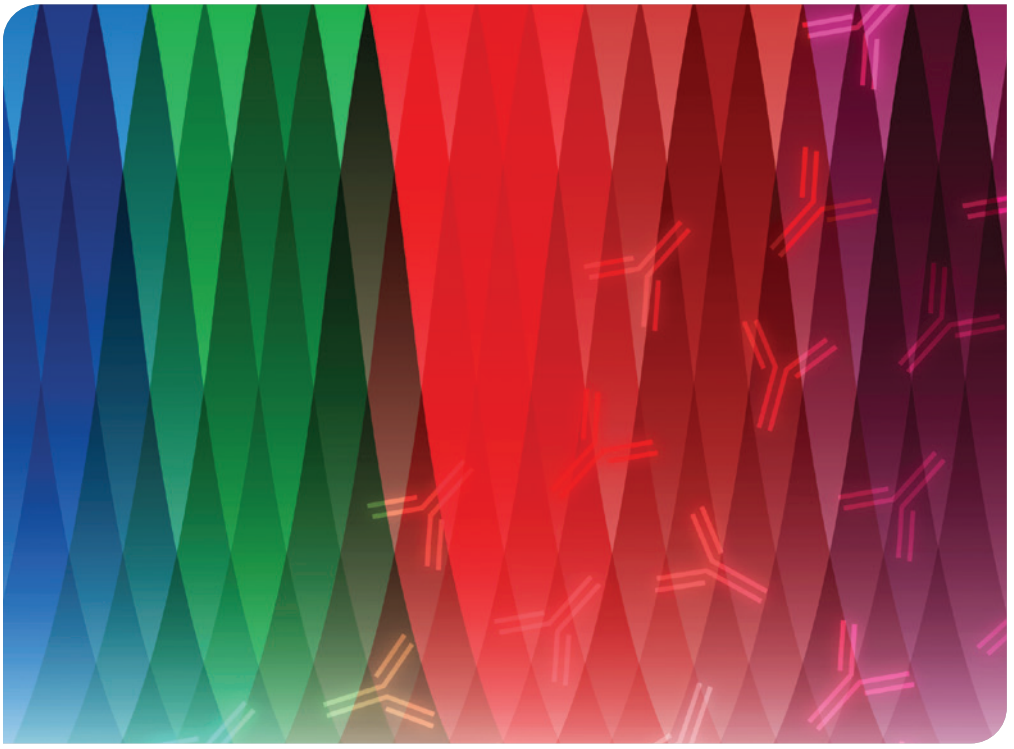


Multiplex Fluorescent Blot Detection: A Troubleshooting Guide

BIO-RAD

Western blotting is a technique widely used to identify and quantify proteins. Several different detection and imaging methods are available. Chemiluminescence detection has been popular for many years due to its compatibility with film technology and widespread familiarity in the research community. Since the early- to mid-2000s, digital technology has made acquiring, managing, and analyzing western blot image data significantly easier. Digital imaging has also enabled the use of fluorescence detection, which offers several advantages compared to chemiluminescence:

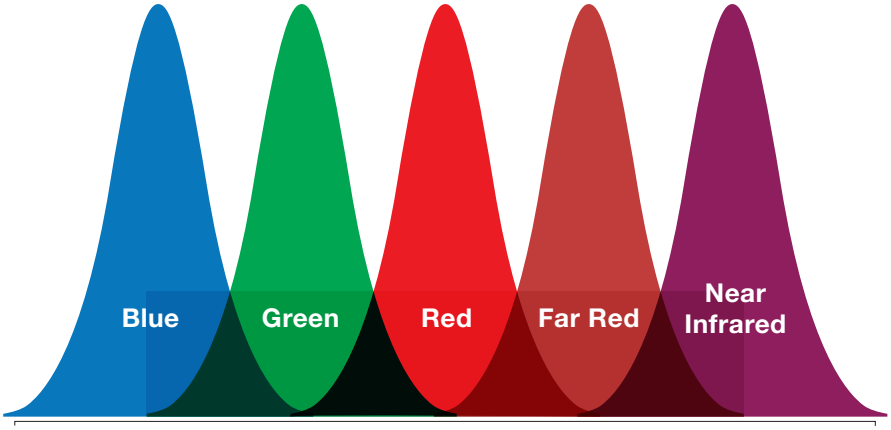
Multiplexing:	**Dynamic Range:**
Using multiple fluorophores for simultaneous detection of several target proteins makes stripping and re-probing unnecessary.	Fluorescence detection has a wider dynamic range than chemiluminescence detection and offers better linearity within detection limits.
Quantitation:	**Stability:**
Fluorescence detection is more quantitative than enzyme-based chemiluminescence detection.	Most fluorophores are highly stable, allowing blots to be archived and re-imaged at a later date.

