

Fluorescent western blotting— a guide to multiplexing

Improvements in imaging and fluorescent probes provide scientists with enhanced tools to maximize the utility of western blot technology. Fluorescent western blotting provides accurate, quantitative results, stable signals, and the ability to evaluate multiple protein targets on a single blot, which is known as multiplexing. Multiplexing helps make research more efficient and productive. For example, one can visualize a protein of interest simultaneously with a loading control protein (Figure 1), differentiate proteins of similar molecular weights (Figure 2), and evaluate complex biological pathways (Figure 3). In multiplexing terminology, probing for 2 proteins of interest is known as a 2-plex experiment, probing for 3 proteins of interest is known as a 3-plex experiment, etc. With the Invitrogen™ iBright™ FL1000 Imaging System, one can perform up to a 4-plex fluorescent western blot with the appropriate experimental setup (Figure 4). Here we share best practices and tips for success in multiplex fluorescent western blotting.

Important considerations before you get started

Before setting up your multiplexing experiment, consider these points to help minimize background fluorescence:

- To eliminate a major source of background fluorescence, use transfer membranes with low autofluorescence, such as Thermo Scientific™ nitrocellulose membranes (Cat. No. 88018 or LC2001) or low-fluorescence polyvinylidene difluoride (PVDF) membranes (Cat. No. 22860)).
- Only handle membranes with gloved hands and clean blunt forceps to limit contamination and scratches on the membrane, which can contribute to background fluorescence and other fluorescent artifacts.
- Use high-quality, filtered buffers (e.g., Thermo Scientific™ Blocker™ FL Fluorescent Blocking Buffer (Cat. No. 37565)). Particles and contaminants in blocking and wash buffers can settle on membranes and create fluorescent artifacts. In addition, limit the use

of detergents during blocking steps, as common detergents can autofluoresce, possibly increasing nonspecific background.

- Sample buffers may contain components that fluoresce (such as bromophenol blue), which can contribute to increased background. We recommend using fluorescence-compatible sample buffers (e.g., Invitrogen™ Fluorescent Compatible Sample Buffer, Cat. No. LC2570).
- If molecular weight markers contain proteins labeled with fluorophores, or that are stained with compounds that may fluoresce at certain wavelengths, decrease the volume of markers loaded onto the gel. Overloading may obscure the signal in adjacent lanes. Consider the Invitrogen™ iBright™ Prestained Protein Ladder (Cat. No. LC5615) for your multiplexing setup. Typically, 2–4 μL of the iBright Prestained Protein Ladder is sufficient for visualization and fluorescence detection.

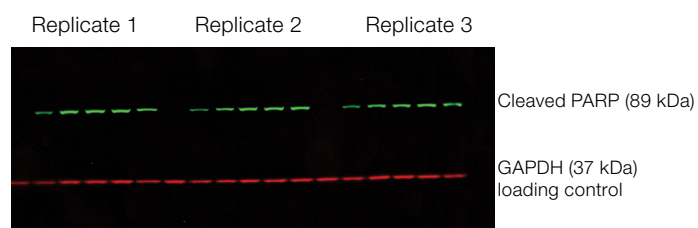


Figure 1. Simultaneous detection of protein of interest and loading control protein. The signal of each protein is captured in a different fluorescence channel, which enables the detection of two proteins on a single blot without stripping and reprobing. Composite image shown, overlaying the signals from each probe.

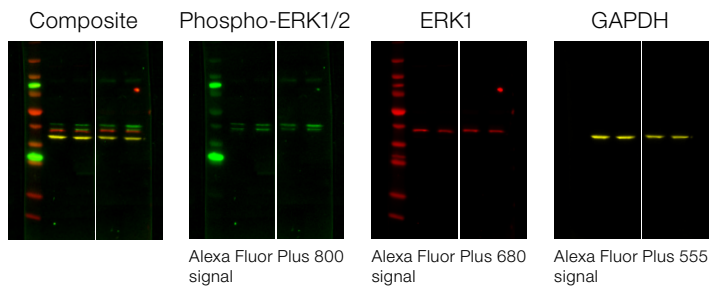


Figure 2. Detection of targets of similar molecular weights. Fluorescent multiplexing allows for clear distinction of multiple targets on the same blot, even when they are of similar molecular weights. A composite image is shown along with images showing the single-color signals of individual proteins. Visualizing the individual signals can sometimes enable assessment of details that may be harder to see in a composite.

