# Comprehensive Guide to Fluorescent Western Blotting with StarBright and hFAB Rhodamine Antibodies

## Instruction Manual

Catalog numbers

| StarBright Blue 520 Antibodies | StarBright Blue 700 Antibodies | hFAB Rhodamine Antibodies |
|---|---|---|
| 12005866 | 12004158 | 12004163 |
| 12005867 | 12004159 | 12004164 |
| 12005869 | 12004161 | 12004165 |
| 12005870 | 12004162 | 12004166 |
| | | 12004167 |
| | | 12004168 |

BIO-RAD

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# Section 1
# **Introduction**

StarBright Blue Secondary Antibodies are labeled with novel fluorescent nanoparticles that give a signal that has exceptionally bright and stable fluorescence. Their fluorescence emission is restricted to a narrow spectral band, making them ideal for multiplex fluorescence imaging for western blotting (Rong et al. 2013). Secondary antibodies (goat anti-mouse or goat anti-rabbit) conjugated to StarBright labels allow detection and quantitation of specific targets with high specificity and sensitivity. These labeled secondary antibodies are used in western blotting where the target is first challenged with an unlabeled primary antibody. Bound primary antibody is subsequently visualized with a StarBright Blue Secondary Antibody that binds to it.

StarBright Secondary Antibodies are excited with blue light (440–470 nm) and are available in two colors: StarBright Blue 520 emits in the green range of the spectrum (emission maximum: 520 nm) and StarBright Blue 700 maximally emits in the far red/near infrared region of the spectrum (700 nm) (Figure 1). They can be used together with other infrared or visible light emitting dye–conjugated antibodies for multiplex imaging applications (Figure 2).

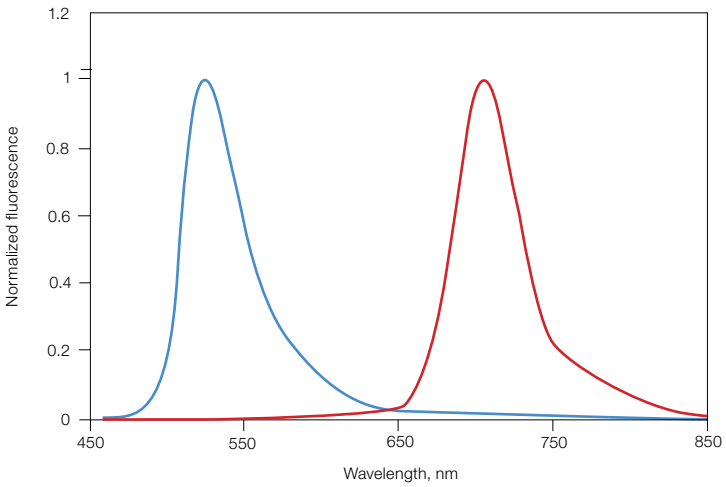


**Fig 1. Fluorescence excitation and emission spectra for the StarBright label.** StarBright Blue 520 (—); StarBright Blue 700 (—).

## What Is Special about the StarBright Label?

- **Brighter and more sensitive** — has a much higher absorbance cross section than conventional fluorescent dyes, which, along with a high quantum yield, contributes to very bright fluorescence. This in turn results in exceptional detection sensitivity
- **Low nonspecific interaction** — particles are surface modified such that there is minimal nonspecific interaction with proteins or blotting membranes. Conjugation with antibodies is through a stable covalent linkage
- **Low background and no interference from autofluorescence** — utilizes intraparticle-amplified energy transfer, which enables a very wide Stokes shift (difference between excitation and emission wavelength). This unique feature brings the fluorescence emission into a range where blotting membrane autofluorescence is minimal. Low background detection is thus possible, further enhancing detection sensitivity. This also permits multiplex imaging with conventional short Stokes shift dyes
- **Highly photostable** — considerably more photostable than conventional dyes, allowing repeated imaging without fading

