AnaTag™ Protein Labeling Kit for TR-FRET

Revision number:1.2	*Last updated: September, 2017*
Catalog #	**AS-72247**
Kit Size	2x1 Conjugation Reactions

- This kit is optimized to label proteins for TR-FRET assay development
- It provides ample materials to perform 1 protein conjugation with Europium and 1 protein conjugation with HiLyte™ Fluor 647.
- One conjugation reaction can label up to 200 µg proteins (IgG).
- The entire process only takes about an hour.

Kit Components, Storage and Handling

Component	Function	Quantity
A. Europium isothiocyanate (MW = 674.45)	Amino-reactive dye	1 vial
B. HiLyte™ Fluor 647, SE (MW = 1302.7)	Amino-reactive dye	1 vial
C. Reaction buffer	For pH adjustment of the conjugation reaction	0.8mL
D. Solvent Buffer	For dissolving Europium	50µL
E: DMSO	For dissolving HiLyte™ Fluor 647, SE	200µL
F. Spin column	To purify dye-protein conjugate	2 pre-packed columns
G. Elution buffer	Buffer for eluting dye-protein conjugate	20 mL
H. Wash tube	Holds buffer for spin column	2 tubes
I. Collect tube	Collects dye-protein conjugate	2 tubes

Storage and Handling

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

Introduction

TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) involves an energy transfer between two fluorophores in close proximity: a donor molecule with long-lived fluorescence and an acceptor molecule with short-lived fluorescence (Fig.1).

Lanthanide metals, such as terbium (Tb) and europium (Eu), serve as energy donors. Their fluorescence is long-lived and is characterized by a large Stoke's shift providing a high signal-to-noise ratio due to minimal crosstalk between excitation and emission wavelengths.

Conventional fluorescent dyes and fluorescent proteins, serve as energy acceptors. TR-FRET pairs are chosen to achieve overlap of the donor emission and the acceptor absorbance spectra. Eu is usually paired with red-shifted dyes (Allophycocyanine, Cy5, HiLyte™ Fluor 647, or Alexa Fluor 647) as energy acceptors.

An important condition for TR-FRET measurement is the short distance between donor and acceptor molecules (up to 100 Å). Since TR-FRET requires proximity of donor and acceptor to occur, it becomes an important tool in assay development for studies of protein-protein, protein-peptide interactions, biomarkers and analytes. Since all these assays are performed in homogenous formats they are ideal tools for HTS.

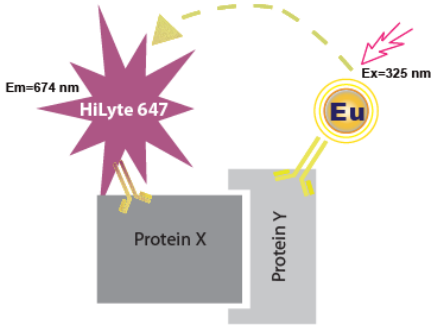


Figure 1. Schematic representation of TR-FRET energy transfer from Eu-labeled donor to HiLyte™ Fluor 647-labeled acceptor.

I. Properties of Europium reactive dye:

AnaSpec Europium (Eu) is an excellent tool for generating Eu-protein conjugates, and offers advantages over conventional fluorophores. Europium's long-lived fluorescence and large Stoke's shift allow for no background interference during assay measurements from the sample's auto-fluorescence. The signal of Europium can be monitored at excitation/emission wavelengths = 325 nm/620 nm. When Eu conjugates are used for TR-FRET with HiLyte™647-labelled reagents the signal can be detected at excitation/emission wavelengths = 325 nm/670 nm.

This kit provides a convenient way to label proteins by using the isothiocyanate reactive form of Europium which couples to free amino groups on proteins to make a covalent thiourea bond (Fig.2).

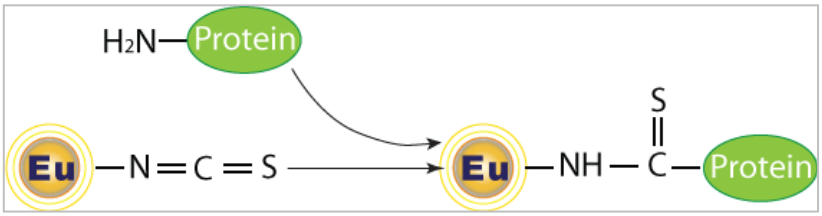


Figure 2. Europium Labeling of an amino group (for instance, a lysine) on a biopolymer (i.e., a protein) with an isothiocyanate reactive group on Europium.

