FluoReporter® FITC Protein Labeling Kit

Catalog no. F6434

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Reactive FITC dye (Component A)	5 vials, each containing 370 µg	• 4°C • Protect from light • Dessicate	When stored as directed, the product is stable for at least 6 months.
Dimethylsulfoxide (DMSO), anhydrous (Component B)	0.7 mL	≤25°C	
Reaction tubes (Component C)	10 columns		
Spin columns (Component D)	10 columns		
Collection tubes, 2 mL capacity (Component E)	10 tubes		
Purification resin (Component F)	2 × 10 mL	• 4°C • Do not freeze	
Approximate fluorescence excitation/emission maxima: FITC: 494/518 in nm.			
Number of reactions: Each kit contains enough reactive dye to perform 5–10 labeling reactions using 0.2–2 mg of protein per reaction.			

Introduction

The FluoReporter® Protein Labeling Kits provide a convenient means for fluorescently labeling proteins—particularly monoclonal and polyclonal antibodies. The versatile FluoReporter® Kits can be used with a wide range of protein concentrations and with virtually any protein larger than 30 kDa. The FITC (fluorescein isothiocyanate) dye provided in this kit reacts with the primary amines of proteins to form the desired dye–protein conjugates. The absorption and fluorescence emission maxima of FITC-labeled proteins are approximately 494 nm and 518 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 spin columns, which provide a convenient and efficient method for purifying the fluorophore–protein conjugate from unreacted dye with minimal dilution of the conjugate.

Before Starting

Materials Required but Not Provided

- 1 M sodium bicarbonate buffer, pH 9

Preparing Sodium Bicarbonate Buffer

To prepare 1 M sodium bicarbonate buffer, dissolve 0.84 g of $NaHCO_3$ in ~9 mL of deionized water. Adjust the pH to 9 with NaOH and add deionized water to a final volume of 10 mL. Store the solution at 4°C and use within one week.

Experimental Protocols

Preparing the Protein

- Each labeling reaction requires 200 µL of protein solution (see note **A**).
- Purified protein (see 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 dialysis against PBS or by using one of the provided spin columns (see **Purification**).

Calculations

Perform the following calculation **before** beginning the conjugation reaction outlined in **Conjugation Reaction**.

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 10 mg/mL reactive dye stock solution (prepared in step 1.3) is added to 200 µL of protein solution. Calculate the volume of the 10 mg/mL reactive dye stock solution to be added (in step 1.4) using the following formula (see note **C**):

$$\text{µL reactive dye stock solution} = \frac{\text{mg/mL protein} \times 0.2\ \text{mL}}{MW_{protein}} \times 389 \times 100 \times MR$$

- 0.2 mL is the volume of protein solution
- 389 is the MW of the reactive dye
- 100 is a unit conversion factor
- For most whole IgGs, MWprotein = 145,000
- MR is the molar ratio of dye to protein in the reaction mixture.

For IgG labeling reactions, we recommend the following MRs, 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:

- MR = 40 (60) if the antibody is at 1–3 mg/mL
- MR = 30 (50) if the antibody is at 4–10 mg/mL.

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 2 mL reaction tube containing a stir bar (Component C).

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

1.3 Prepare the 10 mg/mL reactive dye stock solution: Allow DMSO (Component B) and one vial of reactive FITC dye (Component A) to warm to room temperature. **Immediately before starting the reaction**, add 37 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 (see **Calculations**). Discard the remaining reactive dye stock solution.

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

Purification

2.1 While the labeling reaction is taking place, prepare a spin column (Component D) (see notes **E, F**) by removing the top closure and adding purification resin (Component F) until full (approximately 1.5 mL). After the resin has settled, remove the bottom closure, and allow the column buffer to drain by gravity. Discard this flowthrough.

2.2 Place the spin column in a 2 mL collection tube (the collection tubes are the tubes without stir bars). Centrifuge the column for 3 minutes at 1100 × g using a swinging bucket rotor (see note **G**). Discard the buffer from the collection tube, but save the collection tube for use in step 2.5.

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

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

2.5 Place the spin column into the empty collection tube and centrifuge it for 5 minutes at 1,100 × *g*.

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

Determine the Degree of Labeling

3.1 Dilute the purified conjugate into the sodium bicarbonate buffer (pH 9.0) and measure the absorbance in a cuvette with a 1-cm pathlength at 280 nm (A_{280}) and 494 nm (A_{494}).

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

$$\text{protein concentration (M)} = \frac{[A_{280} - (A_{494} \times 0.3)] \times \text{dilution factor}}{\varepsilon}$$

where 0.3 is a correction factor (see note **I**) and ε is the molar extinction coefficient of the protein at 280 nm. For most IgGs, ε = 203,000 $cm^{-1}M^{-1}$.

3.3 Calculate the degree of labeling:

$$\text{dye per protein molecule} = \frac{A_{494} \times \text{dilution factor}}{68{,}000 \times \text{protein concentration (M)}}$$

where 68,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of the dye at pH 8.0 at 494 nm.

Storing the 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 is 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] You may scale the reaction to accommodate other volumes of protein; however, the molar ratios of dye to protein recommended in **Calculations** may not result in optimal labeling. Note that the spin columns 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 molar ratios, using the values given in this procedure as a starting point. Generally, in the case of antibodies, reactions that result in 4–8 FITC 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.

We 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, dissolved 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. Prepare this reagent freshly before use and discard any remaining hydroxylamine.

[E] If the molecular weight of the protein is less than 30,000, do **not** use the provided spin columns. You can remove the free dye 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. You can divide the reaction and apply it 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, you can estimate the protein concentration using the following formula:

$$\text{concentration of labeled protein (M)} = \frac{\text{mg initial protein} \times 0.85}{\text{mL in collection tube} \times MW_{protein}}$$

where 0.85 is the estimated yield (usually 80–90%). For most whole IgGs, $MW_{protein}$ = 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 website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
F6434	FluoReporter® FITC Protein Labeling Kit *5-10 labelings*	1 kit

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