DyLight™ Multiplex Fluorescent Western Blot Detection Kits

Rockland's multiplex fluorescent Western blot kits are designed for simultaneous quantitative analysis of multiple proteins within the same sample. The kits contain all the necessary components that are optimized for the simultaneous detection of multiple proteins on the same blot using fluorescent-labeled secondary antibodies that are visualized in different fluorescence channels (488, 549, 649, and 800 nm). The two or three-color multiplexing abilities of gel Imaging Systems with the high quality digital camera and band-pass filters matched to a large range of fluorochromes allows for simultaneous detection of multiple proteins.

I. KITS AND KIT COMPONENTS

DyLight™ Multiplex Fluorescent Western Blot Kits					
Kit Description	Fluorophores	**Infra-Red**	**Far-Red**	**Blue Green or Yellow Green**	**Catalog #**
	Number of Targets	*Fluorophore A*	*Fluorophore B*	*Fluorophore C*	
DyLight™ Multiplex 649/800 Duo Western Blot Kit	2	Goat anti-Rabbit IgG DyLight™ 800*	Goat anti-Mouse IgG DyLight™ 649*	--	KFA013
DyLight™ Multiplex 549/800 Duo Western Blot Kit	2	Goat anti-Rabbit IgG DyLight™ 800	--	Goat anti-Mouse IgG DyLight™ 549	KFA014
DyLight™ Multiplex 488/800 Duo Western Blot Kit	2	Goat anti-Rabbit IgG DyLight™ 800	--	Goat anti-Mouse IgG DyLight™ 488	KFA015
DyLight™ Multiplex 649/488 Duo Western Blot Kit	2	--	Goat anti-Rabbit IgG DyLight™ 649	Goat anti-Mouse IgG DyLight™ 488	KFA016
DyLight™ Multiplex 649/549 Duo Western Blot Kit	2	--	Goat anti-Rabbit IgG DyLight™ 649	Goat anti-Mouse IgG DyLight™ 549	KFA017
DyLight™ Multiplex 649/549/800 Trio Western Blot Kit	3	Goat anti-Rabbit IgG DyLight™ 800	Goat anti-Mouse IgG DyLight™ 649	Goat anti-Chicken IgG DyLight™ 549	KFA018
DyLight™ Multiplex 649/488/800 Trio Western Blot Kit	3	Goat anti-Rabbit IgG DyLight™ 800	Goat anti-Mouse IgG DyLight™ 649	Goat anti-Chicken IgG DyLight™ 488	KFA019

Additionally Included Kit Components		
Component	**Description**	**Size**
Blocking Buffer (2X)	Blocking buffer(2X) for fluorescent Western blotting	50 ml
Wash Buffer (10x TTBS)	Wash buffer (10X) for fluorescent Western blotting	50 ml
Incubation Box	Western incubation box	7.5 cm x 5 cm
Pre-stained Protein Standards	Rockland Opal pre-stained protein standard 10-245kDa	50 µL

**Secondary antibodies come in 100 µg size.*

II. STORAGE CONDITIONS

Upon receipt, the secondary antibodies can be stored at 4°C prior to reconstitution. For extended storage of antibodies, aliquot contents and freeze at -20°C or below. Avoid freeze/thaw cycles. The wash buffer and blocking buffer can be stored at 4°C prior to opening. The pre-stained protein Western standards should be stored at 2-8°C. The pre-stained protein standards can also be stored at room temperature for up to 6 months or at -20°C for up to two years.

III. INTRODUCTION

Fluorescence based multiplex Western blot assay is suited for simultaneous detection and quantification of specific protein populations in a biological sample. Using a combination of two or three antibodies selected for minimal cross reactivity, fluorescent detection methods enable simultaneous quantitative analysis of multiple proteins within the same sample on the same blot (Figure 1). The fluorescent dyes such as DyLights™ when conjugated to secondary antibodies, offer a variety of benefits over traditional detection methods such as colorimetric and chemiluminescent detection.