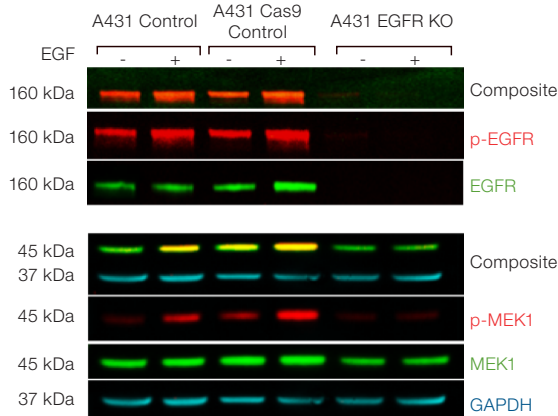


Figure 3. Study complex biological signaling pathways. Western blot analysis of the phosphorylation of EGFR and its downstream target, MEK1, after EGF treatment of A431 EGFR knockout (KO) cells.

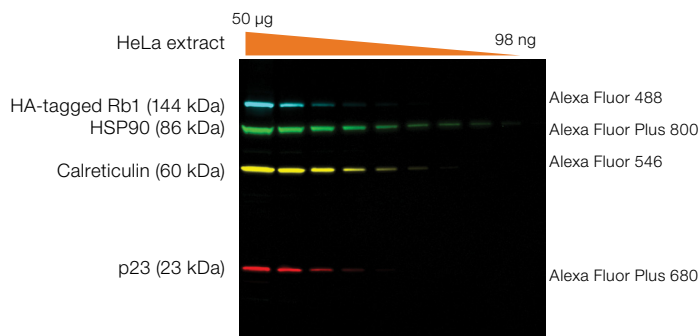


Figure 4. A 4-plex western blot imaged on the iBright FL1000 system.

Selection of antibodies

The selection of appropriate primary antibodies and fluorescently labeled secondary antibodies is critical when designing a fluorescent multiplex western blot experiment. Here are some guidelines to consider:

- Select antibodies designated specifically for western blotting or that list western blotting as an application. Verify the detection of each protein target individually before multiplexing with other targets. This will allow determination of the banding pattern of each antibody prior to a multiplex experiment.



Tip: Look for antibodies for which the supplier provides target-specific verification and validation to ensure the antibodies bind to the right target and to also save time validating. Learn more about the 2-step validation* testing methodology for Invitrogen™ antibodies at thermofisher.com/antibodyvalidation

- Use primary antibodies from different host species for each target being probed. Ideally, use a combination of antibodies from two distantly related species such as rat and rabbit, avoiding combinations like mouse and rat or goat and sheep. This will aid in the selection of appropriate secondary antibodies to minimize potential antibody cross-reactivity, which can lead to confusing results.
- If your protein is epitope tagged (e.g., 6xHis, HA), you may be able to take advantage of a fluorophore-conjugated primary antibody specific to the epitope tag. A primary antibody directly conjugated to a fluorophore does not require the use of a fluorophore-conjugated secondary antibody. This can minimize the number of secondary antibodies needed in a multiplexing experiment, but careful consideration of primary antibody host species is still required to prevent secondary antibody cross-reactivity with the other primary antibodies used in the experiment. Fluorescent labeling kits are also available if particular direct conjugates are not commercially available.
- If differentiation of the primary antibody host species is difficult, consider antibodies that are of a single specific antibody class (IgM, IgG, etc.) or isotype (IgG1, IgG2a, etc.), and a relevant secondary antibody that recognizes only that specific host class or isotype. If you are unsure of the specificity of your antibodies, contact your antibody supplier for more information.
- Additionally, consider using secondary antibodies that are highly cross-adsorbed. Cross-adsorption is a purification process that helps eliminate nonspecific antibodies in an antibody mixture, such as antibodies of specific classes, isotypes, or host species. If you are unsure of the potential cross-reactivity of your secondary antibodies, contact your antibody supplier for more information.