Use this guide to start designing a new fluorescent western blotting experiment, or for optimization and troubleshooting of existing assays.

Assay Design Tips for Fluorescent Western Blotting

General antibody considerations:

- The same primary antibodies can be used for both fluorescence and chemiluminescence detection methods
- When selecting a secondary antibody and its conjugated fluorophore, use fluorophores designated for longer-wavelength fluorescence channels for target proteins that are difficult to detect. Fluorophores that fluoresce in the far red and near infrared (near-IR) ranges can be detected with greater sensitivity than those with traditional blue and green fluorescence. Use antibodies conjugated to fluorophores fluorescing at shorter wavelengths (for example, 550 nm) only for very abundant targets, such as housekeeping proteins (HKPs)
- Follow manufacturers' recommendations for making dilutions of primary and secondary antibodies. Further optimization may be needed if results are not satisfactory (see Troubleshooting, pp. 7–9)
- Primary antibody concentration may need to be increased when paired with a fluorophore that fluoresces at a lower wavelength

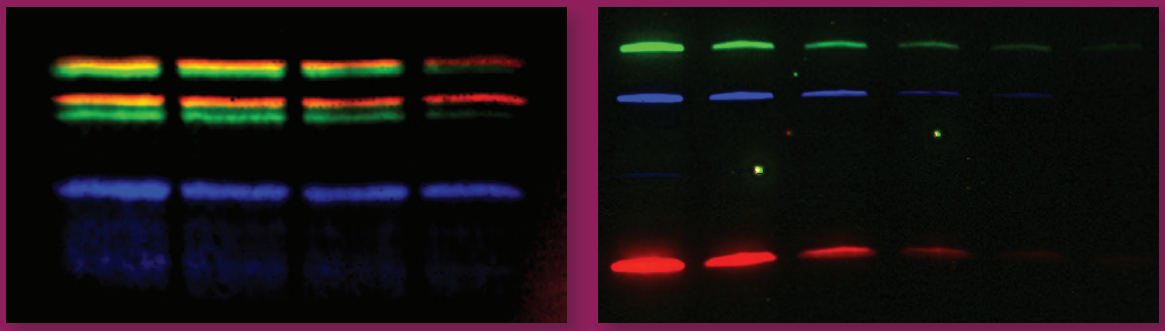


StarBright Blue 520*	hFAB Rhodamine*		StarBright Blue 700*	
DyLight 488	DyLight 550	DyLight 650	DyLight 680	DyLight 800
Alexa Fluor 488	Alexa Fluor 546	Alexa Fluor 647	Alexa Fluor 680	Alexa Fluor 790
Cy2	Cy3	Cy5	Cy5.5	Cy7
		SYPRO Ruby	IRDye 680	IRDye 800CW

Abundant target protein — Limited target protein

* Bio-Rad exclusive.

Note: This is a partial list. Other fluorophores with similar excitation and emission profiles may also be compatible, depending on imager configuration. Visit bio-rad.com/ChemiDocMP to learn more about Bio-Rad's ChemiDoc MP Imaging System.



Sample multiplex western blot (left), and multiplex phosphorylation western blot (right) using 800 nm, 650 nm, and rhodamine channels.

