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Product Information

FluoroTag™ FITC Conjugation Kit

Catalog Number **FITC1**
Storage Temperature 2−8 °C

TECHNICAL BULLETIN

Product Description

The FluoroTag FITC Conjugation Kit is suitable for the conjugation of polyclonal and monoclonal antibodies with fluorescein isothiocyanate (FITC) for use in immunohistochemistry and immunofluorescence studies utilizing flow cytometry. It may also be used for conjugation of FITC to peptide hormones, cytokines, growth factors, and other proteins. The kit contains sufficient reagents for at least 5 conjugations.

Fluorescein isothiocyanate (FITC), Isomer I is among the most widely used fluorescent labeling reagents due to the fluorophore's high quantum efficiency and conjugate stability. FITC has an absorption maximum at 495 nm and emission maximum at 525 nm. FITC reacts with free amino groups of proteins to form stable conjugates (see Figure 1). FITC-protein conjugates, in particular FITC labeled antibodies, are used as specific probes in immunocytochemistry and flow cytometry applications.[1,2] Biologically active FITC-conjugates of peptide hormones and growth factors have been successfully prepared which identify receptors on target cells.[3,4] FITC has also been used as a site-specific probe for several other proteins.[5,6,7]

The use of optimal labeling conditions is recommended. Overlabeling of proteins generally results in altered specificity, aggregation and/or precipitation of the protein. Fluorescent labeling of antibodies with high fluorophore to antibody ratios (molar F/P >6) usually results in increased non-specific binding (fluorescent background) and decreased quantum yield due to the fluorophore self-quenching effect.

The FluoroTag Kit includes detailed procedures for both small and large scale conjugation of FITC to antibody. Small scale FITC conjugations are performed using three different molar ratios of FITC to antibody. Based on the molar ratio that gives the most satisfactory result, the large scale procedure can then be performed to optimally label the protein.
The labeled protein is purified from the unconjugated fluorescein by a quick Sephadex® G-25M column. The F/P molar ratio of the purified protein is then determined by measuring the absorbance at 280 nm and at 495 nm.

Reagents and Materials Provided

- Fluorescein isothiocyanate, Isomer I (FITC), F7250KC-2MG. Five amber vials each containing 2.0 mg of lyophilized fluorescein isothiocyanate.
- 0.1 M Sodium Carbonate-Bicarbonate Buffer, pH 9.0, C0688. Five capsules containing powder.
- Phosphate Buffered Saline (PBS), P3813. Five packages containing powder.
- Gel filtration columns. Two columns prepacked with Sephadex G-25M. These serve to separate unreacted FITC from the conjugate and for buffer exchange. The columns are preswollen in water containing 0.15% Kathon® CG/ICP II as preservative. The gel filtration columns are supplied for two different reaction scales:
  Small Scale (Column A), B7533: One column prepacked with Sephadex G-25M. The bed volume of the column is 3.5 ml and the bed height is 2.6 cm. The maximal sample volume is 0.3 ml. Large Scale (Column B), B4783: One column prepacked with Sephadex G-25M. The bed volume of the column is 9.1 ml and the bed height is 5 cm. The maximal sample volume is 1.5 ml.

Reagents and Equipment Required but Not Provided

- Standard glass vials (1.5−2 ml capacity) equipped with stirring bars.
- Two dilution vials are needed for each conjugation.
- Vortex mixer.
- Standard glass tubes (12 x 75 mm or 13 x 100 mm) to collect fractions from Sephadex G-25M columns.
- Aluminum foil - To protect reaction and FITC labeled protein from intense light.

- Quartz cuvette - 1 cm path length
- UV/Visible spectrophotometer.

Precautions and Disclaimer
For R&D use only. Not for drug, household, or other uses.

Preparation Instructions
0.1 M Sodium Carbonate-Bicarbonate Buffer, pH 9.0, C0688 - Add contents of one capsule to 50 ml of deionized water to make 0.1 M sodium carbonatebicarbonate buffer, pH 9.0. This is used to dissolve FITC and to buffer the conjugation reaction.

Phosphate Buffered Saline (PBS), P3813 - Mix contents of one package with 800 ml of deionized water. Adjust volume to 1 liter to make 10 mM sodium phosphate buffer, 27 mM KCl, 138 mM NaCl, pH 7.4. This serves as an equilibration buffer for the Sephadex G-25 columns, for the elution of the labeled protein from the column, and for the final dilution of the labeled protein.