* The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

Filter sets and fluorophore selection

In a multiplex western blot, ideally each target protein is captured independently in separate images under conditions that eliminate any cross-talk between the fluorescent probes. Therefore, it is essential to know the configuration of the western blot imaging instrument before you begin, most importantly the available excitation and emission filter sets. Many imaging systems use a combination of excitation and emission filter sets that can be chosen to allow a narrow range of light wavelengths to pass through to excite the fluorophore and for the specific fluorophore emission to enter the camera's detector. Instead of filter sets, some instruments may use independent narrow-spectrum light sources for excitation. The specific combination of excitation and emission conditions used is often referred to as a "channel" or "layer" and determines what fluorescent probes can be imaged separately. Depending on the instrument, the available filters may come preinstalled or require installation (see Table 1 for the preinstalled filter sets of the iBright FL1000 Imaging System). Use a tool like the Fluorescence SpectraViewer (thermofisher.com/spectraviewer) to determine excitation and emission spectral overlap among the fluorophores available to you, in the context of the specific imaging instrument's equipped excitation and emission filters. Ideally, the fluorophores used in a multiplex experiment have distinct regions of either excitation or emission spectra that are compatible with the imaging system's filters. Note, only a region of either the excitation

or the emission spectrum needs to be distinct (not both). An example of two fluorophores with a high degree of excitation and emission spectral overlap is shown in Figure 5. An example of a combination of fluorophores with minimal excitation spectral overlap is shown in Figure 6. An example of a combination of fluorophores with minimal emission spectral overlap is shown in Figure 7.

Table 1. iBright FL1000 Imaging System filter sets.

Excitation channel	Filter range (nm)	Emission channel	Filter range (nm)	Examples of compatible fluorophores
EX1	455–485	EM1	510–555	Alexa Fluor 488, Alexa Fluor Plus 488
EX2	515–545	EM2	565–615	Alexa Fluor 546, Alexa Fluor Plus 555
EX3	610–635	EM3	675–720	Alexa Fluor 647, Alexa Fluor Plus 647
EX4	655–680	EM4	725–750	Alexa Fluor 680, Alexa Fluor Plus 680
EX5	745–765	EM5	810–850	Alexa Fluor 790, Alexa Fluor Plus 800

Note: Avoid using the channels EX3/EM3 and EX4/EM4 together in a multiplex experiment, because of the high degree of spectral overlap of fluorophores that would be captured in these channels.



Tip: When imaging with the iBright FL1000 Imaging System, use the brighter, low-wavelength fluorophores for low-abundance targets (e.g., Invitrogen™ Alexa Fluor™ 546 and Alexa Fluor Plus 647 probes) and, conversely, higher-wavelength fluorophores (e.g., Alexa Fluor Plus 680 and Alexa Fluor Plus 800 probes) for high-abundance targets.

