AnaTag™ HiLyte Fluor™ 488 Protein Labeling Kit

Catalog #	72047
Kit Size	3 Conjugation Reactions

- This kit is optimized to conjugate HiLyte Fluor™ 488 SE to proteins (e.g., IgG).
- It provides ample materials to perform three protein conjugations and purifications.
- One conjugation reaction can label up to 5 mg protein.
- The entire process only takes about half an hour.

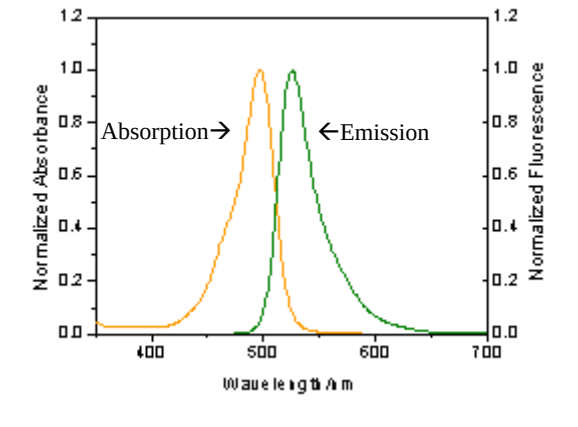
Kit Components, Storage and Handling

Component	Function	Quantity
A. HiLyte Fluor™488 SE	Amino-reactive dye	3 vials
B. Reaction buffer	For pH adjustment of the conjugate reaction	0.5 mL
C. Desalting column	Purify dye-protein conjugate	3 Pre-packed columns
D. DMSO	Solvent for preparing dye solution	1 mL
E. 10X Elution buffer	Solution for eluting dye-protein conjugates	30 mL

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 HiLyte Fluor™488 SE:

- **Fluorescence: Green**
- **Molecular weight: 698.6**
- **Maximal absorption: 499 nm**
- **Maximal emission: 523 nm**
- **Reactive form: Succinimidyl ester (amine-reactive)**

HiLyte Fluor™ 488 SE is an excellent amine-reactive fluorescent labeling dye for generating protein conjugates. The spectrum of HiLyte Fluor™488 is similar to fluorescein (FITC), resulting in an optimal match to filters designed for fluorescein. More photostable than FITC, HiLyte Fluor™ 488-labeled proteins allow additional time for capturing image.

The AnaTag™ HiLyte Fluor™ 488 Protein Labeling Kit provides a convenient way to label proteins by using the succinimidyl ester (SE) reactive form of HiLyte Fluor™488. 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 (Figure 1). HiLyte Fluor™488-protein conjugates can sustain treatments 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™488 -protein conjugates.

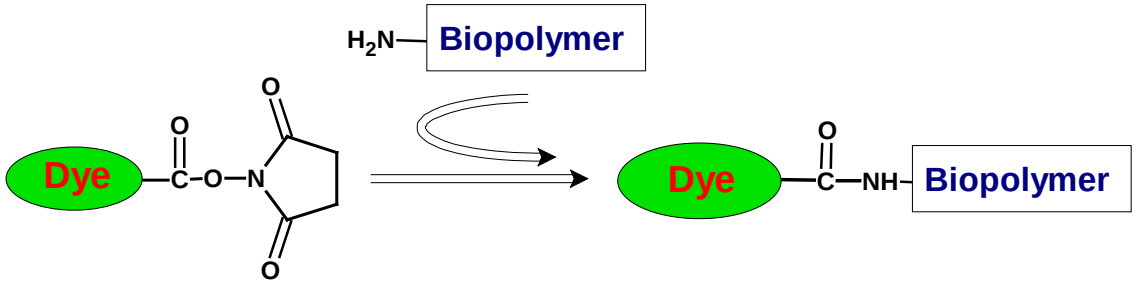


Figure 1. The succinimidyl ester group of fluorophore reacts with amine groups on the protein to form a stable carboxamide bond.