## Advantages of Using StarBright Blue 520 and 700 Secondary Antibodies

- **Highly sensitive fluorescent detection** — 2- to 4-fold lower limit of detection compared to other traditional fluorophores and comparable to the highly sensitive ECL chemiluminescent substrates
- **Short exposure times** — 50- to 100-fold shorter than other traditional fluorophores (seconds vs. minutes)
- **Easy multiplexing options** — Stain-Free gel and/or hFAB Rhodamine Antibodies and antibodies labeled with other traditional fluorophores emitting in the visible or near infrared region (Figures 2 and 3). Detecting multiple proteins using these antibodies can save valuable sample, effort, and time; no need for cutting, stripping, or reprobing membranes

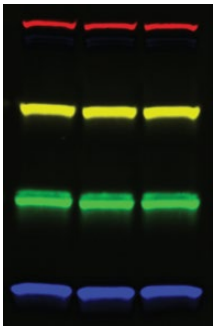

**Fig. 2. Composite blot image for quadruplex western blotting.**

| Color | Primary Antibody | Secondary Antibody |
|---|---|---|
| Red | Rabbit anti-human ATG7 | Goat anti-rabbit IgG StarBright Blue 700 |
| Yellow | — | Anti-tubulin hFAB Rhodamine Antibody |
| Green | Mouse anti-human AKR1C2 | Goat anti-mouse IgG StarBright Blue 520 |
| Blue | Goat anti-human TP1 (biotinylated) | DyLight 800 Streptavidin |

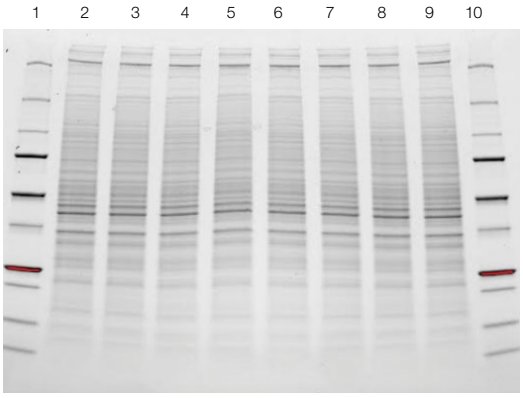


**Fig. 3. Stain-Free gel image.** Lanes 1 and 10: 3 µl Precision Plus Protein All Blue Prestained Protein Standards/well; lanes 2–9: 10 µg HeLa lysate protein/well. Activation time: 45 sec; exposure time: 15 sec.

# Section 2
# **Storage Conditions**

Store the lyophilized product at –20ºC (shelf life: 1 year). Once reconstituted, store the solution at 2–8ºC (shelf life: 6 months after reconstitution). Keep protected from light.

# Section 3
# **Instructions for Use**

### Preparation

Resuspend the lyophilized contents of the tube in the indicated volume of distilled or deionized water and leave on ice for at least 30 minutes prior to use. The resuspended solution may be stored at 4ºC in the dark for up to 6 months. Do not freeze the solubilized material.

Brief centrifugation (pulse spin for 2–3 sec at maximum speed in a tabletop microcentrifuge) may be employed to collect the contents to the bottom of the tube. But do not use prolonged centrifugation (more than 10 sec).

### General Guidelines

StarBright Blue Secondary Antibodies may be used for detection on low fluorescence polyvinylidene fluoride (LF PVDF) or nitrocellulose membranes. LF PVDF is recommended when using any of the hFAB Rhodamine Antibodies for protein loading normalization.

For blocking and washing, use 15 ml for a mini gel. For primary and secondary antibody incubations, use 10 ml for a mini gel. Use a flat-bottomed tray that is as small as possible while still accommodating the blot.

Protect the blot from light (for example, by using aluminum foil) during incubations with fluorescent antibodies. Image immediately after the final wash for best results (see step 5 in Methods).

Do not allow the blot to dry out at any time during antibody incubations or washes.

Shake or rock well (without spilling) during incubations with StarBright Blue Secondary Antibodies.

Dilute StarBright Antibodies into incubation solution immediately prior to use.

StarBright Blue Secondary Antibodies may be used successfully in many different immunodetection protocols. The protocol in Methods is recommended for detection with high sensitivity, low background, and minimal nonspecific cross-reactivity.

