AnaTag™ 5-FAM Protein Labeling Kit

Catalog #	71001
Unit Size	1 Kit
Kit Size	5 Conjugation Reactions

This kit is optimized to conjugate 5-FAM (5-Carboxyfluorescein) to proteins (e.g., IgG). It provides ample materials to perform five protein conjugations and purifications. One conjugation reaction can label up to 5 mg protein. The entire process only takes about half an hour.

- ***Convenient Format:*** Complete kit includes all the assay components.
- ***Optimized Performance:*** Optimal conditions for conjugation and purification.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

USA and Canada Ordering Information

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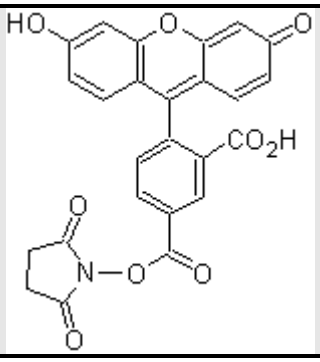
International Ordering Information

A list of international distributors is available at www.anaspec.com.

Introduction



Physical and Spectral Properties of 5-FAM, SE:

Fluorescent color: Green

Molecular weight: 473.39

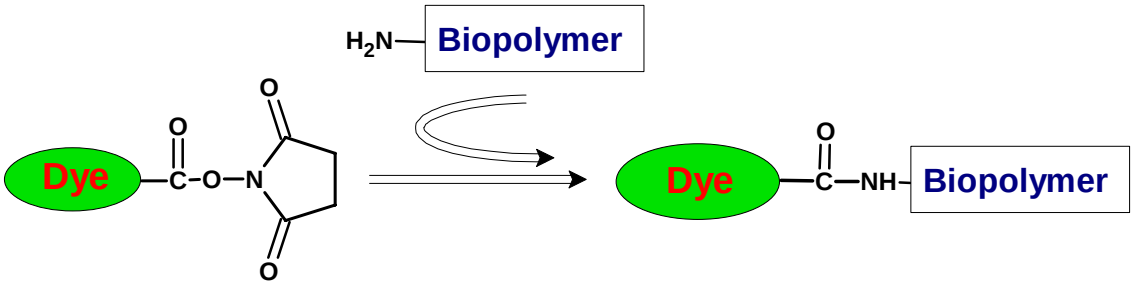
Maximum absorption: 495 nm

Maximum emission: 520 nm

Reactive form: Succinimidyl esters (amine-reactive)

The AnaTag™ 5-FAM Protein Labeling Kit provides a convenient way to label proteins with 5-FAM. 5-FAM (5-Carboxyfluorescein) is one of the most popular green fluorophores used in labeling proteins. It shares the same excitation (495nm) and emission (520nm) wavelengths as FITC. But unlike FITC, whose thiourea bond with protein is unstable resulting in the release of free dye from the protein conjugates over time, the succinimidyl ester (SE) reactive form of 5-FAM reacts with the amine group on the protein and forms stable carboxamide bonds, which is identical to natural peptide bonds. As a result, 5-FAM SE is a better fluorophore than FITC for protein labeling. 5-FAM protein conjugates can withstand treatments during immunofluorescent staining, fluorescence *in situ* hybridization, flow cytometry and other biological applications without hydrolysis.

5-FAM SE is generally stable if it is properly stored, and it shows good reactivity and selectivity towards aliphatic amines. It has a very low reactivity towards aromatic amines, alcohols, phenols (including tyrosine) and histidine. The kit has all the essential components for performing the conjugation reaction and for purifying the conjugate.



KIT COMPONENTS, STORAGE AND HANDLING

Note: Store all kit components at 4℃, and keep component A away from light.

Component	Function	Quantity
A. 5-FAM, SE	Amino-reactive dye	5 vials
B. Reaction buffer	For pH adjustment of the conjugate reaction	0.5 mL
C. Desalting column	Purify dye-protein conjugate	5 Pre-packed columns
D. DMSO	Solvent for preparing dye solution	1 mL
E. 10X Elution buffer	Solution for eluting dye-protein conjugates	30 mL
F. Buffer reservoir	Hold buffer for desalting column	1

Standard Operating Protocol (SOP)

1. Preparing the protein solution

Add reaction buffer (component B) at 1/10 (v/v) ratio to your target protein (e.g. antibody) solution (2-10 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) or protein stabilizers (e.g. BSA). 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 2 mg/mL.

2. Preparing the dye solution

Add 20 µL of DMSO (component D) to one vial of 5-FAM SE (component A) to get 10 mg/mL of 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 should be kept from light.

3. Performing the conjugation reaction

3.1 Add the dye solution to the IgG solution at a dye: protein molar ratio of 10:1. Table 1 gives a quick reference for labeling IgG. Mix the reaction mixture completely.

Table 1. The volume of dye solution needed for different amount of IgG.

