

FluoReporter[®] Rhodamine Red[™]-X Protein Labeling Kit (F-6161)

Quick Facts

Storage upon receipt:

- 4°C
- Do not freeze the spin columns
- Protect the reactive dye from light

Abs/Em: 570/590 nm

Storage and Handling

Upon receipt, store the kit at 4°C. DO NOT FREEZE THE SPIN COLUMNS. Protect the reactive dye from light. The reactive dye and DMSO should be protected from moisture. When stored properly, the kit components should be stable for at least six months.

Materials Required but Not Provided

- **1 M Sodium bicarbonate buffer, pH 8.3** — Dissolve 0.84 g of NaHCO₃ in 10 mL deionized water; the pH should be ~8.3. This solution should be stored at 4°C and used within one week.

Introduction

Molecular Probes' FluoReporter[®] Protein Labeling Kits provide a convenient means for fluorescently labeling proteins — particularly monoclonal and polyclonal antibodies. In addition, the versatile FluoReporter Kits can be used with a wide range of protein concentrations and with virtually any protein larger than 30 kDa. The Rhodamine Red[™]-X dye provided in this kit has a reactive succinimidyl ester moiety, which reacts efficiently with the primary amines of proteins to form stable dye–protein conjugates. The reactive dye also has a seven-atom aminohexanoyl spacer between the fluorophore and the reactive group. This spacer separates the fluorophore from the biomolecule to which it is conjugated and is designed to reduce the quenching that typically occurs upon conjugation. The absorption and fluorescence emission maxima of Rhodamine Red-X dye–labeled proteins are approximately 570 nm and 590 nm, respectively.

Each kit contains enough reactive dye to perform 5–10 labeling reactions using 0.2–2 mg of protein per reaction. In addition to the reactive dye, dye solvent and reaction tubes with stir bars, each kit also contains ready-to-use spin columns, which provide a convenient and efficient method for purifying the fluorophore–protein conjugate from unreacted dye with minimal dilution of the conjugate.

Materials

Kit Components

- **Reactive Rhodamine Red-X dye** (Component A), five vials of 500 µg each
- **Dimethylsulfoxide (DMSO), anhydrous** (Component B), 0.7 mL
- **Reaction tubes** (Component C), ten 2 mL tubes with stir bars
- **Spin columns** (Component D), ten columns containing 30,000 MW size exclusion resin in phosphate-buffered saline (PBS) with 2 mM sodium azide
- **Collection tubes** (Component E), ten 2 mL tubes

Protein Preparation

A 200 µL volume of protein solution will be used in each labeling reaction (note **A**). Purified protein (note **B**) should be at a concentration of 1–10 mg/mL in buffer. The buffer should not contain any ammonium ions or primary amines. The presence of low concentrations of sodium azide (≤3 mM) or thimerosal (≤1 mM) will not significantly affect the conjugation reaction. If the protein is in an unsuitable buffer (e.g. Tris or glycine), the buffer must be replaced by either dialysis against PBS or by using one of the provided spin columns (see *Purification*).

Calculations

The following calculation should be performed *before* beginning the conjugation reaction outlined below.

The amount of reactive dye to be used for each reaction depends upon the concentration of protein to be labeled. In the labeling procedure, a small volume of 5 mg/mL reactive dye stock solution (prepared in step 1.3) is added to 200 µL of protein solution. The volume of this 5 mg/mL dye stock solution to be added (in step 1.4) can be calculated as follows (note **C**):

µL dye stock solution =

$$\frac{\text{mg/mL protein} \times 0.2 \text{ mL}}{\text{MW}_{\text{protein}}} \times 771 \times 200 \times \text{MR}$$

- 0.2 mL is the volume of protein solution
- 771 is the MW of the reactive dye
- 200 is a unit conversion factor
- For most IgGs, MW_{protein} = 145,000
- MR is the molar ratio of dye to protein in the reaction mixture. For IgG labeling reactions using antibodies at 1–10 mg/mL, we recommend MR = 5 (8), where the number

in parentheses is a second MR that can be used if you choose to perform two reactions at different MRs to better ensure optimal labeling. The amount of reactive dye in each vial is sufficient for at least two conjugations, and we have included ten spin columns to allow purification of two different protein conjugates per vial of reactive dye.

Conjugation Reaction

1.1 Transfer 200 μL of protein solution to a reaction tube. (Note, the reaction tubes are the tubes with stir bars.)

1.2 Add 20 μL of 1 M bicarbonate solution to the protein-containing reaction tube.

1.3 Prepare the 5 mg/mL reactive dye stock solution: Allow DMSO (Component B) and one vial of reactive Rhodamine Red-X dye (Component A) to warm to room temperature. *Immediately before starting the reaction*, add 100 μL of DMSO to the vial of reactive dye. Pipet up and down to completely dissolve the contents of the vial.

1.4 While stirring the protein solution in the reaction tube, add the appropriate amount of dye (calculated in *Calculations*). The remaining reactive dye stock solution should be discarded.