Storage/Stability
Store at 2−8 °C.

Procedure
The protocol outlined describes the labeling of 1 mg of IgG at 5 mg/ml with FITC (Small Scale Conjugation Procedure). The procedure can be scaled up to 5 mg of IgG maintaining the same concentration and molar ratio of the reagents (Large Scale Conjugation Procedure). It is important to consider that the number and surface availability of amine groups (primarily amine groups of lysine residues) vary greatly among proteins and even among different IgGs. This may result in a large variability of the level of labeling. Testing different FITC to antibody molar ratios to determine the optimal levels of labeling of the antibody is recommended.

Small Scale Conjugation Procedure (1.0 mg IgG)
This procedure describes the conjugation of FITC to 1 mg of IgG, using one of the following molar ratios in the reaction mixture: 5:1, 10:1 and 20:1 of FITC (MW 389) to IgG (MW 150,000). The labeling is performed in a final reaction volume of 0.25 ml. In general, these reaction molar ratios result in fluorescein-antibody conjugates with F/P ratios of 1−2, 2−4 and 3−6, respectively. This procedure can be modified if a protein of a different molecular weight (or a different amount of IgG) is used in the labeling reaction. (See Table 2 reaction mixture ratios for whole IgG, IgM, or antibodyfragments.)

1. Dissolve the contents of one sodium carbonatebicarbonate capsule (C0688) in 50 ml of deionized water. The pH of this buffer should be 9.0 ± 0.1 (See Application Notes 1 & 2).
2. Prepare at least 0.25 ml of antibody solution at 5.0 mg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9.0 (See Application Notes 3, 4 & 5). The A_{280} of an IgG solution at 1.0 mg/ml is 1.4 (1.0 cm path length).
3. Add 0.2 ml (1.0 mg) of the antibody solution into each reaction vial labeled “5:1”, “10:1”, or “20:1”. 4. Reconstitute one vial of FITC (F7250) in 2 ml of 0.1 M carbonate-bicarbonate buffer and vortex until all FITC has dissolved. Label vial “20:1 FITC”. The solution should be freshly prepared before each conjugation and used within 5 minutes (See Application Note 6). The "20:1 FITC" solution is further used to prepare the "5:1" and "10:1" solutions.
5. Prepare the required dilution of FITC in 0.1 M carbonate-bicarbonate buffer as directed in Table 1.

Table 1.
Dilution of FITC in 0.1 M Carbonate-Bicarbonate Buffer

Expected F/P Ratio	Label	0.1 M Sodium Carbonate-Bicarbonate Buffer	Reconstituted FITC
3 to 6	20:1	2.0 ml	FITC vial
2 to 4	10:1	0.5 ml	0.5 ml of 20:1
1 to 2	5:1	0.75 ml	0.25 ml of 20:1

6. Add 50 µl of the appropriate FITC dilution dropwise while stirring to the correspondingly labeled reaction vial.
7. Completely cover the reaction vial with aluminum foil to protect from light.
8. Incubate all reaction vials for 2 hours at room temperature with gentle stirring.

Isolation of labeled protein
1. Empty contents of PBS package (P3813) into a suitable container. Add 800 ml of distilled or deionized water and mix. Adjust to final volume of 1,000 ml.
2. Label Sephadex G-25M, column A (B7533), “5:1”, “10:1”, or “20:1”. Support column over a suitable (100 ml) beaker.

3. Remove cap from the top of the column, cut open lower tip of column and let excess of liquid flow through. The column will not run dry.
4. Equilibrate the column with 12 ml of PBS solution (6 x 2 ml). If the column is not immediately used, close with top and bottom caps and store at 2–8 °C.
5. Apply reaction mixture to top of column gel bed and collect the flow through (Fraction 1)
6. Elute the column with 2.5 ml of PBS, collecting 0.25 ml fractions (10 x 0.25 ml). Monitor the absorbance of each fraction at 280 nm. Two bands will be visible during elution. The conjugate is present in the first band (fractions 6–8) (See Application Note 7).
7. Pool the main fractions. Do not collect fractions with A_{280} <0.2.
8. Wash the column with 35 ml (10 x column volumes) of PBS to remove unbound fluorophore. This is sufficient to regenerate the column.
9. For prolonged storage, wash the column with 10 ml of PBS containing 0.05% sodium azide and store capped at 2–8 °C, with 1 ml buffer above the gel.
10. Determine the fluorescein/protein ratio (F/P) of the conjugate using a spectrophotometer as described in Determination of Fluorescein/Protein Molar Ratio (F/P).
11. For storage of the conjugate after determination of the F/P molar ratio, add 1% (w/v) BSA and 0.1% (w/v) sodium azide to the conjugate. Store at 2–8 °C, protected from light.

