

# Chemiluminescent Western Blot Kit: for RFP

## Chemiluminescent Kit for Western Blotting

### for recombinant proteins containing Red Fluorescent Protein (RFP)

Catalog # KCA379

#### I. Overview

Rockland Immunochemicals' Chemiluminescent Western Blot Kit for RFP combines all of the necessary reagents with a rapid proven protocol and the extremely high signal detection of our luminol chemiluminescent substrate for the detection of recombinant proteins containing wild type Red Fluorescent Protein (DsRed or RFP) and its variants such as mBanana, mCherry, DsRed2, E2-Crimson, mOrange, mOrange2, mPlum, mRaspberry, mStrawberry and tdTomato. The Chemiluminescent Western Blot Kit design includes straightforward procedures and color-coding to allow for ease of use. This kit contains sufficient substrate for up to 30 mini blots at 7.5 x 8 cm<sup>2</sup> (1,800 cm<sup>2</sup>) and is stable for at least 1 year when stored as indicated.

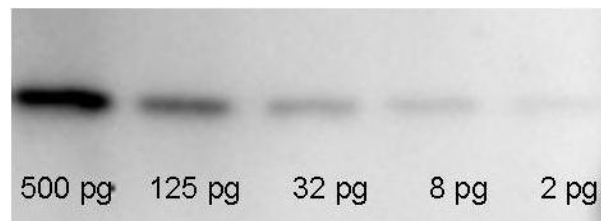
**Please read the entire product insert prior to use.**

#### II. Kit Principle

This Chemiluminescent Western Blot Kit allows for the detection of RFP-tagged recombinant proteins present in cell lysates provided by the user. After protein separation by SDS-PAGE and transfer, the membrane is probed with polyclonal **Anti-RFP**. Detection of the membrane bound antibody-antigen complex is achieved by the addition of a secondary antibody conjugated to the enzyme horseradish peroxidase. The enzyme reacts with a specialized formulation of luminol, an extremely sensitive, non-radioactive substrate that emits light and allows visualization using X-ray film or other imaging methods, including highly sensitive CCD cameras and imaging systems.

#### III. Intended Use

Use Rockland Immunochemicals' **Anti-RFP Chemiluminescent Kit for Western Blotting** for detection of RFP-tagged recombinant proteins by western blot. This kit is useful for both "western blotting" and "dot blotting" methods. If you require additional assistance please call or e-mail technical service at 800-656-7625 or [tech@rockland-inc.com](mailto:tech@rockland-inc.com).



**Western Blot:** Known amounts of recombinant RFP protein (p/n 000-001-379) were spiked into a HeLa cell derived lysates (p/n W09-000-364) and separated by SDS-PAGE using a 4-20% gradient gel under reducing conditions. Proteins were transferred to a nitrocellulose membrane for 1 h at 100 mV. The membrane was blocked for 1 hr at 4° C in 3% BSA (p/n BSA-50) in TBS buffer (p/n MB-012) and probed overnight at 4° C with Anti RFP antibody (p/n 600-401-379) diluted 1:2,000 in Blocking Buffer (p/n MB-070). Primary antibody was detected using secondary antibody (p/n 611-1322) for 1 h at room temperature. **FemtoMax™** Super Sensitive Chemiluminescent Luminol Substrate was used for signal detection (see below).

