AnaTag™ HiLyte Fluor™ 680 Microscale Protein Labeling Kit

Catalog #	72118
Kit Size	3 Conjugation Reactions

- This kit is optimized to conjugate HiLyte Fluor™ 680, SE 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.
- The entire process can take as short as half an hour.

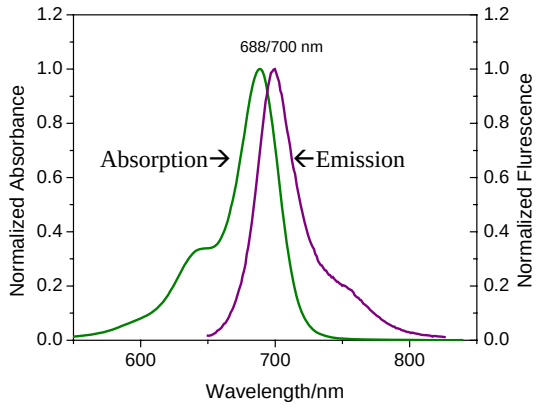
Kit Components, Storage and Handling

Component	Function	Quantity
A. HiLyte Fluor™ 680, SE	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.
- Do not freeze Component C, the Spin column.

Introduction



Physical and Spectral Properties of HiLyte Fluor™ 680, SE:

- **Fluorescence: Red**
- **Molecular weight: 1556.02**
- **Maximal absorption: 678 nm**
- **Maximal emission: 699 nm**
- **Reactive form: Succinimidyl esters (amine-reactive)**

HiLyte Fluor™ 680, SE is an excellent amine-reactive fluorescent labeling dye that can be used for generating protein conjugates. HiLyte Fluor™ 680 has spectral characteristics similar to those of Cy™ 5.5 (Invitrogen), resulting in an optimal match to filters designed for Cy5.5 dyes. Fluorescence emission of HiLyte Fluor™ 680 is well separated from that of other commonly used red fluorophores, such as TAMRA, R-phycoerythrin and HiLyte Fluor™ 647 dyes, making it ideal for multi-color labeling.

The AnaTag™ HiLyte Fluor™ 680 Protein Labeling Kit provides a convenient way to label proteins by using the succinimidyl ester (SE) reactive form of HiLyte Fluor™ 680. 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™ 680-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™ 680 -protein conjugates.

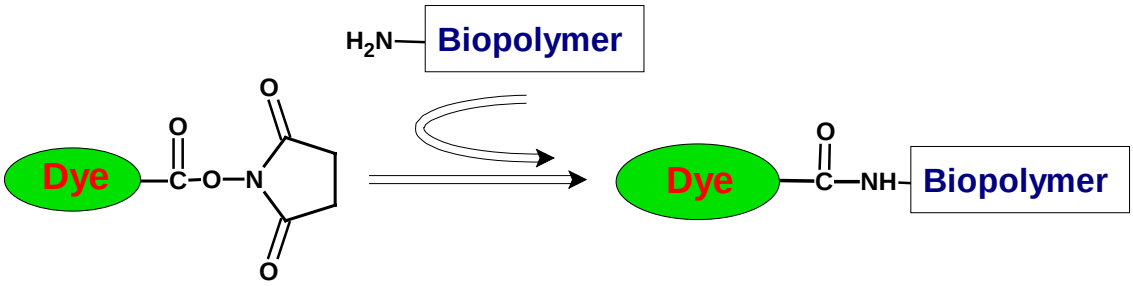


Figure 1. Labeling of an amino group (for instance, a lysine) on a biopolymer (i.e., a protein) with a succinimidyl ester of a dye.

Protocol

Note: Warm all kit components until room temperature before using the kit.

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: 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. The purification column included in this kit can maximally purify 100µl conjugate solution. Protein solution may be concentrated using a speed vacuum or a centrifugal filter (Millipore, Cat# 42407).

2. Preparing the dye solution

Add 10 µL of DMSO (Component D) to one vial of HiLyte Fluor™ 680, SE (Component A). 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 HiLyte Fluor™ 680, SE. For proteins other than IgG, the optimal conditions may need to be determined.

3.1 Add the dye solution to the solution of IgG or your protein. Mix thoroughly. Table 1 gives a quick reference for labeling IgG.

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

Ig G	Dye solution
50 µg	2 µL
100 µg	4 µL
150 µg	6 µL
200 µg	8 µL

3.2 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 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 1,000x 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 1,000x 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 1,000x 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. To determine the DOS of HiLyte Fluor™ 680 labeled proteins:

1. Read absorbance at 280 nm (A_{280}) and 678 (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_{280nm}). The maximal absorption of HiLyte Fluor™ 680 (A_{max}) is approximately at 678 nm.

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

$[Dye] = (A_{max} \times \text{dilution factor}) / \varepsilon_{\text{HiLyte Fluor™680}}$ $\quad \varepsilon_{\text{HiLyte Fluor™680}} = 190{,}000\ cm^{-1}M^{-1}$

ε is the extinction coefficient.

Molar concentration of protein:

$[Protein] = ((A_{280} - 0.09 \times A_{max}) \times \text{dilution factor}) / \varepsilon_{protein}$ $\quad \varepsilon_{IgG=} 203{,}000\ cm^{-1}M^{-1}$

DOS = [Dye]/[Protein]

Protein concentration in mg/mL for IgG:

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

For effective labeling, the degree of substitution should fall within 2-6 moles of HiLyte Fluor™ 680 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. *Methods Mol. Biol.* **45,** 205 (1995).
3. Brinkley, M. *Bioconjug Chem.* **3,** 2 (1992).
4. Banks, PR. and DM. Paquette *Bioconjug Chem.* **6**, 447 (1995).