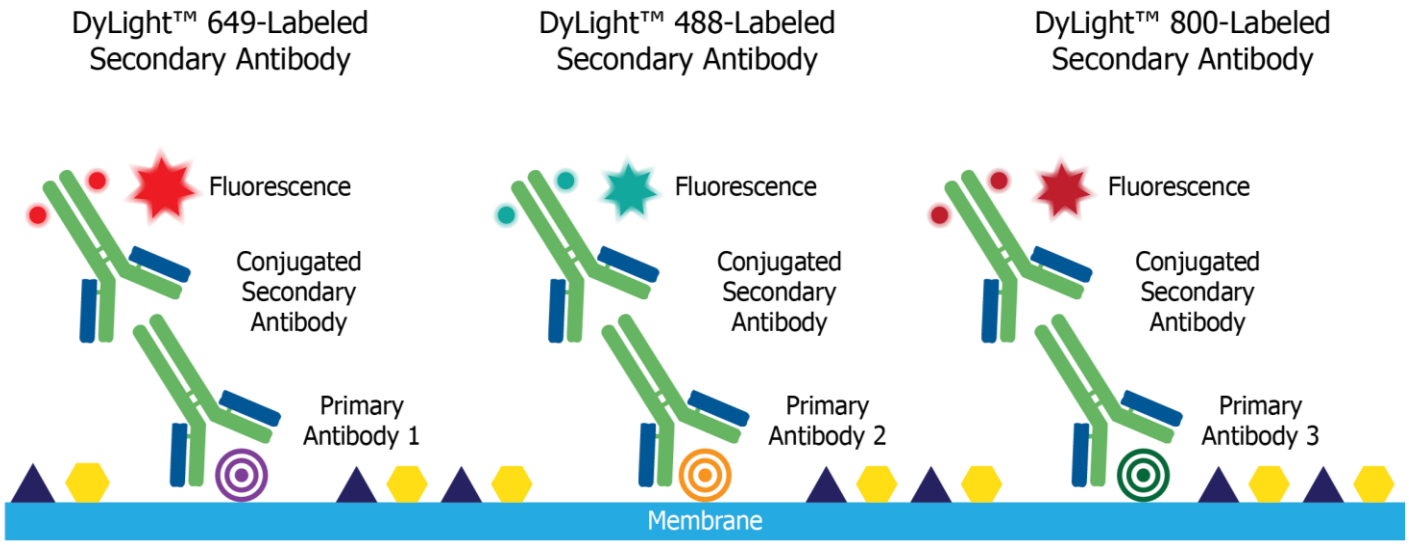


Figure 1: Schematic of Multiplex Fluorescent Western Blotting Using DyLight™ Dye-Conjugates

Using protein-specific primary antibodies and DyLight™-labeled secondary antibodies, a single Western blot was probed for two and three proteins of interest (α-Tubulin & GFP; and α-tubulin, β-actin & GFP) (Figure 2 & 3). In this example, multiplex detection using the correct lighting and filter conditions, distinguishes proteins that co-migrate in a single gel lane, enabling the identification and quantitation of antigens with specific primary antibodies. This type of multicolor analysis not only reduces complete workflow by minimizing the requirement for multiple blots, but also allows the quantitation of multiple proteins more reliable. Other benefits of fluorescent Western blotting include increased sensitivity, excellent signal stability over time as well as precise quantitative analysis with broader dynamic range and high linearity. Multiplex fluorescent Western blotting also reduces or eliminates the need to strip and re-probe. In addition, due to their exceptional photostability, DyLight™ dye conjugates can be archived and visualized several times without a decrease in signal.