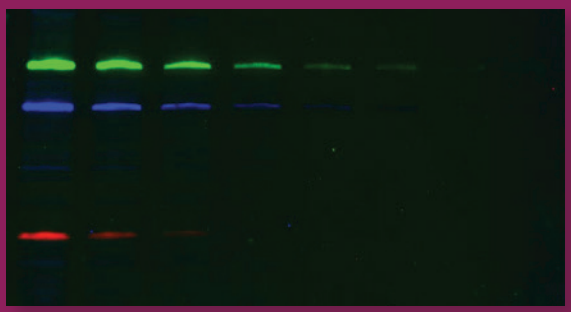
Special antibody considerations for multiplex western blotting:

- Use primary antibodies produced in different host species (for example, mouse and rabbit). Using antibodies produced in two closely related species, such as rat and mouse, often results in cross-reactivity, even when the antibodies are cross-adsorbed
- Assign fluorophores depending on the abundance of each target protein relative to the others. Shorter-wavelength excitation light could lead to high background during long exposures. Therefore, assign your most abundant target to the blue or green channels, your moderately abundant target to the red channel, and reserve the far red and near-IR channels for your least abundant targets
- Use fluorophore conjugates with optically distinct spectra to avoid cross-channel fluorescence. For example, pair a fluorophore fluorescing at 800 nm with one fluorescing at 650 nm rather than one fluorescing at 700 nm
- When using an antibody for the first time, optimize the detection of each target individually before simultaneously detecting multiple targets. Some primary antibodies are nonspecific and will produce multiple bands on a blot; therefore, it is necessary to determine the banding pattern of each antibody prior to a multiplex experiment
- Use secondary antibodies that have been cross-adsorbed against other species to avoid cross-reactivity

General tips for fluorescence detection

- Use a membrane with low autofluorescence, such as an Immun-Blot Low Fluorescence PVDF Membrane, to maximize signal-to-background ratio. An alternative is to use non-supported nitrocellulose membranes. Regular PVDF membranes are not recommended for use with fluorescence detection
- Many blocking buffers can be successfully used for fluorescence detection. We recommend a blocking buffer that has been optimized for fluorescence detection, such as EveryBlot Blocking Buffer. Visit bio-rad.com/everyblot to learn more
- Particulates in buffers can settle on membranes and create fluorescent artifacts. Use only high-quality reagents and filter sterilize all buffers
- Use powder-free nitrile gloves when handling the membrane to minimize artifacts and fingerprints on the blot
- Use a pencil to mark membranes because many inks fluoresce
- Use blunt forceps to handle the membrane from the edges. Avoid scratching or creasing artifacts during fluorescent detection
- Perform immunodetection in the dark
- Store stocks of fluorescently labeled antibodies in the dark
- Finished fluorescent western blots should be flash dried in methanol (low-fluorescence PVDF) or air dried (nitrocellulose) and stored in the dark at –20°C
- Bromophenol blue will fluoresce. Ensure that the dye front has migrated away from the sample and cut off the portion of the gel containing the dye front, or omit bromophenol blue from the sample buffer