# Section 4
# **Methods**

SDS-PAGE gels are run in the conventional manner and transblotted onto LF PVDF or nitrocellulose using equipment and methods of choice (for general western blotting guidelines, see bulletin 2895). Precision Plus Protein All Blue Prestained Protein Standards are recommended (suggested loading volume: 5 µl). We do not recommend standards that include pink or multicolor bands since these can exhibit fluorescence that may interfere with imaging.

The following immunodetection procedure has been found to produce clear, highly sensitive western blots. Alternate protocols could work but would need optimization. All steps are at room temperature (22–25°C) except the overnight primary incubation.

TTBS wash buffer (Tris buffered saline with 0.05% Tween 20) is prepared using 10x Tris buffered saline and 10% Tween 20 stock solutions.

1. **Block:** 1 hr at room temperature with Tris buffered saline (TBS) with 1% casein.
   **Incubate in primary antibody:** Dilute and incubate the primary antibody as specified in your protocol or by the vendor. If the primary antibody is not provided with a protocol, it may be diluted in TBS with 1% casein.
2. **Wash:** 5 x 5 min, room temperature with TTBS.
3. **Incubate with StarBright Blue Secondary Antibody:** Dilute secondary antibody into TBS with 1% casein. The recommended starting dilution is 1:2,500 (dilution range: 1:2,500–1:5,000). Incubate for 1 hr at room temperature.

   **Note:** At this stage, hFAB Rhodamine Antibody (recommended dilution range: 1:1,000–1:10,000) may also be added for detection of housekeeping protein (HKP; actin, tubulin, or GAPDH) for protein loading normalization.
4. **Wash:** 6 x 5 min at room temperature with TTBS.
5. **Image:** Use the ChemiDoc MP Imaging System with Image Lab Touch Software. Choose the **StarBright B520** and/or **StarBright B700** option under **Application** > **Blots** in Image Lab Touch Software, version 2.3 or later.

   **Note:** If hFAB Rhodamine Antibody is being used for protein loading normalization, configure a multichannel imaging protocol using the StarBright B520 and/or StarBright B700 option and the Rhodamine option in Image Lab Touch Software, version 2.3 or later.

   Keep the blot moist until it has been imaged for best results. The blot may be air-dried after soaking in methanol or ethanol for 2–5 min. The dried blot can be archived.

# Section 5
# **hFAB Rhodamine Antibodies**

Bio-Rad hFAB Rhodamine Antibodies are fluorescently labeled primary antibodies raised against the human forms of the HKPs actin, tubulin, and GAPDH (Figure 4). They are intended to be used for normalizing protein loading in western blotting experiments. hFAB Rhodamine Antibodies are themselves sufficient for detection and do not need any secondary antibody for detecting the HKP. For fluorescence multiplexing, these hFAB Rhodamine Antibodies can be incubated along with a variety of fluorescent secondary antibodies, including our StarBright Blue Antibodies. hFAB Rhodamine Antibodies are recombinant human Fab antibody fragments that are not recognized by conventional secondary antibodies, which means they can be used with primary or secondary antibodies of any species.

hFAB Rhodamine Antibodies are designed to give a linear response to HKPs in a range typical for their presence in cellular lysates. They are labeled with a rhodamine derivative that has minimal spectral cross talk with StarBright Blue Secondary Antibodies. The fluorophore is excited around 530 nm (green light) and emits maximally around 580 nm.

hFAB Rhodamine Antibodies may be used successfully with StarBright Blue and other fluorescent secondary antibodies with compatible fluorescence properties in many different immunodetection protocols. The following protocol is recommended for detection with high sensitivity and low background with minimal nonspecific cross-reactivity.

At step 3 in Methods, incubate with hFAB Rhodamine Antibody and StarBright Blue Secondary Antibody. Dilute both reagents in TBS with 1% casein. The recommended starting dilution for hFAB is 1:1,000 (dilution range: 1:1,000–1:10,000).* The recommended dilution for StarBright Blue is 1:2,500 (dilution range: 1:2,500–1:5,000). Incubate for 1 hr at room temperature. These antibodies have been used together successfully for multiplex experiments.

* When using dilutions greater than 1:1,000, quantitation and normalization can be affected.