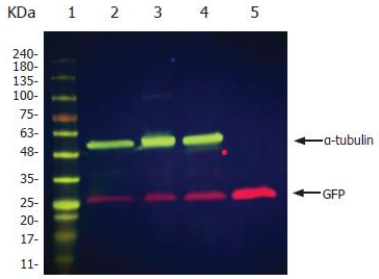


Figure 2: Simultaneous detection of α-tubulin and GFP on a single blot using Rockland DyLight™-labeled secondary antibody conjugates. Protein lysates from HeLa (lane 1), PC12 (lane 2) and K562 (lane 3) cells and 100ng GFP protein (Lane 4) were run on a gel. The cell lysates were spiked with 25ng, 50ng and 75ng GFP protein. Probing of cell lysates and GFP with mouse anti-α-tubulin and chicken anti-GFP antibodies followed by DyLight™ 649 goat anti-mouse IgG (pseudocolored green) and DyLight™ 800 goat anti-chicken IgG (red) conjugates, and then imaged using Syngene G:BOX Imaging System resulted in comparable patterns of detection. Lane 5: Rockland Opal Prestained Protein Standard 10-245kDa.

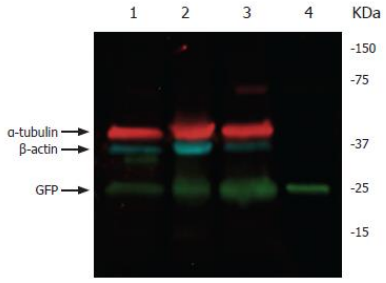


Figure 3: Simultaneous detection of three proteins on a single blot using Rockland DyLight™-labeled secondary antibody conjugates. Protein lysates from HeLa (lane 1), PC12 (lane 2) and K562 (lane 3) cells and 50ng GFP protein (Lane 4) were run on a gel. The cell lysates were spiked with 50ng, 75ng and 150ng GFP protein. Probing of cell lysates and GFP with anti-α-tubulin (mouse), anti-β-actin (rabbit), and anti-GFP (chicken) followed by DyLight™ 649 goat anti-mouse IgG (red), DyLight™ 800 goat anti-rabbit IgG (pseudocolored aqua) and DyLight™ 488 goat anti-chicken IgG (pseudocolored green) conjugates, and imaged using Syngene G:BOX Imaging System resulted in comparable patterns of detection.

IV. FLUOROPHORE SELECTION

Fluorophores	Infra-Red	Far-Red	Blue Green or Yellow Green
Number of Targets	Fluorophore A	Fluorophore B	Fluorophore C
2	DyLight™ 800	DyLight™ 649	--
3	DyLight™ 800	DyLight™ 649	DyLight™ 488 or 549

Table 1: Fluorophore Selection Guide

V. REQUIRED EQUIPMENT AND REAGENTS

- SDS-PAGE precast gels (4-20% or any gel percentage recommended)
- Vertical electrophoresis chambers for precast gels
- Western blotting transfer system
- Power supply
- Low fluorescent PVDF membrane
- Western blotting filter paper
- Forceps to handle the membrane
- Rotary or rocking platform shaker for agitation of membrane during incubations
- 2x SDS-PAGE sample buffer (cat# MB-018)
- DTT or β-mercaptoethanol
- 10x SDS-PAGE running gel buffer (cat # MB-017)
- Loading controls (cell lysates or similar)
- Express transfer buffer (cat # MB-024-1000)
- 10x TBS pH 7.5 (cat # MB-012)
- 10x TTBS pH 7.5 (cat # MB-013)
- Tween 20
- Methanol

VI. GUIDELINES FOR MULTIPLEX FLUORESCENT DETECTION

Two or three different antigens can be detected simultaneously on the same blot using antibodies labeled with Rockland DyLight™ dyes that are visualized in different fluorescence channels (488, 549, 649 and 800 nm). Two or Three-color detection requires careful selection of primary and secondary antibodies.

