AnaTag™ 5-FITC Microscale Protein Labeling Kit

Revision number: 1.3	*Last updated: April 2018*
Catalog #	**AS-72060**
Kit Size	3 Conjugation Reactions

- This kit is optimized to conjugate 5-FITC (Fluorescein - 5- isothiocyanate) to proteins (e.g., IgG).
- It provides ample materials to perform three protein conjugations and purifications.
- One conjugation reaction can label up to 200 μg proteins.

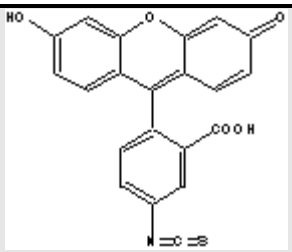
Kit Components, Storage and Handling

Component	Function	Quantity
A. 5-FITC	Amino-reactive dye	3 vials
B. Reaction buffer	For pH adjustment of the conjugation reaction	0.5 mL
C. Spin column	Purify dye-protein conjugate	3 pre-packed columns
D. DMSO	Solvent for preparing dye stock solution	150 μL
E. Elution buffer	Buffer for eluting dye-protein conjugate	20 mL
F. Wash tube	Holds buffer for Spin column	3 tubes
G.Collect tube	Collects dye-protein conjugate	3 tubes

Storage and Handling

- Store all kit components at 4°C.
- Keep Component A away from light and protect from moisture.
- Component A may be frozen.

Introduction



Physical and Spectral Properties of 5-FITC:

- **Fluorescent color: Green**
- **Molecular weight: 389.38**
- **Maximum absorption: 494 nm**
- **Maximum emission: 519 nm**
- **Reactive form: isothiocyanate (amine- reactive)**

The AnaTag™ 5-FITC Protein Labeling Kit provides a convenient way to label proteins with 5-FITC. Despite the availability of alternative amine-reactive fluorescein derivatives that yield conjugates with superior stability and comparable spectra, fluorescein isothiocyanate (FITC) remains one of the most popular fluorescent labeling reagents, probably due to the low cost of the material. 5-FITC and 6-FITC have very similar absorption and fluorescence spectra. However, the isomers may differ in their binding and reactivity to proteins, and the conjugates may elute under different chromatographic conditions or migrate differently in electrophoresis gels. 5-FITC is more widely used than the 6-FITC isomer.

The kit has all the essential components for performing the conjugation reaction and for purifying the conjugate.

Protocol

1. Preparing the protein solution

Add reaction buffer (component B) at 1/10 (v/v) ratio to your target protein (e.g. antibody) solution (3-10 mg/mL is the recommended concentration range).

Note 1: The protein can be dissolved in phosphate, carbonate, borate, triethanolamine or MOPS buffer, pH 7.2-7.5, without reducing reagents (e.g. DTT), protein stabilizers (e.g. BSA) or sodium azide. If the protein is dissolved in Tris or glycine buffer, it should be dialyzed against 0.01 M phosphate buffer saline, pH 7.2-7.4 to get rid of free amines. Ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation must also be removed before performing the dye conjugations.
Note 2: The conjugation efficiency is poor when the concentration of protein is less than 3 mg/mL. Meanwhile, the purification column included in this kit can maximally purify 100 µl conjugate solution. You may concentrate the protein solution using a speed vacuum or a centrifugal filter (Millipore, Cat# MRCPRT010).

2. Preparing the dye solution

Add 10 µL of DMSO (component D) to one vial of 5-FITC (component A). This gives a 2mM dye solution. Completely dissolve all the dye contents by vortexing.

Note: Dye solution must be prepared fresh for each conjugation reaction. Extended storage of the dye solution may reduce dye activity. Any solutions containing the dye must be kept from light.

3. Performing the conjugation reaction

Note: The procedure given here is optimized for IgG (MW ~ 150,000) labeling with 5-FITC. The dye: protein molar ratio is 12:1. For proteins other than IgG, the optimal dye/protein molar ratio may need to be determined. It will normally be between 2:1 and 20:1.