Large Scale Conjugation Procedure (5.0 mg IgG) This procedure describes the scale-up of the conjugation reaction of FITC to 5 mg of IgG, using the molar ratio, which gives the most satisfactory results as obtained from the "Small Scale Conjugation Procedure." The labeling is performed maintaining the same concentrations and ratios of reagents used previously. The reaction is performed in a final reaction volume of 1.25 ml.

1. Add 1.0 ml (5.0 mg) of the antibody solution to a reaction vial labeled "5:1", "10:1", or "20:1".
2. Reconstitute one vial of FITC (F7250) in 2 ml of 0.1 M carbonate-bicarbonate buffer and vortex until all FITC has dissolved.
3. Prepare 10:1 or 5:1 dilution of FITC in 0.1 M carbonate-bicarbonate buffer as directed in Table 1, if necessary.
4. Add 250 µl of the appropriate FITC dilution dropwise while stirring to the reaction vial.
5. Completely cover the reaction vial with aluminum foil to protect from light.
6. Incubate reaction vial for 2 hours at room temperature with gentle stirring.

Isolation of labeled protein

1. Label Sephadex G-25M, column B (B4783), "5:1", "10:1", or "20:1". Support the column over a suitable (100 ml) beaker.
2. Remove cap from the top of the column, cut open lower tip of column and let excess of liquid flow through. The column will not run dry.
3. Equilibrate the column with 30 ml of PBS solution (6 x 5 ml). If the column is not immediately used, close with top and bottom caps and store at 2–8 °C.
4. Apply reaction mixture to top of the column gel bed and collect flow through (Fraction 1).
5. Elute column with 10 ml of PBS, collecting 1.0 ml fractions (10 x 1 ml). Monitor the absorbance of each fraction at 280 nm. Two bands will be visible during elution. The conjugate is present in the first band (fractions 3–5). (See Application Note 7.)
6. Pool the main fractions. Do not collect fractions with A_{280} <0.4.
7. Wash the column with 50 ml of PBS solution to remove unbound fluorophore. This is sufficient to regenerate the column.
8. Preserve the column as instructed in step 9 of the small scale "Isolation of Labeled Protein" section.

Determination of Fluorescein/Protein Molar Ratio (F/P)
The F/P molar ratio is defined as the ratio of moles of FITC to moles of protein in the conjugate. To determine this ratio, it is necessary to first determine the absorbance of the conjugate sample at 280 nm and then at 495 nm.

1. Place the conjugate sample in a quartz cuvette. For the large scale conjugation, dilute 0.1 ml of the FITC conjugate in 0.9 ml of PBS containing sodium azide. Read the absorbance of the conjugate sample at 280 nm and 495 nm. The absorbance reading of the conjugate sample should be between 0.2 and 1.4 at 280 nm. If the absorbance reading is outside this range, adjust the sample dilution accordingly.
2. From the absorbance readings (A_{280} and A_{495}) of the conjugate sample, calculate the F/P of the fluorescein-IgG conjugate according to the equation:[8]

$$2.77 \times A_{495}$$ (For FITC-IgG

Molar F/P = $\frac{}{A_{280} - (0.35 \times A_{495})}$ conjugates only)

The protein concentration of the fluorescein-IgG conjugate is calculated from the following formula:

$$\text{IgG (mg/ml)} = \frac{A_{280} - (0.35 \times A_{495})}{1.4}$$

Where 1.4 is the A_{280} of IgG for most species at a concentration of 1.0 mg/ml at pH 7.0.

3. When any protein other than IgG is conjugated to FITC, use the general formula below, substituting the appropriate values for the particular protein:

Molar F/P =

$$\frac{MW}{389} \times \frac{A_{495}/195}{[A_{280} - (0.35 \times A_{495})]/ E^{0.1\%}} =$$

$$\frac{A_{495} \times C}{A_{280} - [(0.35 \times A_{495})]}$$

Where: $C = \frac{MW \times E^{0.1\%}_{280}}{389 \times 195}$

C is a constant value given for protein
MW is the molecular weight of the protein.
389 is the molecular weight of FITC.
195 is the absorption $E^{0.1\%}_{280}$ of bound FITC at 490 nm at pH 13.0.