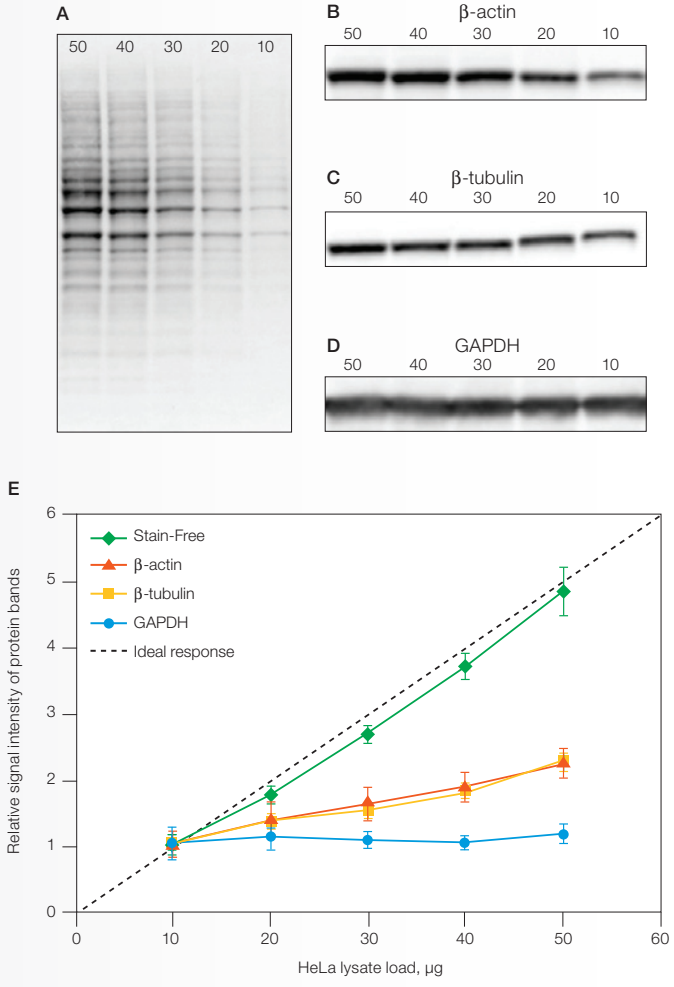
Other commercial fluorescence-optimized blocking buffer, 1 hr.



EveryBlot Blocking Buffer, 5 min.

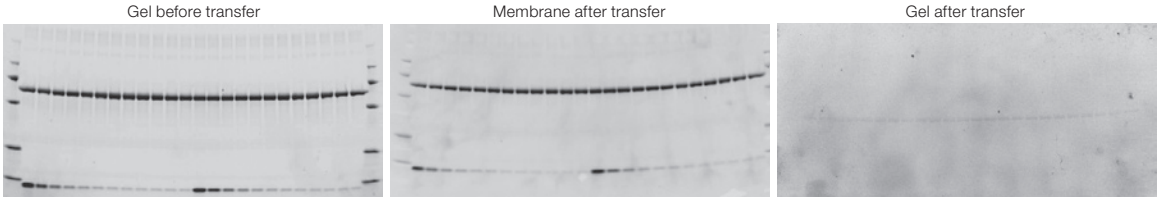
Multiplex Fluorescent Western Blots Using Stain-Free Gels

Stain-Free gels are formulated with a trihalo compound that becomes covalently bound to tryptophan residues in proteins when the gel is activated with UV light. The labeled tryptophans then fluoresce when excited by UV light, allowing for direct visualization of total protein in gels and on blots.



Linearity comparison of Stain-Free total protein measurement and immunodetection of three housekeeping proteins in 10–50 µg of HeLa cell lysate. Representative images of: **A,** Stain-Free blot; chemi blots for **B,** β-actin; **C,** β-tubulin; **D,** GAPDH. Lane labels correspond to total protein load (µg). **E,** although the actin and tubulin signals appear linear, the densitometric ratio was far below the predicted quantitative response of actual loading, whereas the Stain-Free signal closely matched the expected result.

After UV light activation, Stain-Free fluorochromes are covalently bound to protein molecules in the gel, allowing them to be imaged repeatedly on the gel or on a membrane after transfer without staining and destaining steps. This characteristic enables rapid imaging at checkpoints in the separation and transfer steps of western blotting to ensure optimal results.

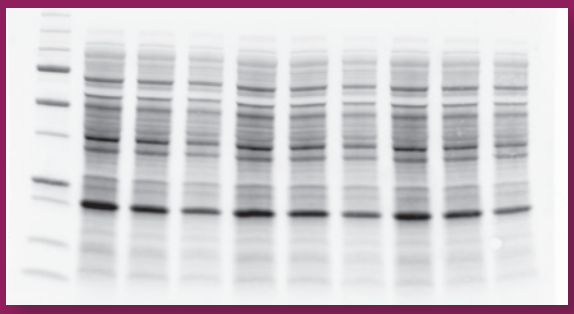


Assessment of protein transfer using a Stain-Free–compatible imaging system. Serial 1:2 dilutions of hemoglobin (starting quantity, 80 ng) with 1.8 µg of BSA/lane as a carrier (top band) were electrophoretically separated on a 4–20% 26-well Criterion TGX Stain-Free Precast Gel.