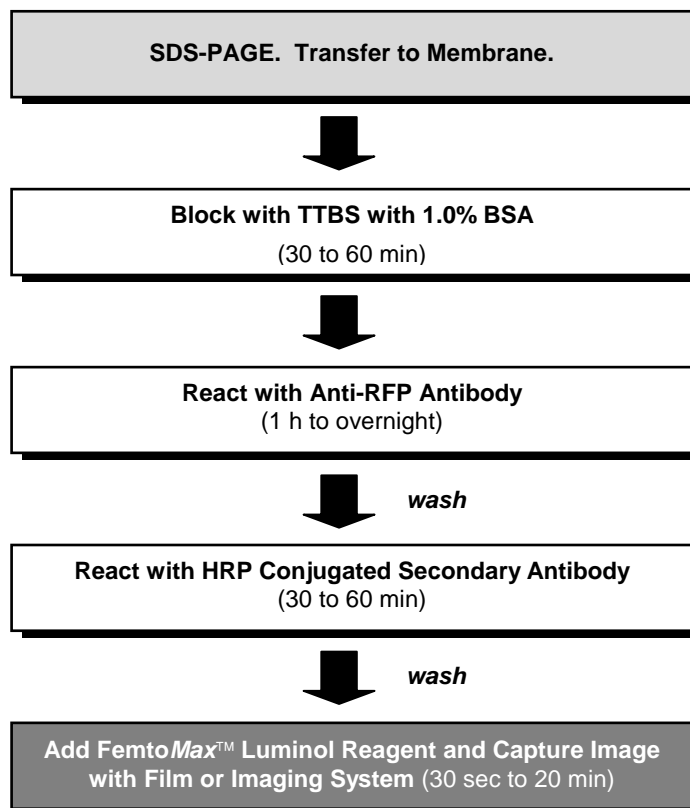
#### IV. Storage and Stability

This kit is stable for at least one year when stored as indicated upon receipt. Individual components are stable for 3-4 weeks after dilution when stored at +4°C. The **FemtoMax™** luminol chemiluminescent reagent is stable for up to 8 h at room temperature after mixing with buffer.

#### V. Number of Assays

Components in this kit are sufficient to run approximately 30 mini blots at 7.5 x 8 cm<sup>2</sup> (1,800 cm<sup>2</sup>). The amount of peroxidase conjugated secondary antibody supplied when diluted as recommended in our protocol will yield in excess of 200 mL of working solution. Adjustments in volumes for larger or smaller blots will affect the number of blots detected.

## VI. Flow Diagram for Chemiluminescent Western Blot Procedure



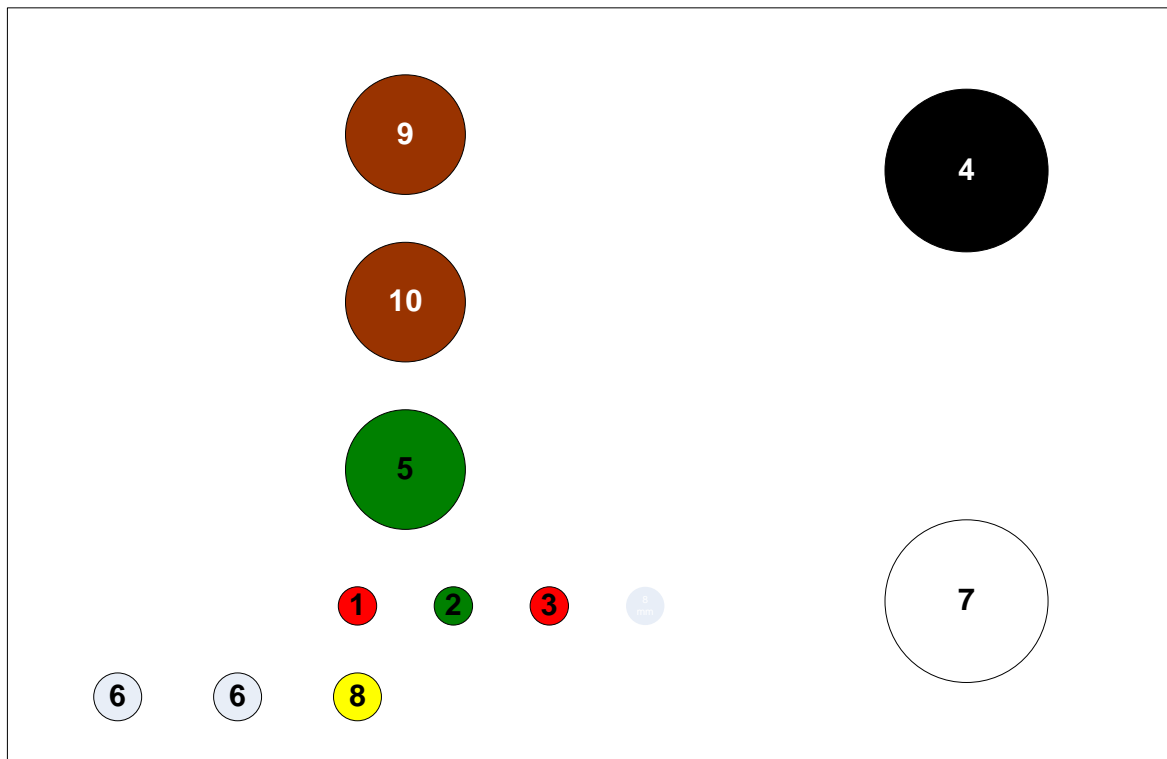
## VII. Materials Required but Not Supplied