Ig G	Dye solution	Ig G	Dye solution
0.5 mg	1.6 µL	3 mg	9.5 µL
1 mg	3.2 µL	3.5 mg	11.0 µL
1.5 mg	4.7 µL	4 mg	12.6 µL
2 mg	6.3 µL	4.5 mg	14.2 µL
2.5 mg	7.9 µL	5 mg	15.8 µL

Note 1: The molecular weight of IgG is 150 kDa.

Note 2: The dye: protein molar ratio of 10:1 is optimized for labeling IgG with 5-FAM SE. For proteins other than IgG, the optimal dye/protein molar ratio needs to be determined. The desired dye/protein molar ratio usually should be between 2:1 and 20:1.

3.2 Keep the reaction mixture from light and shake for 15 minutes at room temperature on a rotator or a shaker.

4. Purify dye-protein conjugates

Note: Desalting column (component C) is best suited for purifying proteins of MW>5,000. For smaller proteins, we recommend using Sephadex LH-20 or dialysis. HPLC may also be used to purify the smaller protein conjugates.

4.1 Dilute 10X elution buffer (component E) to 1X in deionized water.

4.2 Hold the desalting column (component C) upright. Remove the top cap of the column, and then cut its bottom tip. Place the buffer reservoir (component F) on the top of the column.

4.3 Add 25 mL 1X elution buffer into the buffer reservoir to pre-equilibrate the column.

4.4 As soon as the liquid runs just below the top solid surface, load the column with the reaction mixture (directly from step 3.2.).

4.5 As soon as the reaction mixture runs just below the top gel surface, add 10 mL 1X elution buffer into the column.

4.6 When the reaction mixture runs down the column, you should see it separated into two bands. The faster-running band (lower band) contains the desired dye-labeled protein, while the slower-running band (upper band) contains the free dye.

4.7 Collect the faster-running band only. Avoid the slower-running band, which will contaminate your conjugate.

4.8 The degree of substitution (DOS) of the conjugate can be determined according to Appendix I.

Appendix I. Characterizing the Dye-Protein Conjugate

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

1. Read absorbance at 280 nm (A_{280}) and A_{max}

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 range. The maximal absorption of protein is at 280 nm (A_{280nm}). The maximal absorption of 5-FAM amide (A_{max}) is approximately at 496 nm (Figure 1 and 2).
Note: The maximal absorbance of 5-FAM 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:

$[Dye]=A_{max} / \varepsilon_{5\text{-}FAM}$ X dilution factor

$\varepsilon_{5\text{-}FAM}$= 80,000 $cm^{-1}M^{-1}$
ε is the extinction coefficient.

Molar concentration of protein:

$[Protein]=(A_{280} - 0.2 \text{ X } A_{max}) / \varepsilon_{protein}$ X dilution factor

$\varepsilon_{IgG=}$ 203,000 $cm^{-1}M^{-1}$

DOS = [Dye]/[Protein]

Protein concentration (mg/mL):

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-FAM to one mole of protein.

The Storage of 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. 0.1% 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.

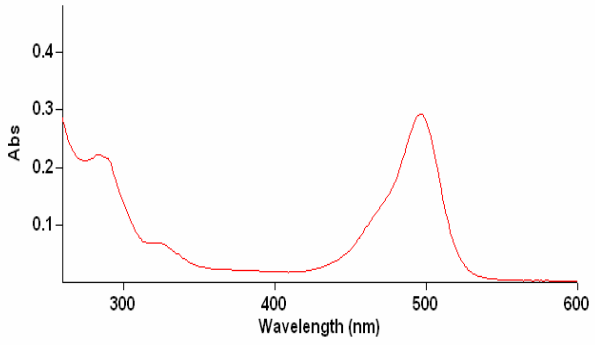


Figure 1. The absorbance spectrum of 5-FAM-Ig G conjugate.

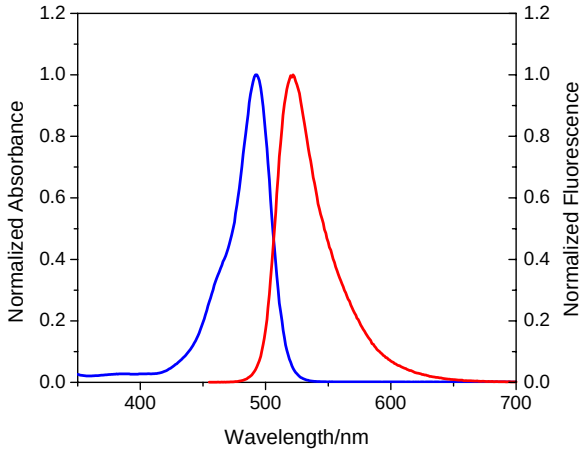


Figure 2. The spectrum of 5-FAM - absorption (left curve), fluorescence (right curve).

Reference

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.
3. Brinkley M (1992). A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjug Chem* **3,** 2-13.