The following guidelines will help design multi-color experiments:

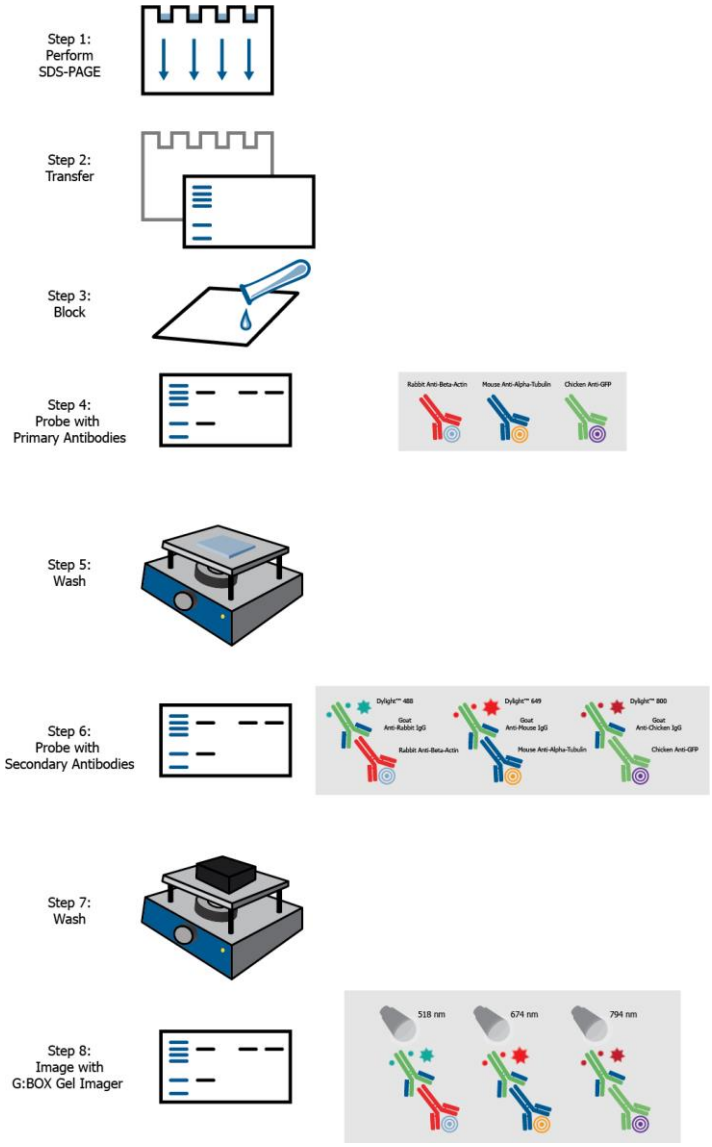
- Use primary antibodies from distantly related host species such as mouse, rabbit or chicken to minimize non-specific binding when using in direct detection. Avoid using primary antibodies derived from mouse and rat together. Cross-reactivity may occur because these species are so closely related.
- Before combining primary antibodies in a two or three-color experiment, always perform separate preliminary blots with each primary antibody to determine the expected banding pattern and possible background bands. The more proteins combined on a blot, the more complex optimization becomes. Single target detection helps determine the banding pattern of each antibody prior to multiplex analysis.
- For the primary antibodies that are derived from different host species (for example, mouse and chicken), Dylight™ IgG secondary antibodies derived from the same host and labeled with different fluorophores must be used (for example, Dylight™ 800 Goat anti-Mouse and Dylight™ 488 or Dylight™ 649 goat anti-Rabbit can be used).
- Use highly cross-adsorbed secondary antibodies. Failure to use highly cross-adsorbed antibodies may result in cross-reactivity.
- Use fluorophore conjugates with non-overlapping emission spectra to avoid cross-channel fluorescence or bleed-through. Due to higher wavelength and lower auto-fluorescence, optimal detection of lower abundance targets may be achieved by detecting targets in the 800 channel, with normalization in the 649 channel.
- To minimize auto-fluorescence, substitute standard PVDF membranes for low fluorescence PVDF membranes. These membranes increase signal-to-background ratios per channel for transferred proteins, producing bright clean bands. To additionally minimize fluorescent artifacts, avoid using inks to mark membranes, don't handle while wearing powdered nitrile gloves, and make sure to be gentle when handling membranes.
- While there's no risk to photo-bleaching fluorescently labeled antibodies in working daylight conditions, all fluorescently labeled antibody stocks should be stored in the dark.