Nearly all components required for the detection of RFP-tagged recombinant protein by western blot are provided for your convenience in Rockland Immunochemicals' Chemiluminescent Western Blot Kit for RFP. Some additional materials not included are required:

- ◆ SDS-PAGE electrophoresis equipment and related materials
- ◆ Nitrocellulose, PVDF (polyvinylidene difluoride) or other membranes for protein transfer and transfer materials
- ◆ Microfuge tubes
- ◆ Rocker platform for gentle mixing during incubations
- ◆ X-ray film, cassettes and related materials or camera based imaging system
- ◆ Deionized water and molecular biology grade Tris base and sodium chloride

### VIII. Kit Components and Layout

1. (1) x 100  $\mu$ L **anti-RFP rabbit polyclonal antibody** in clear plastic 0.5 mL vial with **RED** cap. Store at  $-20^{\circ}$  C.
2. (1) x 100  $\mu$ g Peroxidase anti-Rabbit IgG antibody in amber plastic 0.5 mL vial with **GREEN** cap. Store at  $+4^{\circ}$  C.
3. (1) x 100  $\mu$ g of control recombinant RFP protein in clear plastic 0.5 mL vial with **RED** cap. Store at  $-20^{\circ}$  C.
4. (1) x 50 mL Ultra Pure Tween-20 in an amber glass bottle with **BLACK** cap. Store at room temperature.
5. (1) empty dropper bottle with **GREEN** cap labeled "Diluted Secondary antibody".
6. (2) empty microfuge tubes for intermediate dilution of secondary antibody.
7. (1) x 10 g Bovine Serum Albumin (BSA) in a plastic bottle with a **WHITE** cap. Store at  $+4^{\circ}$  C.
8. (1) x 0.5 mL Control Rabbit IgG in plastic vial with **YELLOW** cap. Store at  $+4^{\circ}$  C.
9. (1) x 10 mL **FemtoMax** Reagent A - Luminol - in an amber plastic bottle with **BROWN** cap. Store at  $+4^{\circ}$  C.
10. (1) x 10 mL **FemtoMax** Reagent B - Reaction Buffer - in an amber plastic bottle with **BROWN** cap. Store at  $+4^{\circ}$  C.
11. Incubation box with lid.
12. Instruction Manual.



## IX. Solutions Required but Not Supplied

The user is to prepare the following buffers for this procedure. The exact volume of buffers required depends on the size of the membranes to be processed. We suggest preparation of 1.0 L of Tris Buffered Saline with Tween (TTBS) and 100 ml of TTBS with BSA. Prepare all solutions using ultra pure reagents and deionized (or equivalent) water. Filter the solutions and store at +4° C. Warm solutions to room temperature prior to use. Do not store solutions for more than one (1) month.

**!** **Note** - wash buffers **MUST NOT** contain **SODIUM AZIDE** or other inhibitors of peroxidase activity.

### **Buffer I Tris Buffered Saline with Tween-20 (TTBS)**

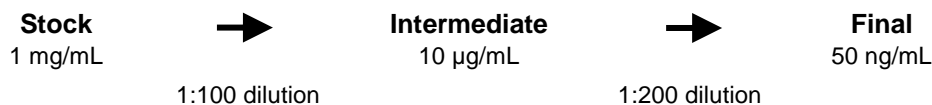
Add 800 mL of deionized water.  
Dissolve 12.1 g of Tris base.  
Dissolve 8.8 g Sodium Chloride (NaCl).  
Adjust pH to 7.5 with HCl.  
Add 1.0 mL of Tween-20 (provided).  
Adjust volume to 1.0 L with deionized water.

### **Buffer II TTBS with 1.0% (w/v) BSA**

Add 100 mL of TTBS.  
Dissolve 1.0 g of BSA (provided).  
Use solution immediately.