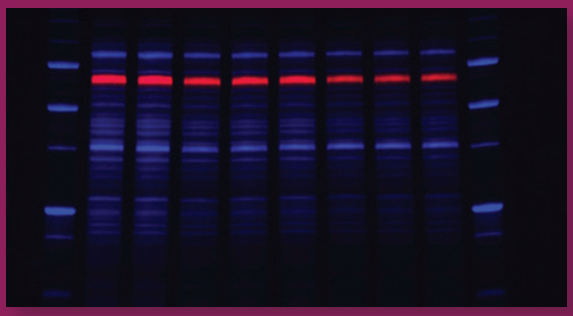
Visit bio-rad.com/StainFree to learn more about Stain-Free technology.

Special considerations for Stain-Free western blotting:

Stain-Free technology allows the user to obtain truly quantitative western blot data by normalizing bands to total protein in each lane, eliminating the inherently problematic use of housekeeping proteins as loading controls. For multiplex western blot applications, use the red, far red, and near-IR channels for detecting proteins of interest and reserve the blue channel for total protein detection using Stain-Free technology.

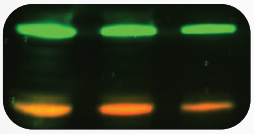


Stain-Free image used as loading control for total protein normalization.



Multiplex western blot imaged with the Stain-Free (total protein, in blue) and near-IR (target protein, DyLight 680, in red) channels.

Troubleshooting



Extra Bands

Several factors can contribute to unwanted bands on a fluorescent western blot, especially when multiplexing. Identify and eliminate these issues using the following tips.

Cross-channel fluorescence:

- **Fluorophores are not optically distinct.** This occurs when signal from an individual fluorophore is detected in neighboring channels in a multiplex western blot

  Solution: Use secondary antibody combinations that are conjugated to fluorophores with non-overlapping emission spectra

- **Inappropriate emission filters**

  Solution: Ensure appropriate emission filters are used during image acquisition

Antibody cross-reactivity:

- **Primary antibody host species are related.** Primary antibodies from the same or related species may bind to the same secondary antibody in a multiplex western blot

  Solution: Check that each primary antibody in the multiplex assay is from a different species. Avoid using closely related species, such as rat and mouse, as even cross-adsorbed antibodies from similar species may still cross-react

Nonspecific binding:

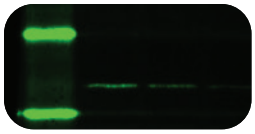
- **Primary antibodies bind to non-target proteins.** Off-target binding may produce multiple bands

  Solution: If more specific antibodies are not available, optimize the detection of each target individually before simultaneously detecting multiple targets. Single-target detection will help determine the banding pattern of each antibody prior to a multiplex experiment

- **Secondary antibodies bind to non-target proteins.** Check for this issue by running control blots without primary antibody. Visible bands indicate non-specific secondary antibody binding

  Solution: Use appropriately cross-adsorbed secondary antibodies

Troubleshooting (contd.)



Weak Signal

Antibody concentrations:

- **Antibody concentrations are too low.** When adapting a chemiluminescence protocol for fluorescence detection, antibody concentrations may need to be increased

  Solution: Optimize by testing several different dilutions of each antibody

- **Concentration of secondary antibody/antibodies is too high.** Extremely high concentrations of fluorescently labeled secondary antibody may yield low signal due to quenching via Förster resonance energy transfer (FRET)

  Solution: Optimize by testing several dilutions of each secondary antibody

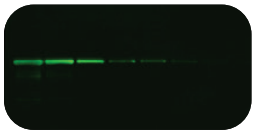
Other issues:

- **Incomplete transfer of proteins to blot.** High molecular weight proteins may require a longer transfer time

