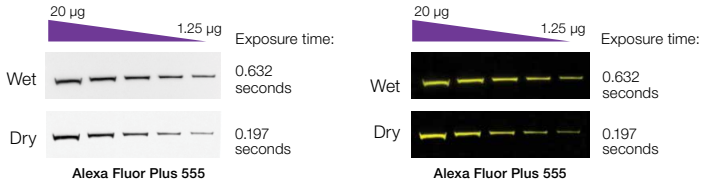
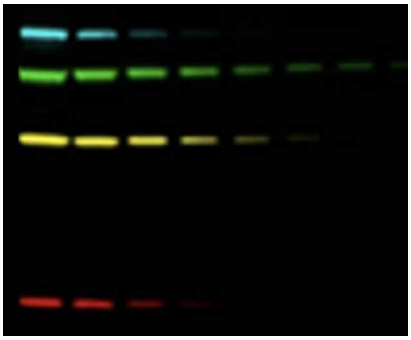


Figure 2. Dry or wet membranes provide comparable fluorescence imaging results. HeLa cell lysates were serially diluted (to load 20 to 1.25 µg per lane), run on SDS-polyacrylamide gels, and transferred to PVDF membranes using iBlot 3 Transfer Stacks (Cat. No. IB34002X3) on the iBlot 3 Western Blot Transfer Device (Cat. No. IB31001). The blot was processed overnight using the Bandmate Automated Western Blot Processor (Cat. No. BW1000). Membranes were blocked in 1X Blocker FL Fluorescent Blocking Buffer (Cat. No. 37565), then probed with a 1:20,000 dilution of HSP90 alpha Polyclonal Antibody (Cat. No. PA3-013) followed by a 1:10,000 dilution (0.2 µg/mL) of Goat Anti–Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555 conjugate (Cat. No. A32732). Images were captured on the iBright FL1500 Imaging System using the Smart Exposure tool; exposure times for each membrane are shown. All the images were adjusted separately to the same black, white, and gamma levels. The Alexa Fluor Plus 555 signal was pseudocolored yellow (right panels) and in grayscale (left panels) to facilitate comparison.

Recommended secondary antibodies

The wide range of Invitrogen Alexa Fluor and Alexa Fluor Plus conjugated secondary antibodies for western blotting provide nonoverlapping spectra to enable multiplex analysis. Multiple targets can be detected independently in the same lane and blot with clearly distinguishable colors. Our Alexa Fluor Plus secondary antibodies combine enhanced sensitivity and low background for better multiplexing results. Below are examples of secondary antibodies for western blotting.



Ordering information

Antibody	Conjugate	Quantity	Cat. No.
Goat Anti–Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody	Alexa Fluor Plus 488	1 mg	A32723
	Alexa Fluor Plus 555	1 mg	A32727
	Alexa Fluor Plus 647	1 mg	A32728
	Alexa Fluor Plus 680	1 mg	A32729
	Alexa Fluor Plus 800	1 mg	A32730
Goat Anti–Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody	Alexa Fluor Plus 488	1 mg	A32731
	Alexa Fluor Plus 555	1 mg	A32732
	Alexa Fluor Plus 647	1 mg	A32733
	Alexa Fluor Plus 680	1 mg	A32734
	Alexa Fluor Plus 800	1 mg	A32735

Discover the complete line of Alexa Fluor Plus secondary antibodies for western blotting

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Troubleshooting

Problem	Possible cause	Solution
High overall background levels	High concentration of secondary antibody resulting in increased background	• Decrease antibody concentrations
	High concentration of primary antibody causing nonspecific bands to be detected	
	Insufficient washing can result in high background and low signal-to-noise ratio	• Increase the number of washes and/or the volume of buffer used per wash • Add Tween-20 detergent to the wash buffer, to a final concentration of 0.05%
	Membrane background fluorescence contamination	• Use a low-fluorescence PVDF membrane
	Exposure time is too long	• Reduce exposure time • On the iBright FL1500 system, use the Smart Exposure tool to obtain an optimal image
	Low signal may be present, limited detection above background noise	• Increase primary antibody concentration and incubation time • Use a higher secondary antibody concentration • For targets with very low abundance, increase sample load on the gel to 10–20 µg
Uneven background levels	Low wash and incubation volumes	• For typical incubation trays, volumes should be at least 15 mL for mini blots and 30 mL for midi blots to fully cover the membrane
	PVDF membrane not properly pre-wetted with methanol or ethanol, or not kept fully wet during blotting	• Ensure membrane is fully wetted in methanol or ethanol before protein transfer and equilibration in transfer buffer • If the membrane gets dry during blotting, re-wet the membrane in 100% methanol or ethanol for a few seconds, and rinse with deionized water, before proceeding to the blocking step
Weak or no signal	Insufficient amount of primary antibody	• Increase primary antibody concentration
	Antibody may have lost activity	• Perform a dot blot to determine activity
	Exposure time is too short	• Increase exposure time • On the iBright FL1500 system, use the Smart Exposure tool to obtain an optimal image
	Incorrect instrument settings	• Ensure the correct excitation and emission wavelengths have been selected for the fluorophore

Learn more about how an iBright Imaging System can empower your success in fluorescent western blotting at **thermofisher.com/ibright**

To explore all of our western blotting products, visit **thermofisher.com/western**

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