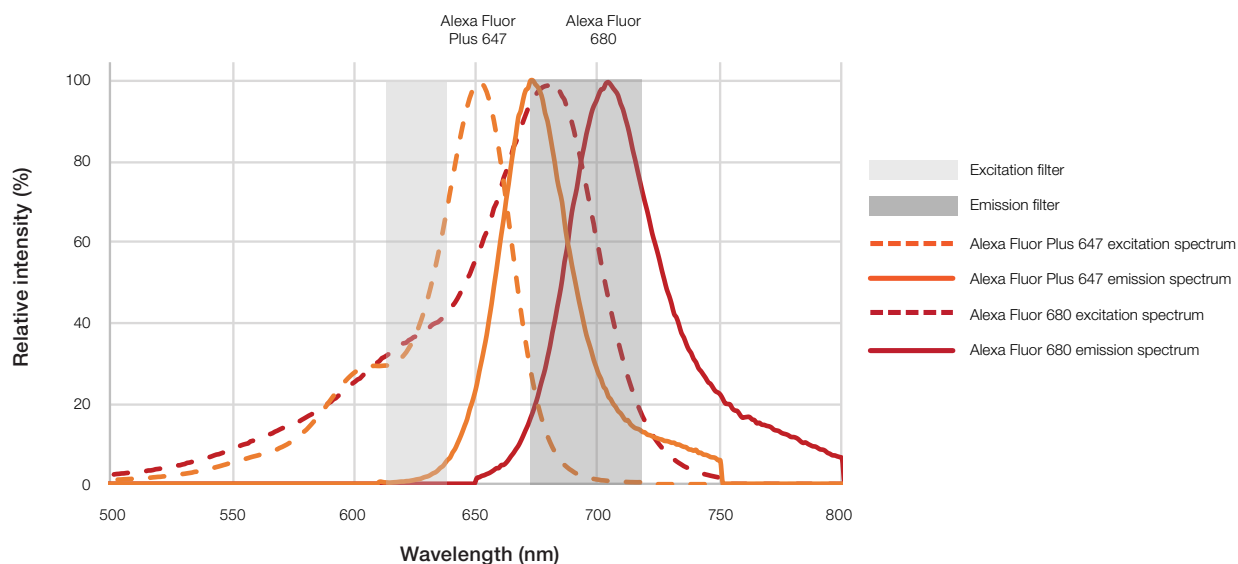


Figure 5. Example of significant spectral overlap. In this example generated on our online Fluorescence SpectraViewer, portions of the excitation and emission spectra of both Alexa Fluor Plus 647 and Alexa Fluor 680 fluorophores are within the ranges of the excitation and emission filters, so both fluorophores would be excited and their emissions would reach the camera's detector under these conditions, making it difficult to distinguish the source of the detected fluorescence.