## X. Preparation of Working Solutions

The Chemiluminescent Western Blot Kit for RFP -tagged recombinant protein comes with a concentrated stock of **anti-RFP rabbit polyclonal antibody** (primary) and peroxidase conjugated anti-Rabbit IgG antibody (secondary). Prior to use, dilute the primary antibody to a final concentration of 1µg/mL. **Reconstitute and dilute the secondary antibody in a two-step process to a working solution.** The working solution is ready for use. Reconstitute the peroxidase conjugated anti-Rabbit IgG antibody by adding 100 µL of deionized water to the **GREEN** capped amber vial. Mix thoroughly and maintain this stock at 4 °C. Prepare an intermediate dilution (1:100) by pipetting 5 µL of the stock solution to 0.5 mL of Buffer I (TTBS) in a clean microfuge tube. Prepare a second dilution (1:200) by pipetting 50 µL of the intermediate solution to the **GREEN** capped dropper bottle labeled "Diluted Secondary Antibody." Add 10 mL of Buffer I (TTBS). Mix thoroughly. The final dilution will contain 50 ng/mL peroxidase conjugated goat anti-Rabbit IgG antibody and will represent a 1:20,000 dilution of the stock solution.



- **FemtoMax™ Luminol Substrate Reagent.** Just prior to use, prepare **FemtoMax™** Super Sensitive Chemiluminescent Substrate by mixing 1 mL of the Luminol chemiluminescent reagent (Reagent A) with 1 mL of the Reaction Buffer (Reagent B). Mix well. Protect from intense light. Keep working solution in an amber bottle. Normal laboratory light will not harm the working solution. Larger or smaller volumes of the substrate can be prepared by mixing components at the same 1:1 ratio.

## XI. Western Blot Method

The following method is suggested as a **guideline** for the use of Rockland Immunochemicals' Chemiluminescent Western Blot Kit for RFP-tagged recombinant protein. Each researcher must optimize Western blotting conditions for their protein of interest. Membranes composed of nitrocellulose or PVDF can be used. Nylon membranes may also be used (see "Additional Notes"). After your antigen has been immobilized onto the membrane by transfer, dotting or filtration, follow the numbered steps below to process the blot. All reactions occur at room temperature. Use a rocking platform set at low speed for gentle agitation. Always add enough solution to cover the membrane. Never let a membrane air dry during this process. Add the suggested volumes or just enough volume to cover the membrane to keep it wet. Do not touch the membrane with your skin!

**!** Note - some antigens in combination with the **anti-RFP polyclonal antibody** may require specific conditions other than those stated below. If so, use these recommendations as a starting point for further optimization.

1. After transfer is complete, block the membrane by immersing in *Buffer II* (TTBS with BSA) and incubate at room temperature for 1 h with gentle agitation. The addition of 1.0% BSA (provided) as a blocking agent increases the signal-to-noise ratio when compared to using TTBS alone. Other blocking agents may be used (see "Additional Notes").
2. Aspirate or decant the blocking solution<sup>1</sup>. Immediately add 5 ml of **anti-RFP polyclonal antibody** (primary) solution diluted in *Buffer I* (TTBS) to the membrane. The appropriate dilution should be determined by the end user. We would recommend a starting dilution of 1:1,000. Greater dilutions often result in lower backgrounds but may require longer incubation times. Incubate for 1 h at room temperature with gentle agitation. If desired, the membrane can be incubated with **anti-RFP polyclonal antibody** (primary) overnight at 4° C.
3. Aspirate or decant the **anti-RFP polyclonal antibody** (primary) solution. Wash the blot with 3 changes of *Buffer I* for 5 min each with gentle agitation. Increasing the wash buffer volume or the number of washes may decrease background.
4. Aspirate or decant the wash solution and add 5 ml of the "Diluted Secondary Antibody" solution from the **GREEN** capped dropper bottle (see above for preparation). Incubate for 1 h at room temperature with gentle agitation.
5. Aspirate or decant secondary antibody solution. Wash the blot as in Step 3.
6. Prepare **FemtoMax**<sup>™</sup> chemiluminescent reagent as described above just prior to use. Transfer blot to incubation box or film cassette then add 0.75 to 1.0 mL of freshly prepared **FemtoMax**<sup>™</sup> reagent to the membrane (7.5 x 8.0 cm).
7. Immediately visualize the membrane by exposing X-ray film or by other imaging methods, including CCD camera based imaging systems. For film, expose and process the film according to the manufacturer's instructions. Cover the blot with clear plastic wrap or equivalent and remove any excess liquid and any air bubbles to reduce imaging artifacts. Start with a 60 sec exposure. Exposure times may be varied for best results. For imaging systems, follow the manufacturer's instructions and vary exposure times and/or binning for best results. The length of time required to achieve optimum signal varies greatly depending on several factors. Incubation times can range from 30 sec to 20 min or more.