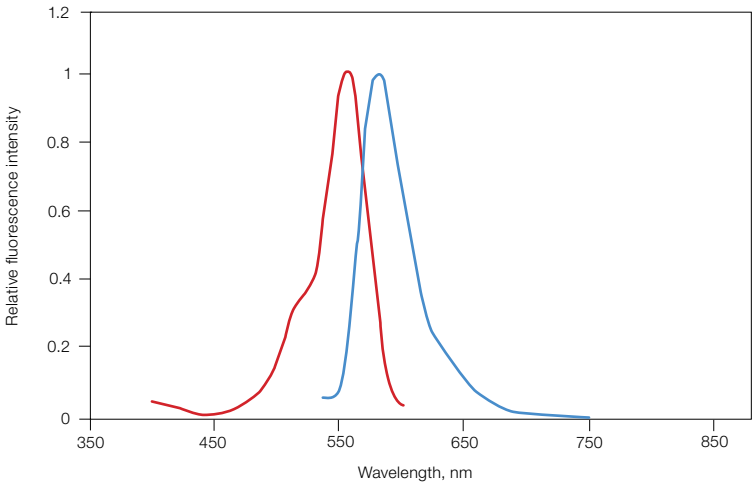


**Fig 4. Fluorescence excitation and emission spectra for the label used to tag hFAB Rhodamine Antibodies.** Excitation (—); emission (—).

# Section 6
# Imaging with the ChemiDoc MP Imaging System and Image Lab Touch Software

## Imaging of StarBright Blue Secondary Antibodies

StarBright Antibodies are imaged using blue light excitation (440–470 nm). StarBright Blue 520 requires a 518–546 nm emission filter and StarBright Blue 700 requires a 720 nm emission filter. This may be enabled in Image Lab Touch Software, version 2.3 and later software releases, by selecting the **StarBright B520** and/or **StarBright B700** option under **Application** > **Blots** (Figure 5). The image exposure may be set up for automatic optimization or the exposure time, or it may be optimized by manually choosing the maximum exposure time that does not result in saturated bands. Optimal exposure time is usually 1–20 seconds or StarBright Antibodies, depending on band intensity. Tap the Camera button to begin the exposure.

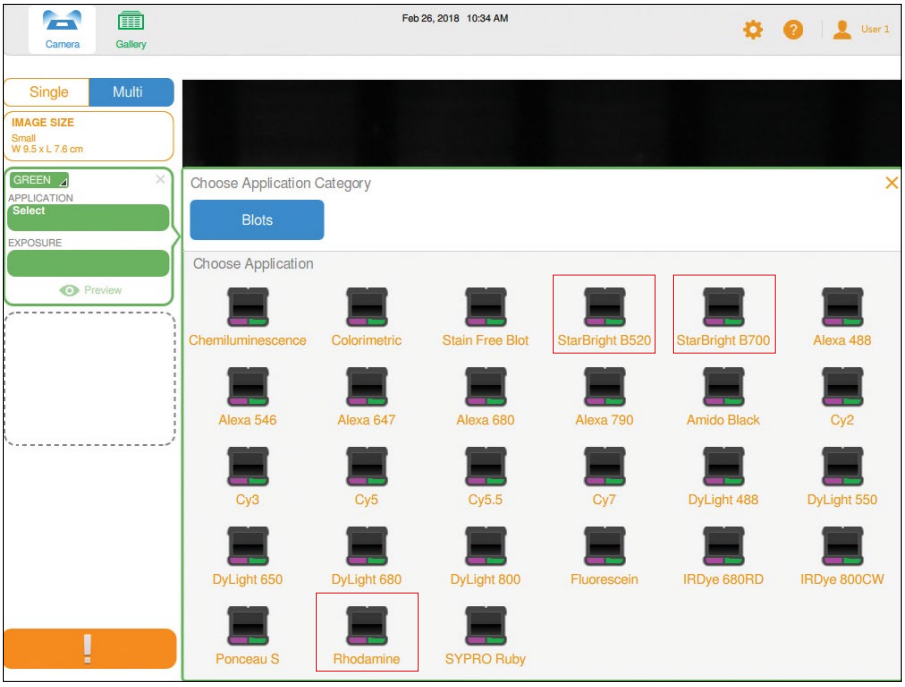


**Fig 5. Application window in Image Lab Touch Software, version 2.3.**

## Imaging of hFAB Rhodamine Antibodies

Select the **Rhodamine** option under **Application** > **Blots** (Figure 5) in Image Lab Touch Software, version 2.0 and later software releases.