1.5 Stir the reaction at room temperature for approximately 1 hour, protected from light (note **D**).

Purification

2.1 Prepare spin column (notes **E**, **F**): Remove the top cap, then remove the bottom closure. Allow the column buffer to drain from the column by gravity. Place spin column in a 2 mL collection tube. (Note, the collection tubes are the tubes without stir bars.) Centrifuge the column for 3 minutes at $1100 \times g$ using a swinging-bucket rotor (note **G**). Discard the buffer from the collection tube, but save the collection tube for step 2.4.

2.2 Carefully inspect the labeling reaction. If any precipitate has formed in the reaction, centrifuge the samples for 5 minutes in a microfuge to remove the particulate matter.

2.3 Load sample, or supernatant if centrifugation was necessary, dropwise to the center of the spin column. Allow the solution to absorb into the gel bed.

2.4 Place the spin column into the empty collection tube and centrifuge for 5 minutes at $1100 \times g$.

2.5 After centrifugation, the collection tube will contain labeled protein in approximately 200–250 μL of PBS with 2 mM sodium azide. Discard the spin column.

Determination of Degree of Labeling

3.1 Dilute the purified conjugate into PBS or other suitable buffer and measure the absorbance in a cuvette with a 1 cm pathlength at both 280 nm (A_{280}) and 570 nm (A_{570}).

3.2 Calculate the protein concentration (note **H**):

$$\text{protein concentration (M)} = \frac{[A_{280} - (A_{570} \times 0.17)] \times \text{dilution factor}}{\epsilon}$$

where 0.17 is a correction factor (note **I**) and ϵ is the molar extinction coefficient of the protein at 280 nm. For most IgGs, $\epsilon = 203,000 \text{ cm}^{-1}\text{M}^{-1}$.

3.3 Calculate the degree of labeling:

$$\text{dye per protein molecule} = \frac{A_{570} \times \text{dilution factor}}{120,000 \times \text{protein concentration (M)}}$$

where $120,000 \text{ cm}^{-1}\text{M}^{-1}$ is the molar extinction coefficient of the dye at 570 nm.

Storage of Conjugates

Store the labeled protein — which will be in PBS with 2 mM sodium azide if the provided spin columns were used — at 4°C , protected from light. If the final concentration of purified protein conjugate is less than 1 mg/mL (see step 3.2), add bovine serum albumin (BSA) or other stabilizing protein at 1–10 mg/mL. In the presence of 2 mM sodium azide, the conjugate should be stable at 4°C for several months. For longer storage, divide the conjugate into small aliquots and freeze at -20°C . **AVOID REPEATED FREEZING AND THAWING. PROTECT FROM LIGHT.**

Notes

[A] The reaction can be scaled to accommodate other volumes of protein, although the molar ratios of dye to protein recommended in *Calculations* may not result in optimal labeling and the spin columns used in *Purification* are designed for a maximum reaction volume of 250 μL .

[B] Impure proteins (e.g. antibodies in crude serum) will not label well.

[C] This procedure is designed primarily for antibody labeling. Because antibodies (and other proteins) react with fluorophores at different rates and retain biological activity at different degrees of dye labeling, the molar ratios recommended here may not always result in optimal labeling. For most purposes, antibody conjugates made using this procedure will be acceptable. To determine the optimal degree of labeling for your protein, we recommend performing test reactions at various MRs, using the values given in this procedure as a starting point. Generally, in the case of antibodies, reactions that result in 2–4 Rhodamine Red-X dye molecules per antibody molecule give the best results.

[D] Hydroxylamine hydrochloride was previously included as a component of this protein labeling kit. The purpose of this component was to terminate the reaction between the reactive dye and the protein by providing an alternate, low-molecular weight substrate that could easily be separated from the derivatized protein.

Scientists at Molecular Probes have determined that the purification procedure is more than sufficient for terminating the reaction. By simply separating the reactive dye from the protein, the reaction is indirectly, yet efficiently terminated. However, the reaction can be stopped by adding 5 µL of freshly prepared 1.5 M hydroxylamine, pH 8.5, to the conjugation reaction. Stir this reaction at room temperature for 30 minutes.

To prepare this reagent, dissolve hydroxylamine hydrochloride at 210 mg/mL in distilled water and adjust the pH to 8.5 with 5 M NaOH. Dilute the resulting solution with an equal volume of distilled water. This reagent should be freshly prepared before use. Any remaining hydroxylamine should be discarded.

[E] If the molecular weight of the protein is less than 30,000, then the provided spin columns should not be used. Free dye can be removed from the conjugate by extensive dialysis.

[F] If the volume of the reaction exceeds 250 µL, a single spin column will not adequately separate the conjugate from the free

dye. The reaction can be divided and applied to multiple spin columns, if desired.

[G] A fixed angle rotor will suffice if a swinging-bucket rotor is not available.

[H] Alternatively, if no precipitation occurred in the reaction, the protein concentration can be estimated by:

$$\text{conc. of labeled protein (M)} = \frac{\text{mg initial protein} \times 0.85}{\text{mL in collection tube} \times \text{MW}_{\text{protein}}}$$

where 0.85 is the estimated yield (usually 80–90%) and, for most IgGs, $\text{MW}_{\text{protein}} = 145,000$.

[I] The correction factor (CF) is included to compensate for absorption of the dye at 280 nm.

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
F-6161	FluoReporter® Rhodamine Red™-X Protein Labeling Kit *5-10 labelings*	1 kit

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

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