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<sup>1</sup> Aspirate using a glass pipette attached to a vacuum. Alternatively, the solution may be poured off away from the blot.

## XII. Additional Notes

- The methods given in these instructions are to be used as a guideline. Experienced users can make deviations from the stated method. Solutions have been optimized for the stated method and any change in reagent concentration, volume, reaction time or temperature will affect the overall performance of the kit. Generally, if a variable is to be modified, only alter one condition at a time.
- Nylon membrane is more difficult to block and may result in higher levels of non-specific staining. Using 10% BLOTTO (non-fat dry milk) (p/n [B501-0500](#)) is suggested to block nylon membranes. Allow the blocking step to proceed for several hours to overnight at 37° C. Do not use Tween-20 when using nylon membranes.
- The blocking of membranes for western blotting can be accomplished with TTBS only (p/n [MB-013](#)). We suggest adding BSA (p/n [BSA-50](#)) to lower non-specific staining. Users may omit BSA from the blocking step or use some other blocking agent, such as 5% normal goat serum, 3% fish gelatin, or other commercially available blocking agent (see p/n [MB-070](#)), depending on previous experience.
- Always use enough solution to cover the membrane. Never let the membrane dry during the process.
- Protect the **FemtoMax**<sup>™</sup> chemiluminescent reagent from light. Precise optimization is required to achieve maximum signal detection including optimizing the membrane, blocking conditions, antigen and antibodies. Detection by **FemtoMax**<sup>™</sup> chemiluminescent reagent requires much less sample and antibody than most commercially available ECL substrates.
- Western blots can be repeatedly exposed to X-ray film to obtain optimal results or stripped of detection reagents and re-probed.
- Use the same blotting conditions for **FemtoMax**<sup>™</sup> chemiluminescent reagent as you would for Amersham ECL Plus<sup>™</sup> Substrate or Pierce SuperSignal<sup>®</sup> West Femto Substrate.
- Use care not to touch the membrane with your skin! Wear gloves. Make certain that all equipment used in the process is free of foreign material.
- All reactions occur at room temperature.
- Use a rocking platform set at low speed for gentle agitation for all incubation steps.
- Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used to dilute the secondary antibody, substrate or any other **FemtoMax**<sup>™</sup> chemiluminescent reagent.
- A positive control Rabbit IgG is provided in a YELLOW capped vial for use as a secondary antibody control. Spot 1 or 2 µL as a control on your Western or dot blot prior to the blocking step.
- Store the components of this kit as specified.
- Individual components of this kit may be ordered separately (see "Replacement Parts List").

### XIII. Troubleshooting Guide

#### Little or no signal

*Incomplete transfer of proteins.* Follow all protocols included with your transfer apparatus. Check for the presence of transferred proteins using India ink stain as described in Reference 1.

*Poor binding of anti-RFP polyclonal antibody (primary).* Use provided control RFP recombinant protein in serial dilution as a control. Decrease the dilution (increase the concentration) **anti-RFP polyclonal antibody**. Increase the incubation time of anti-RFP antibody from 30 minutes to several hours or overnight. Increase the incubation temperature to 37° C.

*Poor binding of peroxidase conjugated anti-IgG.* Include 1 or 2 µL of Rabbit IgG as a control in your western blot or dot blot to ensure that the **FemtoMax™** kit components are performing as described.

*Inactive Peroxidase Conjugate.* Be certain that all buffers are free of sodium azide, which is a strong inhibitor of peroxidase activity.