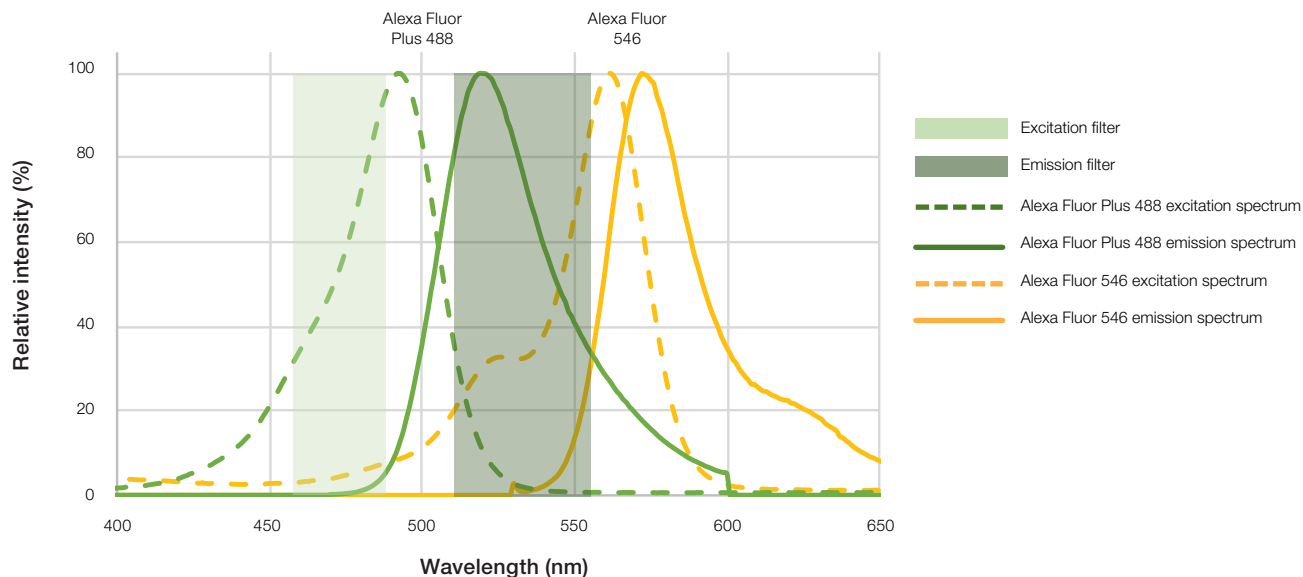


Figure 6. Example of multiplex experiment with carefully chosen fluorophores with distinct excitation spectra. In this example generated on the Fluorescence SpectraViewer, the excitation spectra (dashed lines) of Alexa Fluor Plus 488 and Alexa Fluor 546 fluorophores have minimal overlap within the range of the excitation filter. Despite both fluorophores having part of their emission spectra (solid lines) within the range of the emission filter, Alexa Fluor 546 would not be excited by the excitation filter that has been selected for Alexa Fluor Plus 488, so no fluorescence from Alexa Fluor 546 would be present to go through the emission filter.

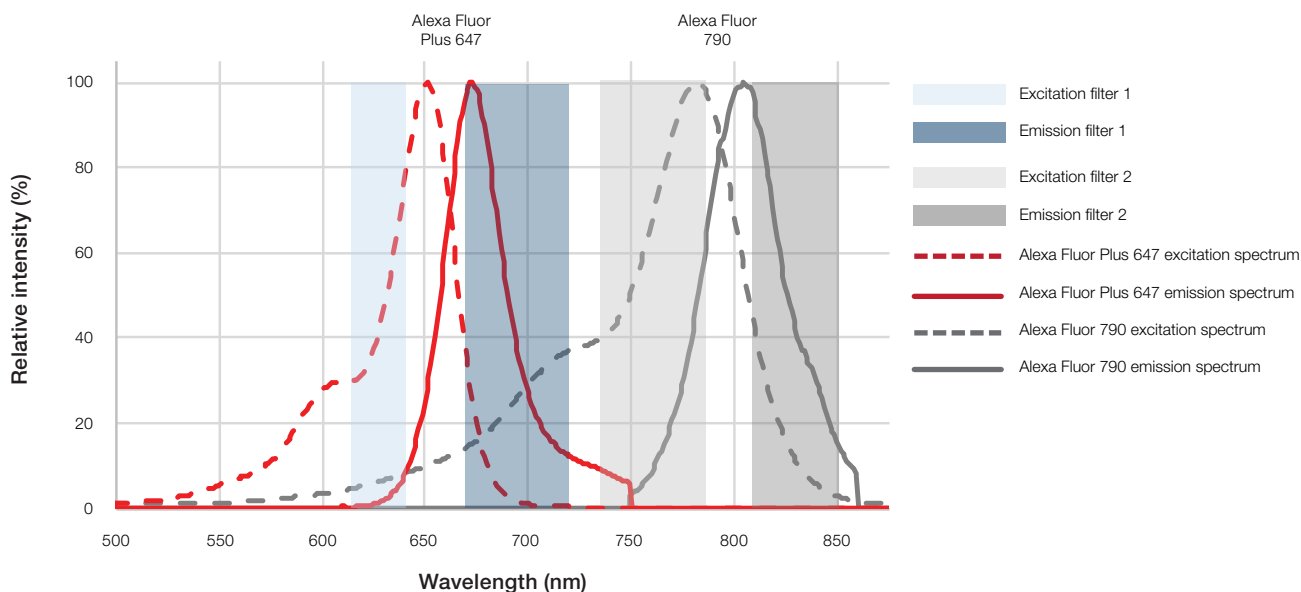


Figure 7. Example of multiplex experiment with carefully chosen fluorophores with distinct emission spectra. In this example generated on the Fluorescence SpectraViewer, the emission spectra (solid lines) of Alexa Fluor Plus 680 and Alexa Fluor 790 have no overlap within the ranges of the two emission filters. Despite both fluorophores having part of their excitation spectra (dashed lines) within the range of excitation filter 1, any Alexa Fluor 790 fluorescence generated by that excitation range is not within the wavelengths allowed to pass through emission filter 1, so no fluorescence from Alexa Fluor 790 would reach the camera detector in that channel.