  Solution: Use Stain-Free gels and monitor gel and membrane post-transfer to ensure that transfer is efficient. Increase transfer time if necessary

- **Protein load on gel is too low.** Some proteins elute less readily from the gel and require higher loads of protein to produce satisfactory blots

  Solution: Use more protein when loading the gel



Uneven Signal

- **Air bubbles between gel and membrane.** Air bubbles prevent protein transfer and will produce blank spots on the membrane

  Solution: Use a roller to expel any trapped air during assembly of the transfer sandwich. Transfer artifacts can be monitored using Stain-Free gels

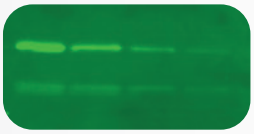
- **Uneven coverage of membrane during antibody incubation**

  Solution: Ensure incubations occur with enough volume to completely cover the membrane. Incubate and wash with continuous, vigorous agitation

- **Membrane autofluorescence**

  Solution: Use a membrane with low autofluorescence

Troubleshooting (contd.)



High Background

- **Inappropriate fluorophore-target protein pairing**

  Solution: Detect low-abundance targets using the 800, 700, or 650 nm channels

- **Insufficient blocking**

  Solution: Increase the concentration of the blocking agent or duration of the blocking step or try an alternate blocking agent

- **Insufficient washing**

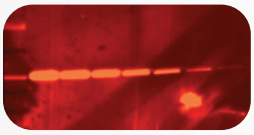
  Solution: Increase the number or duration of wash steps

- **Antibody concentrations too high**

  Solution: Optimize the concentration of primary and secondary antibodies

- **Membrane autofluorescence**

  Solution: Use a membrane with low autofluorescence



Uneven Background

- **Improper wetting of the PVDF membrane or drying during handling**

  Solution: Ensure that the PVDF membrane is fully wetted in methanol prior to equilibration in aqueous solution. Thereafter, ensure that the membrane remains consistently wet

- **Uneven washing**

  Solution: Ensure that incubations occur with enough volume to completely cover the membrane and wash with vigorous agitation

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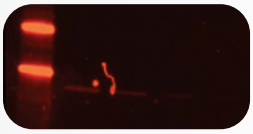

Fluorescent Artifacts

- **Scratches or creases on the membrane**

  Solution: Always use blunt forceps to handle the membrane. Grasp the membrane only at the edges

- **Fingerprints on the membrane.** Bare skin and some gloves can leave fluorescent residue on membranes

  Solution: Do not touch membranes with bare hands. Use forceps and powder-free nitrile gloves to handle the membrane

- **Particulate contaminants in solutions.** Particles may settle and appear as fluorescent speckles on the blot

  Solution: Filter buffers and reagents to remove contaminants

- **Bromophenol blue on the membrane.** Bromophenol blue in gel loading dye can produce a fluorescent signal when transferred to the membrane

  Solution: Ensure that the dye front has migrated away from the protein of interest and cut off the dye front before transfer, or use a loading dye without bromophenol blue