# Section 7
# **Tips**

1. We recommend using Precision Plus Protein All Blue Prestained or Precision Plus Protein Unstained Protein Standards because red or pink colored standards may fluoresce brightly in the Rhodamine imaging channel and can interfere with data acquisition.
2. Use either nitrocellulose or LF PVDF with StarBright Blue Secondary Antibodies. Since the StarBright Antibodies use an imaging channel in which nitrocellulose autofluorescence is minimal, either type of membrane may be used for transfer with little difference in detection sensitivity. However, LF PVDF is recommended when using hFAB Rhodamine Antibodies for load normalization because nitrocellulose and conventional PVDF have considerable autofluorescence in this imaging channel.
3. Other basic western blot processing protocols may provide satisfactory results. The TBS with 1% casein blocking reagent described here was selected for its minimal nonspecific cross-reactivity. Other blocking reagents may provide higher sensitivity but at the expense of selectivity. Antibody dilutions might need optimization based on the blocking conditions.
4. The primary antibody incubation step needs to be optimized based on the target abundance and the overall quality of the selected primary antibody. The blot is best imaged while it is still moist. We recommend imaging right after step 4 in Methods for best results. If not imaged shortly after immunodetection, the blot can be stored between sheets of wet filter paper. If not imaged within a few hours, the blot and wet filter paper sheets should be wrapped in plastic and stored in the dark at 4ºC. If the blot is allowed to dry out, it may be imaged dry, but the spectral quality of the StarBright label might change over time, causing spillover into other fluorescence imaging channels and complicating multiplex imaging applications.
5. When using hFAB Rhodamine Antibodies for load normalization, it is important for the HKP to be in a linear range for detection. If too much sample is loaded such that the housekeeping protein band is outside the linear range of quantitation, the normalization is not valid. The anti-HKP antibodies were selected to have a linear response to HKP quantities typically present in 20 µg of sample when detected by western blotting. It is advisable to verify the linear range by loading a dilution series of lysate and plotting a standard curve of band fluorescence vs. µg protein in the sample.
6. Total protein quantified from a Stain-Free gel may be used for load normalization as well. StarBright Blue 700 and hFAB Rhodamine Antibodies are compatible with Stain-Free imaging. StarBright Blue 520 can affect Stain-Free imaging. It is recommended the Stain-Free image of the blot be captured immediately following transfer and before immunoblotting with StarBright Blue 520. StarBright Blue Antibodies are not compatible with stripping and reprobing.
7. StarBright Blue 700 Secondary Antibodies and hFAB Rhodamine Antibodies may be multiplexed and imaged along with other fluorescent labels. It is critical to select antibody conjugates such that there is little cross talk among the different fluorescent labels within individual imaging channels. In general, any fluorescent label selected for multiplexing should have both excitation and emission characteristics that are distinct from the other labels used and spectral overlap should be minimized as much as possible. The rhodamine-derived label used with anti-HKP hFAB Antibodies is excited with green light and detected with a band pass filter centered around 605 nm. It might therefore be compatible with labels that excite with blue, red, or near infrared light and are detected with a band pass filter other than 605 nm. In all cases, the compatibility of labels used in multiplex imaging has to be determined experimentally for optimal performance.