General procedure for multiplex fluorescent western blotting

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., Thermo Scientific™ Tris-buffered or phosphate-buffered saline with 0.05% Tween™ 20 (Cat. No. 28360 or 28352)

- Incubation trays and containers (e.g., Thermo Scientific™ Mini or Midi Gel Incubation Trays (Cat. No. 22843 or 22841)
- Invitrogen™ primary antibodies from distinct species
- Invitrogen™ fluorescently labeled, highly cross-absorbed secondary antibodies
- iBright FL1000 Imaging System (Cat. No. A32752) or another system designed for imaging fluorescent blots

Protocol

Immunodetection

1. After protein transfer, equilibrate the membrane by washing 4 times with deionized water for 5 minutes each with agitation.
2. Incubate the membrane with a sufficient volume of blocking buffer (to block nonspecific binding sites) for 30 minutes at room temperature with agitation.



Tip: 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. Avoid low volumes, as agitation and coverage differences can produce high or uneven background.

3. Mix all primary antibodies together in blocking buffer at the individually optimized concentrations. Incubate the membrane protein-side up in the primary antibody solution with agitation, for 1 hour at room temperature or overnight at 2–8°C. Ensure the volume of the antibody solution is enough to fully cover the membrane.
4. Wash the membrane 3 times with agitation for 10 minutes each with a wash buffer containing 0.05% Tween 20. If using a fluorescently conjugated primary antibody, proceed to Step 7.



Tip: Using an excess of secondary antibody can result in either high background or signal quenching.

5. Mix all secondary antibodies together in wash buffer at the optimized concentrations. Optimization is required to achieve the best signal-to-noise ratio for each secondary antibody, but the typical recommended range, regardless of the fluorescent conjugate, is 0.4 to 0.1 µg/mL (1:5,000 to 1:20,000) for imaging on the iBright FL1000 system. Appropriate ranges may

be different depending on the instrument. Compared to traditional Alexa Fluor secondary antibodies, Alexa Fluor Plus secondary antibodies (thermofisher.com/alexafluorplus) were designed to provide high signal-to-noise ratios and lower cross-reactivity for fluorescent western blotting, thus requiring less time for optimization (Table 2).

Table 2. Examples of multiplex fluorophore combinations that can be used with the iBright FL1000 Imaging System.

Example fluorophore combinations				
Number of targets	Conjugate 1	Conjugate 2	Conjugate 3	Conjugate 4
1	Alexa Fluor Plus 647			
2	Alexa Fluor Plus 647	Alexa Fluor 546		
3	Alexa Fluor Plus 647	Alexa Fluor 546	Alexa Fluor Plus 488	
4	Alexa Fluor Plus 647	Alexa Fluor 546	Alexa Fluor Plus 488	Alexa Fluor 790

6. Incubate the membrane protein-side up in the secondary antibody solution for 1 hour with agitation at room temperature. Ensure the volume of the antibody solution is enough to fully cover the membrane, and protect the membrane from bright light to prevent photobleaching of the fluorescent dyes.
7. Wash the membrane 6 times with agitation for 5 minutes each in wash buffer to remove any unbound secondary antibodies. It is crucial to thoroughly wash the membrane at this step.
8. The blot can be imaged immediately while still wet or may be dried prior to imaging. To prevent contamination, place the blot in a sheet protector or on a clean surface prior to imaging.
9. Image the blot on the iBright FL1000 system using an appropriate detection channel and the Smart Exposure feature, or an equivalent imager.