#### Multiple signals

*Too much protein on the blot.* Verify the concentration of your protein sample, using Bradford or BCA reagent. For best results, load approximately 10 µg of total protein (lysate) per lane.

*Too high concentration of anti-RFP polyclonal antibody.* Increase the dilution of anti-RFP antibody solution.

*Overexposure of signal.* Decrease exposure time of film or decrease settings on camera system to decrease the signal from minor bands.

#### High background / Poor signal-to-noise ratio

*Insufficient blocking.* Be certain blocking buffer has been properly prepared. In most cases, the addition of 1.0% BSA will decrease background over the use of TTBS alone. In some cases, increased concentrations of BSA (up to 5%) are necessary.

*Insufficient Washing.* Increase the number of wash steps and the volume of TTBS used for each wash.

### XIV. References

*Antibodies, A Laboratory Manual.* Ed Harlow and David Lane, eds. Cold Spring Harbor Press. 1988. Chapter 12 gives an excellent overview of Western Blotting techniques, including India Ink staining.

*Current protocols in Molecular Biology.* J. Ausebel, et al, eds. John Wiley and Sons, New York. Gives a complete protocol of Western Blotting and Dot Blotting.

*Molecular Cloning: A Laboratory Manual.* 2<sup>nd</sup> Edition. J. Sambrook, E.F. Fritsch and T. Maniatis, eds. Cold Spring Harbor Press, 1989. Chapter 18 gives detailed protocols for both the production of cell lysates and electrophoresis and blotting of proteins.

*Antibodies, A Practical Approach.* 2<sup>nd</sup> Edition. Catty, D., ed. IRL Press, Oxford, England. 1990. Volumes I and II represent a detailed and complete reference for most current antibody techniques.

### XV. Trademarks

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## XVI. Disclaimer

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## XVII. Additional Products and Services

### Additional Chemiluminescent Western Blotting Kits

Product	Code	Size	Price
<b>FemtoMax™</b> Chemiluminescent Western Blot Kit for use with Human Primary Antibody	KCA001	1 each	<a href="#">inquire</a>
<b>FemtoMax™</b> Chemiluminescent Western Blot Kit for use with Mouse Primary Antibody	KCA002	1 each	<a href="#">inquire</a>
<b>FemtoMax™</b> Chemiluminescent Western Blot Kit for use with Rabbit Primary Antibody	KCA003	1 each	<a href="#">inquire</a>
<b>FemtoMax™</b> Chemiluminescent Western Blot Kit for use with Goat Primary Antibody	KCA004	1 each	<a href="#">inquire</a>
<b>FemtoMax™</b> Chemiluminescent Western Blot Kit for DYKDDDDK (FLAG®) proteins	KCA383	1 each	<a href="#">inquire</a>
<b>FemtoMax™</b> Chemiluminescent Western Blot Kit for GFP recombinant proteins	KCA215	1 each	<a href="#">inquire</a>

### Replacement Parts for RFP Chemiluminescent Kit for Western Blotting

Anti-RFP polyclonal antibody	600-401-379	100 µg	<a href="#">inquire</a>	BSA, Protease and IgG Free	BSA-10	10 g	<a href="#">inquire</a>
Anti-RFP polyclonal antibody	600-901-379	100 µg	<a href="#">inquire</a>	Ultra Pure Tween-20	TW0020	50 mL	<a href="#">inquire</a>
Peroxidase Anti-Rabbit IgG	611-1322	2.0 mg	<a href="#">inquire</a>	10X TTBS pH 7.5	MB-013	1.0 L	<a href="#">inquire</a>
RFP Control Protein	000-001-379	100 µg	<a href="#">inquire</a>	BLOTTO Immunoanalytical Grade (Non-Fat Dry Milk)	B501-0500	500 g	<a href="#">inquire</a>
<b>FemtoMax™</b> Super Sensitive Chemiluminescent Substrate	FEMTOMAX-110	110 mL	<a href="#">inquire</a>	Western Incubation Box SMALL	WIB-2875-010	10 PACK	<a href="#">inquire</a>
Control Rabbit IgG	KCC003	0.5 mL	<a href="#">inquire</a>	Western Incubation Box LARGE	WIB-4625-005	5 PACK	<a href="#">inquire</a>

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