



AnaTag™ HiLyte Fluor™ 647 Protein Labeling Kit

Catalog #	71008
Unit Size	1 Kit
Kit Size	5 Conjugation Reactions

This kit is optimized to conjugate HiLyte Fluor™ 647 SE to proteins (e.g., IgG). It provides ample materials to perform five protein conjugations and purifications. One conjugation reaction can label 100 µg of 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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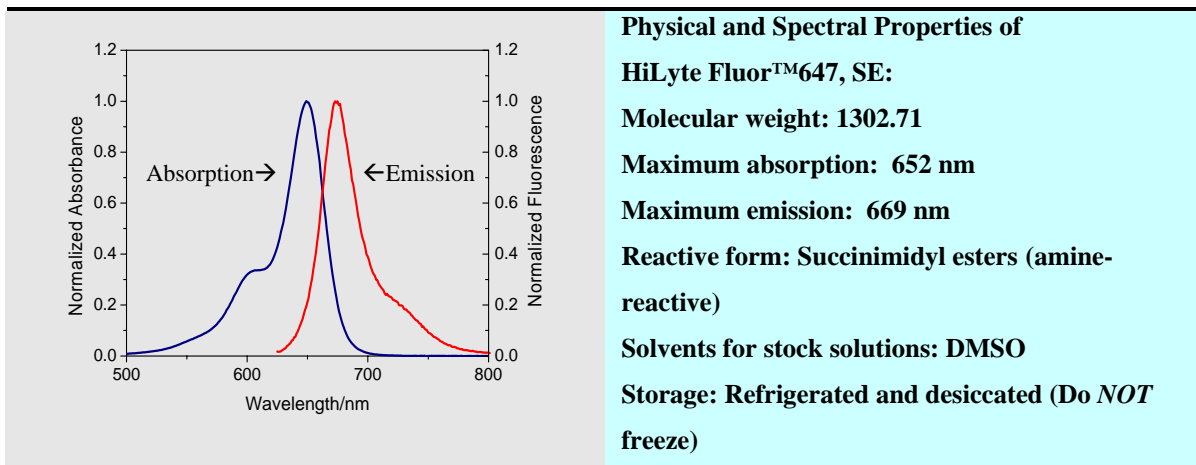
Technical Support

Tel: 408-452-5055
Fax: 408-434-9266
E-mail: assay@anaspec.com

International Ordering Information

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

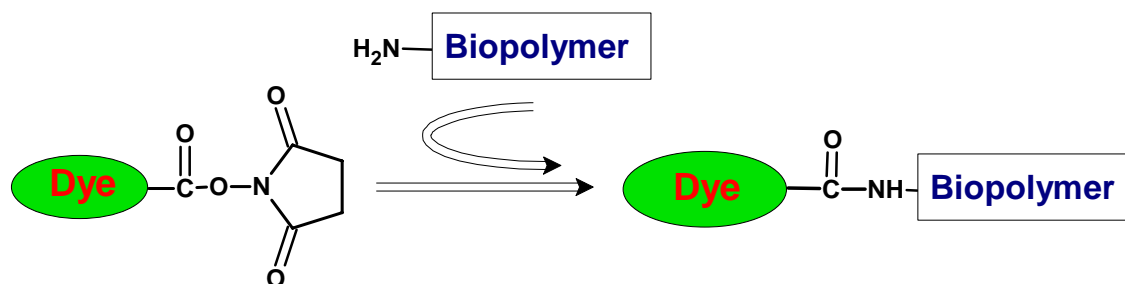
Introduction



HiLyte Fluor™ 647 acid, SE is an excellent fluorescent labeling dye for generating protein conjugates that are slightly red-shifted as compared to those of Cy5 dyes, resulting in an optimal match to filters designed for Cy5. The total fluorescence of the secondary antibody conjugates of HiLyte Fluor™ 647 is significantly higher than that of Cy5 conjugates. Also, unlike Cy5, HiLyte Fluor™ 647 has very little change in absorption or fluorescence spectra when conjugated to most proteins, oligonucleotides and nucleic acids, thus yielding greater total fluorescence at the same degree of substitution.

AnaTag™ HiLyte Fluor™ 647 Protein Labeling Kit provides a convenient way to label proteins by using the succinimidyl ester (SE) reactive form of HiLyte Fluor™647. The succinimidyl ester shows good reactivity and selectivity with aliphatic amines of the protein and forms a carboxamide bond, which is identical to, and is as stable as the natural peptide bond (Scheme 1). HiLyte Fluor™647-protein conjugates can withstand treatments performed during immunofluorescent staining, fluorescence *in situ* hybridization, flow cytometry and other biological applications without hydrolysis.

The kit has all the essential components for performing the conjugation reaction and for purifying the HiLyte Fluor™647 -protein conjugates.