VII. MULTIPLEX PROTOCOL

Two or Three Color Detection

1. Take 10-35µg of protein sample and add equal volume of 2x SDS-PAGE sample buffer. Boil samples at 95°C for 5 minutes and load onto 4-20% (or any gel percentage) precast SDS-PAGE gels.
2. Run protein samples and pre-stained protein standard (5-10µl) using SDS-PAGE and transfer to low fluorescent-PVDF membrane using standard transfer conditions (use methanol to pre-wet dry PVDF membrane).
3. Block membrane overnight at 4°C in blocking buffer for fluorescent Western blotting with continuous agitation (do not include Tween in this step).
4. Probe membrane with a mixture of primary antibodies (e.g. mouse primary antibody against target protein 1, rabbit primary antibody against target protein 2 and/or chicken primary antibody against target protein 3), diluted in blocking buffer for three to four hours at room temperature with continuous agitation. (Note: Optimum dilution of primary antibodies depends on the antibody and should be determined empirically).
5. Wash the membrane 4 times for 5 minutes each at room temperature in wash buffer (1x TTBS, pH 7.5) with continuous agitation.

6. Incubate the membrane with a mixture of Rockland DyLight™-labeled secondary antibodies (DyLight™649 conjugated goat anti-mouse antibody, DyLight™800 conjugated goat anti-chicken antibody and/or DyLight™488 conjugated goat anti-rabbit antibody) diluted 1:2000 in blocking buffer at room temperature for one hour with continuous agitation (incubating more than 60 minutes may increase background).
7. After the incubation, wash the membrane four times with wash buffer (1x TTBS, pH 7.5) for five minutes each wash with continuous agitation, then briefly rinse with deionized water. Transfer the membranes to 100% methanol for 30 seconds and completely dry the membrane at room temperature.
8. Image the dry membrane using gel imaging system. Center the membrane on the sample stage of gel imager and scan the membrane in the appropriate channels. The optical filters used are described in Table 2.



Channel	Excitation	Emission Filter	Compatible Dyes
Far-Red	646	674	DyLight™ 649
Infra-Red	770	794	DyLight™ 800
Blue Green	493	518	DyLight™ 488
Yellow Green	550	568	DyLight™ 549

Table 2: Fluorescent Detection Setting for gel Imaging Systems

VIII. STORAGE OF FLUORESCENT MEMBRANES

After immunodetection, fluorescent membranes can be stored for analysis at a later date. If re-probing is not desired, PVDF membranes can be dipped in methanol for few seconds and allowed to dry. Membranes can then be placed in a plastic sheet protector to prevent contamination. Store the membranes at 4°C in the dark or wrapped in foil to prevent photobleaching. Depending on the fluorophore, membranes can be stored for months and reimaged without significant loss of signal.

IX. GENERAL CONSIDERATIONS

The fluorescent Western blot kit reagents are optimized to function together. For best results, use the primary and secondary antibodies at the recommended dilutions.

Use a clean, dust free, incubation box for each step of the fluorescent Western blotting procedure.

Store the DyLight™ secondary antibody vials at 4°C in the dark. DyLight™ secondary antibodies may be aliquoted and frozen for long-term storage. Minimize exposure to light and take care not to introduce contamination into the vial. Dilute immediately prior to use. If particulates are seen in the antibody solution, centrifuge before use.

Soak the gel in transfer buffer for 10-20 minutes before setting up the transfer. Soaking equilibrates the gel and removes buffer salts that will be carried over into the transfer tank.

To enhance signal, try longer primary antibody incubation at room temperature or overnight at 4°C. Avoid extended incubations in secondary antibody.

Protect membrane from light during secondary antibody incubations and washes.

Always wear powder free gloves and use clean forceps to handle the blot. Please note that directly handling the blot, even with gloves, may introduce artifacts during imaging.

Use pencil when marking the blot, as the fluorescent properties of pen ink may interfere with blot imaging.