$(0.35 \times A_{495})$ is the correction factor due to the absorbance of FITC at 280 nm.[8]

$E^{0.1\%}$ is the absorption at 280 nm of a protein at 1.0 mg/ml.

(For $E^{0.1\%}_{280}$ and C values, see Table 2)

Application Notes

1. Do not store sodium carbonate-bicarbonate buffer more than 1 week at 2–8 °C. The pH of the buffer may change upon storage. It is advised that fresh buffer be made just before use.
2. The conjugation of FITC to proteins is affected by the reaction conditions (concentration, temperature, pH).
3. When conjugating antibodies with FITC, the starting material should be free of contaminating serum proteins. Affinity isolated antibodies, IgG fractions, or Protein A purified immunoglobulin are generally acceptable.
4. Protein solutions should not be prepared in buffers containing amines such as Tris, glycine or sodium azide since they inhibit the labeling reaction. If the buffer contains amines or sodium azide, dialyze protein solution (1 ml) against PBS, pH 7.4 (1,000 ml), overnight at 2–8 °C. Avoid dialysis at high pH values (>8.0–8.5) as this may be harmful to some proteins.
5. If the antibody is in PBS pH 7.4 (without azide), add 1 M carbonate-bicarbonate buffer (1 capsule (C0688) in 5 ml deionized water) to a final concentration of 0.1 M (e.g., 0.1 ml of 1 M carbonate-bicarbonate buffer to 0.9 ml IgG solution at 5.0 mg/ml).
6. FITC is not stable in aqueous solutions. Do not store FITC stock solutions.
7. The second band containing unbound fluorophore is retained on the column and will elute only with subsequent PBS washes.

References

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4. Schwartz, J., and Vale, W., Fluorescent and cytotoxic analogs of corticotropin-releasing factor: probes for studying target cells in heterogeneous populations. *Methods in Enzymol*., **168**, 29 (1989).
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Table2.
Reaction Mixture Ratios for Whole IgG, IgM or AntibodyFragments

Antibodies	MW	$E^{0.1\%}_{280}$	C*	FITC (µl)** per Protein	
				1 mg	5 mg
IgG1, IgG2 IgG3, IgG4	150,000	1.4	2.77	50	250
IgA1, IgA2	160,000	1.32	2.78	49	245
IgD	165,000	1.7	3.70	47	235
IgE	185,000	1.53	3.73	42	210
IgM	900,000	1.18	14.0	50***	250***
F(ab′) 2	100,000	1.5	1.98	78	195****
Fab′	50,000	1.5	0.99	156	195*****
Fc	50,000	1.2	0.79	156	195*****

*C is a constant value for FITC conjugation of a given protein:

$$C = \frac{MW \times E^{0.1\%}_{280}}{389 \times 195}$$

** This corresponds to volume in microliters of the appropriate FITC concentration (5:1, 10:1, or 20:1).

***For IgM, a lower molar ratio is recommended as high levels of FITC may result in overlabeling of the antibody and self-quenching of the fluorophore.

****For F(ab′) 2 use a 2-fold more concentrated FITC solution than used for IgG.

*****For Fab′ and Fc use a 4-fold more concentrated FITC solution than used for IgG.

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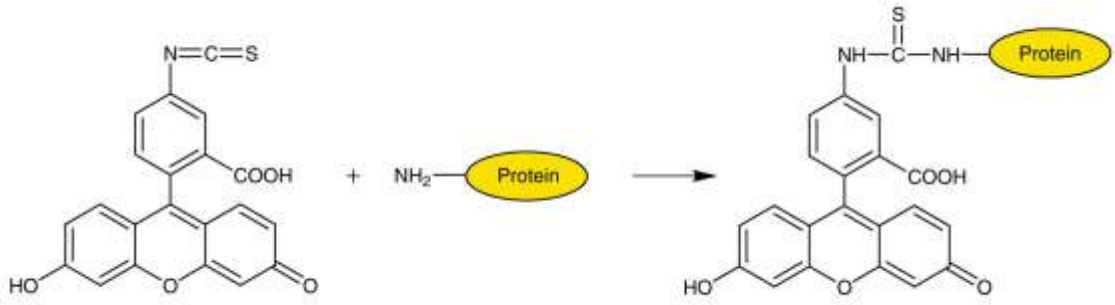
Figure 1.

The FITC Labeling Reaction



FITC conjugation occurs through the free amino groups of proteins or peptides, forming a stable thiourea bond.