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 (2-10 mg/mL is the recommended concentration range of protein).

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 2 mg/mL. Meanwhile, the purification column included in this kit can maximally purify 3 mL conjugate solution. You may concentrate the protein solution using a speed vacuum or a centrifugal filter (Millipore, Cat# 42407).

2. Preparing the dye solution

Add 30 µL of DMSO (component D) to one vial of HiLyte Fluor 488 SE (component A). This gives a 10 mg/mL of dye solution (14 mM). Completely dissolve all the dye contents by vortexing.

Note: Dye solution should 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 HiLyte Fluor 488 SE. 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: protein molar ratio of about 12:1. Mix thoroughly. Table 1 gives a quick reference for labeling IgG.
Note: The molecular weight of IgG is 150 kDa.

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

Ig G	Dye solution
0.5 mg	2.8 µL
1 mg	5.6 µL
1.5 mg	8.4 µL
2 mg	11.2 µL
2.5 mg	14 µL
3 mg	16.8 µL
3.5 mg	19.6 µL
4 mg	22.4 µL
4.5 mg	25.2 µL
5 mg	28 µL

3.2 Keep the reaction mixture from light and shake for 15 minutes – 1 hour at room temperature on a rotator or a shaker.

4. Purify dye-protein conjugates

Note: The desalting column (component C) is best suited for purifying proteins of MW>6,000. For smaller proteins, we recommend using Sephadex LH-20 or dialysis. HPLC may also be used to purify the smaller protein or peptide 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. Pour off the excess buffer above the top frit.

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

4.4 Allow the buffer to drain to the top frit. The column will not run dry. Flow will stop when the buffer level reaches the top frit. Load the column with the reaction mixture (directly from step 3.2.).

4.5 Allow entire sample to enter the column, add 10 mL 1X elution buffer into the column.

4.6 As the reaction mixture runs down the column, you should see the colored material 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 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 HiLyte Fluor™488 labeled proteins:

1. Read absorbance at 280 nm (A_{280}) and 499 (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 ranges. The maximal absorption of protein is at 280 nm (A_{280}). The maximal absorption of HiLyte Fluor™488 (A_{max}) is approximately at 499 nm (Figure 2).

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

Molar concentration of dye:

$[Dye] = (A_{max} \times \text{dilution factor}) / \varepsilon_{\text{HiLyte Fluor™488}}$ $\quad \varepsilon_{\text{HiLyte Fluor™488}} = 68{,}000\ cm^{-1}M^{-1}$

ε is the extinction coefficient.

Molar concentration of protein:

$[Protein] = ((A_{280} - 0.19 \times A_{max}) \times \text{dilution factor}) / \varepsilon_{protein}$ $\quad \varepsilon_{IgG=} 203{,}000\ cm^{-1}M^{-1}$

$DOS = [Dye]/[Protein]$

Protein concentration (mg/mL):

Ig G (mg/mL) = [Ig G] x150,000 $\quad MW_{Ig\ G}=150{,}000$

For effective labeling, the degree of substitution should fall within 2-6 moles of HiLyte Fluor™488 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.

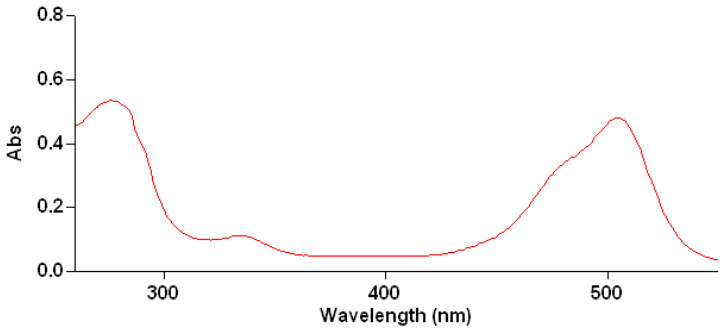


Figure 2. The absorbance spectrum of HiLyte Fluor™488-Ig G conjugate.

References

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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.