TROUBLESHOOTING

High Overall Background

- Membranes have higher inherent fluorescence with short wavelength excitation. Detect the weakest target using the red channel.
- Blocking may be insufficient. Block the membrane for at least one hour at room temperature or overnight at 4°C or try an alternate blocking agent.
- The concentration of the primary and secondary antibody may be too high. The concentration of the antibodies may have to be optimized, dilute the primary and secondary antibodies within the indicated dilution range.
- The primary antibody may be non-specifically binding. Ensure by other methods that primary antibody is specific for the protein of interest and has minimal non-specific binding.
- Washing may be insufficient. Increase the number or duration of wash steps. Make sure that 0.1% Tween 20 is present in buffer and increase if needed.
- Inadequate antibody volume used. Increase antibody volume so that entire surface of the membrane is sufficiently covered with the solution at all times.
- Membranes may have been touched or bent. Only use forceps to touch the membrane after hydration and do not bend the membrane.

Weak or No Signal

- Inadequate quantities of antigen or primary antibody loaded. Increase the amount of antigen or primary antibody.
- Insufficient protein transfer. Optimize transfer conditions. Use pre-stained molecular weight standards to monitor transfer, and stain gel after transfer to make sure proteins are not retained in gel.
- Protein lost from membrane during detection. Extended blocking or high concentrations of detergent in diluted antibodies may result in loss of protein from the blotted membrane.

Non-Specific Bands

- The primary antibody concentration may be too high. Dilute the primary antibody within the indicated range. Reduce antibody incubation times. Increase Tween-20 in diluted antibodies.
- Not using optimal blocking solution. Try a different blocking reagent.
- Cross-reactivity between antibodies. Verify the sources and specificities of the primary and secondary antibodies used (see V. Guidelines for Multiplexing Fluorescent Detection). Use only highly cross-adsorbed secondary antibodies.

X. RELATED PRODUCTS

Component	Catalog #	Size
2x SDS-PAGE Sample Buffer without DTT or β-MeOH	MB-018	100 ml
10x SDS-PAGE Running Gel Buffer	MB-017	1000 ml
Express Transfer Buffer	MB-024-1000	1000 ml
Blocking Buffer for Fluorescent Western Blotting	MB-070	500 ml
10x TBS pH 7.5	MB-012	1000 ml
10x TTBS pH 7.5	MB-013	1000 ml
Goat Anti-Chicken IgG (H&L) Antibody DyLight™800 Conjugated Pre-adsorbed	603-145-126	100µg
Goat Anti-Chicken IgG (H&L) Antibody DyLight™488 Conjugated Pre-adsorbed	603-141-126	100µg
Goat Anti-Chicken IgG (H&L) Antibody DyLight™549 Conjugated Pre-adsorbed	603-142-126	100µg
Goat Anti-Mouse IgG (H&L) Antibody DyLight™649 Conjugated pre-Adsorbed	610-143-121	100µg
Goat Anti-Mouse IgG (H&L) Antibody DyLight™549 Conjugated pre-Adsorbed	610-142-121	100µg
Goat Anti-Mouse IgG (H&L) Antibody DyLight™488 Conjugated pre-Adsorbed	610-141-121	100µg
Goat Anti-Rabbit IgG (H&L) Pre-adsorbed Antibody DyLight™549 conjugated	611-142-002	100µg
Goat Anti-Rabbit IgG (H&L) Antibody DyLight™649 conjugated Pre-adsorbed	611-143-122	100µg
Goat Anti-Rabbit IgG (H&L) Antibody DyLight™800 conjugated Pre-adsorbed	611-145-122	100µg
Opal Pre-stained Protein Standard 3.5-245kDa	MB-211-0500	500µl
Opal Pre-stained Protein Standard 10-245kDa	MB-210-0500	500µl
Opal Pre-stained Protein Standard 10-180kDa	MB-209-0500	500µl
Western Incubation Box	WIB-2875-010	7.5 x 5 cm

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