3.1 Add the dye solution to the solution of IgG or your protein at a dye to protein molar ratio of 12:1. For 200 µg IgG, add 8 µl of 2 mM dye solution.

Note: The molecular weight of IgG is 150 kDa.

3.2 Keep the reaction mixture away from light and shake for 1 hour at room temperature on a rotator or a shaker.

4. Purify dye-protein conjugates

4.1 Resuspend the gel in the spin column (component C) by inverting sharply several times. Avoid bubbles.

4.2 Remove the top cap of the column, and then cut its bottom tip. Place the column into a wash tube (component F) and centrifuge at 1,000 x g for 2 min. Discard the eluted buffer.

4.3 Exchange the gel-packing buffer by adding 500 µL of elution buffer (component E) to the spin column and centrifuge at 1,000 x g for 1 min. Discard the eluent. Repeat the above step three times.

4.4 Place the spin column into a clean collection tube (component G). Apply the reaction mixture from Step 3 to the center of gel bed surface. Centrifuge the column at 1,000 x g for 4 min.

4.5 The dye-protein conjugate is in the collection tube.

4.6 The degree of substitution (DOS) of the conjugate should be determined according to the Appendix.

Appendix. Characterizing The Dye-Protein Conjugate

The degree of substitution (DOS) is important for characterizing dye-labeled proteins. Proteins of lower DOS usually have weaker fluorescence intensity, but proteins of higher DOS (e.g. DOS>6) tend to have reduced fluorescence due to fluorescence quenching. The optimal DOS recommended for most antibodies is between 2 and 6. To determine the DOS of 5-FITC labeled proteins:

1. Read absorbance at 280 nm (A_{280}) and 494 nm (A_{494})
For most spectrophotometers, dilute a small portion of conjugate solution in 0.1 N NaOH so that the absorbance readings are in the 0.1 to 0.9 ranges. The maximal absorption of protein is at 280 nm (A_{280}). The maximal absorption of 5-FITC is approximately at 494 nm (A_{494}).

> Note: The maximal absorbance of 5-FITC is pH sensitive, so an aliquot of conjugate solution should be diluted in 0.1 N NaOH before reading the absorbance.

2. Calculating the DOS using the following equations for IgG labeling
Molar concentration of dye:

$[Dye] = (A_{494} \times \text{dilution factor}) / \varepsilon_{5\text{-FITC}}$ $\varepsilon_{5\text{-FITC}} = 80{,}000\ cm^{-1}M^{-1}$

ε is the extinction coefficient.

Molar concentration of protein:

$[Protein] = ((A_{280} - 0.32 \times A_{494}) \times \text{dilution factor}) / \varepsilon_{protein}$ $\varepsilon_{IgG} = 203{,}000\ cm^{-1}M^{-1}$

* 0.32 is correction factor for the fluorophore's contribution to A_{280}

DOS = [Dye]/[Protein]

Protein concentration in mg/mL for IgG:

Ig G (mg/mL)=[Ig G] x 150,000 $MW_{Ig\ G} = 150{,}000$

For effective labeling, the degree of substitution should fall within 2-6 moles of 5-FITC per one mole of protein.

Storage of Dye - Protein Conjugates
The dye-labeled protein should be stored at > 0.5 mg/mL or in the presence of a carrier protein (e.g., 0.1% Bovine Serum Albumin). We recommend adding preservative (e.g. 0.01% sodium azide). The dye-labeled protein can be stored at 4°C for two months without significant changes if kept from light. For extended storage, it can be aliquoted or lyophilized and stored at -20°C in the dark.

References

1. Hermanson GT (1996). *Bioconjugate Techniques*, Academic Press, New York.
2. Haugland RP (1995). Coupling of monoclonal antibodies with fluorophores. *Methods Mol Biol* **45,** 205-21.
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4. Banks PR, Paquette DM (1995). Comparison of three common amine reactive fluorescent probes used for conjugation to biomolecules by capillary zone electrophoresis. *Bioconjug Chem*. **6**, 447-58.