APPLICATION NOTE

iBright FL1000 Imaging System

Fluorescent western blotting—an introduction for new users

Western blotting is an important technique in life science research. While most life science researchers use chemiluminescent substrates for detection, more scientists are now taking advantage of fluorescence detection because of an increased need for multiplexing. With the introduction of advanced digital imaging instruments like the Invitrogen™ iBright™ FL1000 Imaging System, and improvements in fluorescent conjugate technologies, scientists now have the necessary tools to take advantage of the range of fluorescent dyes and antibodies for western blot detection. These advancements provide access to fluorescence detection with reduced cost and improved sensitivity. 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 help enable your success with fluorescent western blotting.

Considerations for optimal fluorescence detection

When getting started with fluorescent western blotting, some reagents and steps will need to be optimized to help ensure background fluorescence does not interfere with detection of the protein of interest. Here are some tips for getting started:

- 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).

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 signal.

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 fluorescent background • Longer exposure times can produce high background because of the small amount of excitation light passing through the emission filters

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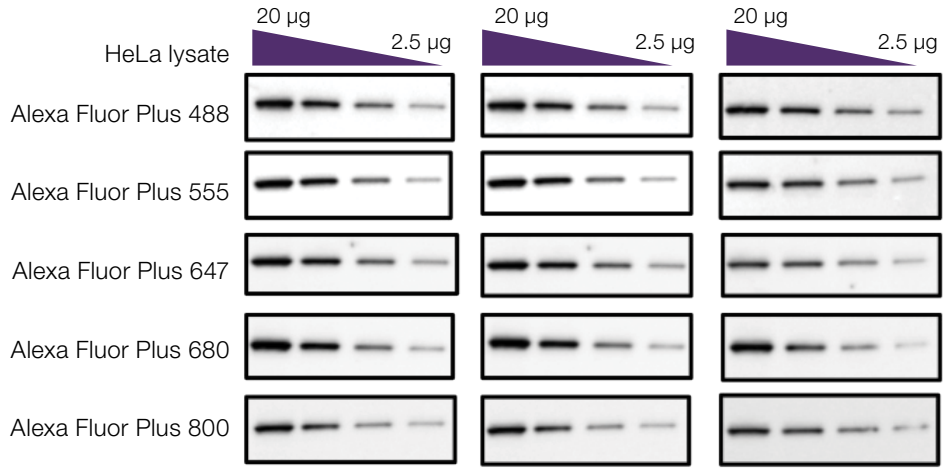
- Decrease the amount of molecular weight markers loaded onto the gel. Standard prestained molecular weight markers can be used, but the loading amount will need to be optimized if the marker contains fluorescent bands since overloading can increase background fluorescence and signal bleed-through to adjacent lanes. The Invitrogen™ iBright™ Prestained Protein Ladder (Cat. No. LC5615) provides prestained proteins as well as fluorescent bands for detection. Typically, 2–4 µL of the iBright Prestained Protein Ladder is sufficient for visualization and fluorescence detection.
- To eliminate a major source of background fluorescence, use membranes with low autofluorescence, including nitrocellulose and specialty low-fluorescence PVDF membranes such as Thermo Scientific™ Nitrocellulose Membrane (Cat. No. 88018) and Low-Fluorescence PVDF Transfer Membrane (Cat. No. 22860).

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. In addition, limit the use of detergents during blocking steps, as common detergents can autofluoresce and increase nonspecific background.
- Only handle membranes with gloved hands and clean blunt forceps to limit contamination and scratches on the membranes, which can contribute to background fluorescence and artifacts.
- 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 FL1000 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.



Smart Exposure time (seconds)		
0.05	0.97	1.36
0.77	1.25	1.59
1.49	2.43	2.84
11.06	11.03	17.24
12.74	19.54	27.55

Secondary antibody dilution	1:5,000	1:10,000	1:20,000
Secondary antibody conc.	0.4 µg/mL	0.2 µg/mL	0.1 µg/mL

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 0.4 and 0.1 µg/mL (1:5,000–1:20,000), exposure times are less than 30 seconds. HeLa cell lysates were serially diluted (to load 20 to 2.5 µg per lane), run on SDS-PAGE gels, and transferred to nitrocellulose membranes. The blots were incubated overnight with a 1:1,000 dilution of Invitrogen™ HDAC1 Polyclonal Antibody (Cat. No. PA1-860) followed by either a 1:5,000, 1:10,000, or 1:20,000 dilution of Invitrogen Alexa Fluor Plus secondary antibody (Cat. No. A32731, A32732, A32733, A32734, or A32735). Images were captured on the iBright FL1000 Imaging System using the Smart Exposure tool; exposure times for each membrane are tabulated on the right. All 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. 88018, 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
- Incubation trays or containers (e.g., Thermo Scientific™ Mini or Midi Gel Incubation Trays, Cat. No. 22843 or 22841)
- iBright FL1000 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.

Tip: Do not add detergent to blocking buffer, as this may increase background fluorescence.

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.
5. Incubate the membrane with gentle agitation in the primary antibody 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 of 0.4 to 0.1 µg/mL in an appropriate volume 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). Place each blot in a sheet protector or on a clean surface prior to imaging to prevent contamination. Image on the iBright FL1000 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/noise ratio; signal will remain stable for days to weeks with proper storage of the membrane.

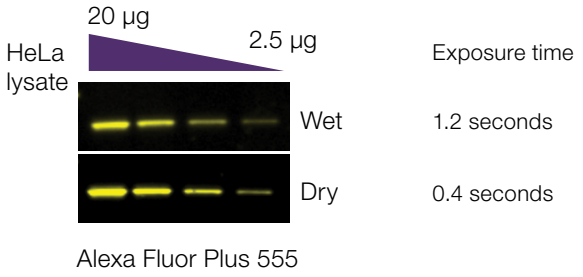


Figure 2. Dry or wet membranes provide comparable fluorescence imaging results. HeLa cell lysates were serially diluted (to load 20 to 2.5 µg per lane), run on an SDS-PAGE gel, and transferred to a nitrocellulose membrane. The blot was probed overnight with a 1:1,000 dilution of HDAC1 Polyclonal Antibody (Cat. No. PA1-860) followed by a 1:10,000 dilution (0.2 µg/mL) of Invitrogen Alexa Fluor Plus 555 Goat Anti–Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (Cat. No. A32732) for 1 hour at room temperature. Images were captured on the iBright FL1000 Imaging System using the Smart Exposure tool. Images were adjusted separately to the same black, white, and gamma levels for each blot. The Alexa Fluor Plus 555 signal was pseudocolored yellow.

Recommended secondary antibodies

The wide range of Invitrogen Alexa Fluor and Alexa Fluor Plus conjugated secondary antibodies for western blotting provides 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.

Alexa Fluor

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

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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	
	Detergent from the blocking step may be a source of background in fluorescence applications	• Use blocking buffers without detergent
	Insufficient washing can lead to 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%
	Exposure time is too long	• Reduce exposure time • On the iBright FL1000 system, use the Smart Exposure tool to obtain an optimal image
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 FL1000 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

Find out more at **thermofisher.com/ibright**

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