Troubleshooting

Problem	Possible cause	Solution
Weak or no signal	Insufficient amount of primary antibody	<ul style="list-style-type: none"> • Increase primary antibody concentration • Ensure primary antibody has a good titer and is specific for the protein/epitope to be detected • Extend the incubation time to overnight at 4°C, or 3–6 hours at room temperature • Try using an antibody enhancer
	Lost activity of antibody	<ul style="list-style-type: none"> • Ensure the antibody was stored appropriately • Check the expiration date of the antibody • Avoid multiple uses of prediluted antibodies
	Exposure time is too short	<ul style="list-style-type: none"> • Increase exposure time • Use the Smart Exposure feature on the iBright FL1000 system, or a comparable autoexposure feature on another instrument
	Incorrect instrument settings	<ul style="list-style-type: none"> • Ensure the correct excitation and emission ranges are selected for the intended fluorophore
	Use of detergent	<ul style="list-style-type: none"> • Too much detergent, or the nature of the detergent, can result in washing away the signal—decrease or eliminate detergent
	Blocking buffer blocks epitope	<ul style="list-style-type: none"> • Some blocking solutions can mask the blot and reduce the availability of the epitope to the antibody, especially if the blocking step is >1 hour • Dilute your primary antibody in wash buffer • Evaluate another blocking buffer
	Quantity of sample loaded on the gel	<ul style="list-style-type: none"> • Too little lysate leads to insufficient availability of the target of interest • Perform serial dilutions of the lysate or sample to determine the optimal amount of protein to load
	Poor transfer of protein, or loss of the protein after transfer	<ul style="list-style-type: none"> • Check transfer conditions to confirm protein transfer • Reoptimization may be required when probing for a new protein
Nonspecific bands	Poor antibody specificity for the target of interest	<ul style="list-style-type: none"> • Evaluate additional primary antibodies; find tips at thermofisher.com/antibodyvalidation
	Poor sample integrity	<ul style="list-style-type: none"> • Sample degradation due to overheating or protease activity results in target breakdown and low target recognition by the antibody
	Antibody cross-reactivity in multiplex detection	<ul style="list-style-type: none"> • Choose primary antibodies raised in distantly related host species • Use highly cross-adsorbed secondary antibody conjugates • Reduce the amount of secondary antibody used while remaining within the optimal performance range
	Fluorescence bleed-through from another channel when multiplexing (appearance of an unexpected band)	<ul style="list-style-type: none"> • Use a tool like Fluorescence SpectraViewer to visualize fluorophore spectra, and avoid spectrally close conjugates, especially when the signal is very strong • Ensure that your fluorescent dyes can be distinctively detected on your imager • Use the autoexposure feature on the instrument to determine the optimal exposure time for each channel
Background issues (high, uneven, or speckled)	High background due to membrane contamination	<ul style="list-style-type: none"> • Handle the membrane carefully using clean dishes, trays, and forceps • Determine the best blocking buffer for your application—primary antibodies will react differently in different blocking buffers; blocking solutions like normal animal sera or milk may result in cross-reactivity
	Artifacts from overloading the protein marker or ladder	<ul style="list-style-type: none"> • Load less of the molecular weight marker onto the gel
	Nonoptimal wash or diluent solutions	<ul style="list-style-type: none"> • Use a wash buffer with 0.1–0.2% Tween 20 • Prepare the secondary antibody diluent with 0.05% Tween 20 • Increase the number or duration of wash steps
	High background from excess secondary antibody	<ul style="list-style-type: none"> • Optimize the secondary antibody dilution depending on the dye being used, following the vendor's recommended dilution and adapting accordingly
	Blotchy or uneven background due to the membrane drying out	<ul style="list-style-type: none"> • Ensure good coverage of the whole blot during all incubation steps • Ensure consistent agitation during every incubation step
	Incorrect choice of membrane	<ul style="list-style-type: none"> • The membrane type can affect background; for example, standard PVDF membranes can autofluoresce and cause high background, so use low-fluorescence PVDF membranes
	Speckles and fingerprints on the membrane	<ul style="list-style-type: none"> • Use clean forceps to handle the membrane, and avoid directly touching the membrane; particulates and contaminants from unclean tools will fluoresce • Use clean incubation trays and dishes—rinsing with methanol followed by water will help dissolve residual dried dyes from previous uses • Clean off transfer devices and dusty consumables (e.g., pads) if using a wet transfer method, as they can introduce speckles • Clean the imager surface with ethanol to remove dust, lint, and residues before placing a blot for imaging

Learn more about the iBright FL1000 system at [thermofisher.com/ibright](https://www.thermofisher.com/ibright)

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