KIT COMPONENTS, STORAGE AND HANDLING

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

Component	Function	Quantity
A. HiLyte Fluor™647, SE	Amino-reactive dye	5 vials
B. Reaction buffer	For pH adjustment of the conjugate reaction	0.5 mL
C. Spin column	Purify dye-protein conjugate	5 columns
D. DMSO	Solvent for preparing dye stock solution	1 mL
E. Elution buffer	Solvent for eluting protein	20 mL
F. Wash tube	Collect eluted waste	5 tubes
G. Collect tube	Collect dye-protein conjugate	5 tubes

Standard Operating Protocol (SOP)

1. Prepare the protein solution

Add reaction buffer (component B) at 1/10 (v/v) ratio to your target protein (e.g. antibody) solution. (1-10 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) and protein stabilizers (e.g. BSA). If the protein is dissolved in Tris or glycine buffer, it should be dialyzed against 0.01 M phosphate buffered 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: Conjugation efficiency is poor when the concentration of protein is less than 1 mg/mL.

2. Prepare the dye solution

Add 10 μ L of DMSO (component D) to one vial of HiLyte Fluor 647 SE (component A) to get 1 mM 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 away from light.

3. Perform the conjugation reaction

- Add the dye solution to the IgG solution at the molar ratio 10:1 (dye: IgG). For 100 μ g IgG, add 6.7 μ L of 1 mM dye solution. Mix the reagents completely, but do not vortex.

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

Note 2: For proteins other than IgGs, 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.

- Keep the reaction mixture away from light and shake for 15 min at room temperature on a rotator or a shaker.

4. Purify dye-protein conjugate

- Resuspend the gel in the spin column (component C) by inverting sharply several times. Avoid bubbles.
- 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.
- Exchange the gel-packing buffer by adding 500 μ L of elution buffer (component E) to the spin column and centrifuge at 1,000 x g for 1 min. Discard the eluent. Repeat above step for three times.
- 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.
- The dye-protein conjugate is in the collection tube.
- The degree of substitution of the conjugate should be determined according to the formula below.

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>8) tend to have reduced fluorescence also. The optimal DOS recommended for most antibodies is between 2 and 8. To determine the DOS of HiLyte FluorTM647 labeled proteins:

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

For most spectrophotometers, dilute a small portion of conjugate solution in phosphate buffered saline so that the absorbance readings are in the 0.1 to 0.9 range. The maximal absorption of protein is at 280 nm (A_{280}). The maximal absorption of HiLyte FluorTM647 (A_{max}) is approximately at 652 nm (Figure 1).

2. Calculate the DOS using the following equations for IgG labeling

Molar concentration of dye:

$$[\text{Dye}] = (A_{\text{max}} \times \text{dilution factor}) / \epsilon_{\text{HiLyte Fluor}^{\text{TM}}647}, \quad \epsilon_{\text{HiLyte Fluor}^{\text{TM}}647} = 250,000 \text{ cm}^{-1}\text{M}^{-1}$$

Molar concentration of protein:

$$[\text{Protein}] = [(A_{280} - 0.05 \times A_{\text{max}}) \times \text{dilution factor}] / \epsilon_{\text{protein}}, \quad \epsilon_{\text{IgG}} = 203,000 \text{ cm}^{-1}\text{M}^{-1}$$

$$\text{DOS} = [\text{Dye}] / [\text{Protein}] \quad \text{where } \epsilon \text{ is the extinction coefficient}$$

Protein concentration (mg/mL):

$$\text{Ig G (mg/mL)} = [\text{Ig G}] \times 150,000 \quad \text{MW}_{\text{Ig G}} = 150,000$$

For effective labeling, the degree of substitution should fall within 2-8 moles of HiLyte FluorTM647 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 should be divided into aliquots and stored at -20°C in the dark. Avoid multiple thaw-freeze cycles.

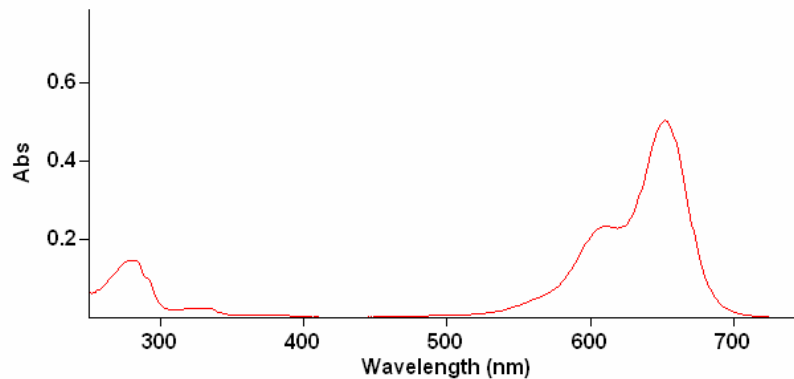


Figure 1. The absorption spectrum of HiLyte FluorTM647-Ig G conjugate.

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.