8. For multiplex experiments, we recommend detecting the target protein with the least abundance using the StarBright Blue 700 Secondary Antibody because the label has enhanced brightness compared to other traditional fluorescent dyes.
9. The presence of detergents can contribute to fluorescence background and, therefore, should be used with caution. Do not add Tween 20 to solutions containing StarBright Antibodies.
10. Membranes should be handled only by their edges and only with clean forceps. Take great care to never touch the membrane with bare or gloved hands. Use powder-free gloves.
11. In general, keep everything clean. Prevent background by thoroughly cleaning all equipment and trays prior to use. If reusing staining trays, clean with laboratory detergent or dish soap prior to reuse.
12. In general, TBS blocking reagents are used for detection of phosphoproteins because the phosphate present in PBS blocking reagents may competitively bind with antibodies to phosphoproteins. Milk-based blockers may contain IgG that can cross-react and create background. We do not recommend using milk.
13. At all blocking and antibody incubation steps ensure that the entire surface of the membrane is covered and keep the trays covered during all incubations.
14. Place aluminum foil over the incubation tray during all incubations after the fluorescent antibody is added. This minimizes photobleaching.

# Section 8
# **Troubleshooting Guide**

### High Background on the Blot

1. Washing may be insufficient. Increase the number or duration of wash steps.
2. Antibody concentration may be too high. The concentration of primary or secondary antibodies may have to be further optimized.
3. When using LF PVDF membrane, SDS at a concentration of 0.02% can be used in the StarBright Antibody Dilution Buffer to improve background. SDS is not recommended for use with nitrocellulose membranes as it could reduce specificity.

### Uneven Background on the Blot

1. LF PVDF membrane may have been improperly wetted or may have dried during handling. Ensure that the membrane is fully wetted in methanol prior to equilibration in aqueous solution and ensure that the membrane stays wet.
2. Wash steps may have occurred in insufficient volume. Ensure that incubations occur with enough volume to completely cover the membrane and wash with vigorous agitation.
3. If the membrane is not completely immersed during antibody incubation steps (primary or secondary or both), then uneven background can occur. The volume of antibody incubation solution can be increased by 1.5- to 2-fold to ensure the membrane is always fully immersed.

# Section 9
# **Ordering Information**

**StarBright Blue 520 Secondary Antibodies**

| | |
|---|---|
| 12005866 | **Goat Anti-Mouse IgG StarBright Blue 520**, 400 µl |
| 12005867 | **Goat Anti-Mouse IgG StarBright Blue 520**, 80 µl |
| 12005869 | **Goat Anti-Rabbit IgG StarBright Blue 520**, 400 µl |
| 12005870 | **Goat Anti-Rabbit IgG StarBright Blue 520**, 80 µl |

**StarBright Blue 700 Secondary Antibodies**

| | |
|---|---|
| 12004158 | **Goat Anti-Mouse IgG StarBright Blue 700**, 400 µl |
| 12004159 | **Goat Anti-Mouse IgG StarBright Blue 700**, 80 µl |
| 12004161 | **Goat Anti-Rabbit IgG StarBright Blue 700**, 400 µl |
| 12004162 | **Goat Anti-Rabbit IgG StarBright Blue 700**, 80 µl |

**hFAB Rhodamine Antibodies**

| | |
|---|---|
| 12004163 | **Anti-Actin hFAB Rhodamine Antibody**, 200 µl |
| 12004164 | **Anti-Actin hFAB Rhodamine Antibody**, 40 µl |
| 12004165 | **Anti-Tubulin hFAB Rhodamine Antibody**, 200 µl |
| 12004166 | **Anti-Tubulin hFAB Rhodamine Antibody**, 40 µl |
| 12004167 | **Anti-GAPDH hFAB Rhodamine Antibody**, 200 µl |
| 12004168 | **Anti-GAPDH hFAB Rhodamine Antibody**, 40 µl |

**Related Products**

| | |
|---|---|
| 12010020 | **EveryBlot Blocking Buffer**, 500 ml |
| 1706435 | **10x Tris Buffered Saline (TBS)**, 1 L |
| 1610781 | **10% Tween 20**, 1 L |
| 1610373 | **Precision Plus Protein All Blue Prestained Protein Standards** |

# Section 10
# **Reference**

Rong Y et al. (2013). Multicolor fluorescent semiconducting polymer dots with narrow emissions and high brightness. ACS Nano 7, 376–384.

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

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***Portugal*** *00 800 00 24 67 23* ***Russian Federation*** *00 800 00 24 67 23* ***Singapore*** *65 6415 3188* ***South Africa*** *00 800 00 24 67 23*
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