- **Markings made using pen.** Some inks are fluorescent

  Solution: Do not use a pen to mark the membrane. Use a pencil instead

Ordering Information

Catalog #	Description
Imaging Systems	
12003154	**ChemiDoc MP Imaging System**, includes blot and gel imaging system and is capable of imaging UV/visible light, chemiluminescence, and 5 fluorescence channels (RGB, far red, near-IR). Includes internal computer, 12" touch-screen display, Image Lab Touch Software, blot/UV/Stain-Free sample trays
Transfer Devices and Kits	
1704150	**Trans-Blot Turbo Transfer System**, blotting instrument, includes base, 2 cassettes to hold 1–2 midi or up to 4 mini blotting sandwiches, blot roller
1704275	**Trans-Blot Turbo RTA Midi LF PVDF Transfer Kit**, for 40 blots
1704274	**Trans-Blot Turbo RTA Mini LF PVDF Transfer Kit**, for 40 blots
Membranes	
1620260	**Immun-Blot Low Fluorescence PVDF/Filter Paper Sets**, pkg of 10, 7 x 8.5 cm, precut low fluorescence PVDF/filter paper for immunoblotting
1620261	**Immun-Blot Low Fluorescence PVDF/Filter Paper Sets**, pkg of 20, 7 x 8.5 cm, precut low fluorescence PVDF/filter paper for immunoblotting
1620262	**Immun-Blot Low Fluorescence PVDF/Filter Paper Sets**, pkg of 10, 8.5 x 13.5 cm, precut low fluorescence PVDF/filter paper for immunoblotting
1620263	**Immun-Blot Low Fluorescence PVDF/Filter Paper Sets**, pkg of 20, 8.5 x 13.5 cm, precut low fluorescence PVDF/filter paper for immunoblotting
1620264	**Immun-Blot Low Fluorescence PVDF Membrane**, pkg of 1 roll, 28 cm x 3.8 m, low fluorescence PVDF membrane for immunoblotting

Catalog #	Description
Blocking Buffer and Detergents	
12010020	**EveryBlot Blocking Buffer**, 500 ml
12010947	**EveryBlot Blocking Buffer**, 50 ml
1610781	**10% Tween 20**, 1 L, detergent
1610783	**1x Phosphate Buffered Saline with 1% Casein**, 1 L, blocking reagent
1662403	**10x Phosphate Buffered Saline**, 100 ml, 10x PBS
1610782	**1x Tris Buffered Saline with 1% Casein**, 1 L, blocking reagent
1706435	**10x Tris Buffered Saline**, 1 L, 10x TBS
StarBright Blue Secondary Antibodies	
12004158	**StarBright Blue 700 Goat Anti-Mouse IgG**, 400 µl
12004159	**StarBright Blue 700 Goat Anti-Mouse IgG**, 80 µl
12004161	**StarBright Blue 700 Goat Anti-Rabbit IgG**, 400 µl
12004162	**StarBright Blue 700 Goat Anti-Rabbit IgG**, 80 µl
12005866	**StarBright Blue 520 Goat Anti-Mouse IgG**, 400 µl
12005867	**StarBright Blue 520 Goat Anti-Mouse IgG**, 80 µl
12005869	**StarBright Blue 520 Goat Anti-Rabbit IgG**, 400 µl
12005870	**StarBright Blue 520 Goat Anti-Rabbit IgG**, 80 µl

TGX Stain-Free Precast Gels





Description	10-Well 30 µl	10-Well 50 µl	12-Well 20 µl	15-Well 15 µl	IPG Well 7 cm IPG Strip
Mini-PROTEAN TGX Stain-Free Precast Gels					
7.5% Resolving Gel	4568023	4568024	4568025	4568026	4568021
10% Resolving Gel	4568033	4568034	4568035	4568036	4568031
12% Resolving Gel	4568043	4568044	4568045	4568046	4568041
4–15% Resolving Gel	4568083	4568084	4568085	4568086	4568081
4–20% Resolving Gel	4568093	4568094	4568095	4568096	4568091
8–16% Resolving Gel	4568103	4568104	4568105	4568106	4568101
Any kD Resolving Gel	4568123	4568124	4568125	4568126	4568121





Description	12+2-Well* 45 µl	18-Well 30 µl	26-Well 15 µl	Prep+2-Well* 700 µl	IPG+1-Well* 11 cm IPG Strip
Criterion TGX Stain-Free Precast Gels**					
7.5% Gel	5678023	5678024	5678025	—	—
10% Gel	5678033	5678034	5678035	—	—
12% Gel	5678043	5678044	5678045	—	—
4–15% Gel	5678083	5678084	5678085	5678082	5678081
4–20% Gel	5678093	5678094	5678095	5678092	5678091
8–16% Gel	5678103	5678104	5678105	5678102	5678101
Any kD Gel	5678123	5678124	5678125	5678122	5678121

* Reference wells accommodate 15 µl of markers/standards.
** Criterion TGX Stain-Free Gels are sold singly.

Visit **bio-rad.com/FluorescentWB** for more information.

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