Protocol A: Conjugation of protein with Europium

1. Preparing the protein solution

1.1 Add reaction buffer (component C) 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 or carbonate 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 0.1 M sodium bicarbonate, pH =9.0. 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: If protein to be labeled can be prepared in 0.1M sodium bicarbonate buffer pH 9.0, step1 should be omitted.

Note 3: The conjugation efficiency is poor when the protein concentration is less than 2 mg/mL. Meanwhile, the purification column included in this kit can purify a maximum of 100 µl of conjugate solution. A concentration step can be performed on the protein solution using a speed vacuum or a centrifugal filter (Millipore, Cat# MRCPRT010), if needed.

2. Preparing the Europium solution

Add 8 µL of solvent buffer (Component D) to one vial of Europium (Component A). Completely dissolve all the Europium contents by vortexing.

3. Performing the conjugation reaction

Note: The procedure given here is optimized for IgG (MW ~ 150,000) labeling with Europium.

3.1 Add 8µl of Europium solution to the 200µg solution of IgG from Step 1, or amount specified in Table 1 below.

Note: The molecular weight of IgG is 150 kDa.

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

Ig G	Europium solution
100 µg	4 µL
150 µg	6 µL
200 µg	8 µL

3.2 Keep the reaction mixture away from light and shake for about half an 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 F) 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 H) and centrifuge at 1,000 x g for 2 min. Discard the eluted buffer.

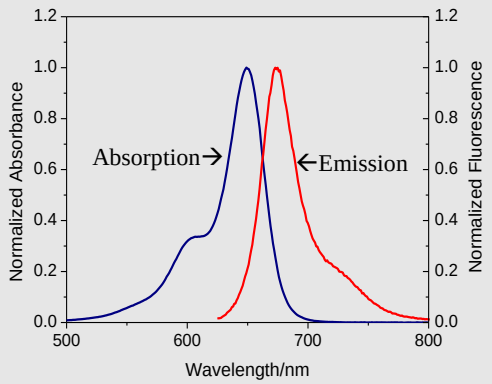
4.3 Exchange the gel-packing buffer by adding 500 µL of elution buffer (component G) to the spin column and centrifuge at 1,000 x g for 1 min. Discard the eluent. Repeat the above step three times.

4.4 Place the spin column into a clean collection tube (component I). 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 Europium-protein conjugate is in the collection tube.

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

II. Properties of HiLyte™ Fluor 647 reactive dye:



- **Fluorescence: Red**
- **Maximum absorption: 649 nm**
- **Maximum emission: 674 nm**
- **Reactive form: Succinimidyl ester (amine-reactive)**

HiLyte™ Fluor 647 is an excellent fluorescent labeling dye resulting in conjugates with a good match to filters designed for Cy5™. Conjugates of protein-HiLyte™ Fluor 647 exhibit higher fluorescence signal and more stability than that of Cy5™ conjugates.

This kit provides a convenient way to label proteins by using the succinimidyl ester (SE) reactive form of HiLyte™ Fluor 647. The succinimidyl ester couples selectively 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 3).

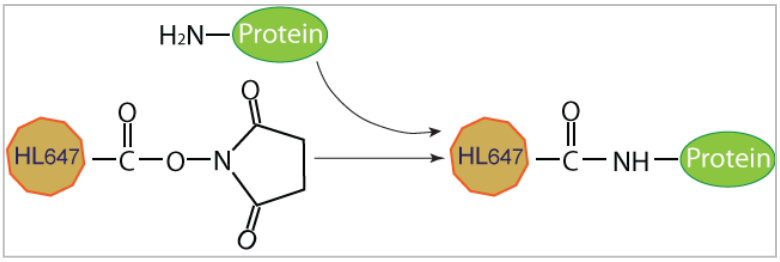


Figure 3. HiLyte™ 647 labeling of an amino group (for instance, a lysine) on a biopolymer (i.e. a protein) with a succinimidyl ester of a dye.

Protocol B: Conjugation of protein with HiLyte™ Fluor 647

1. Preparing the protein solution

Add reaction buffer (component C) 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), 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 protein concentration is less than 2 mg/mL. Meanwhile, the purification column included in this kit can purify a maximum of 100 µl of conjugate solution. A concentration step can be performed on the protein solution using a speed vacuum or a centrifugal filter (Millipore, Cat# MRCPRT010), if needed.

2. Preparing the dye solution

Add 10 µL of DMSO (component E) to one vial of HiLyte™ Fluor 647 SE (component B). 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 away from light.

3. Performing the conjugation reaction

Note: The procedure given here is optimized for IgG (MW ~ 150,000) labeling with HiLyte™ Fluor 647 SE. The dye: protein molar ratio is 10:1. 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 10:1. For 200 µg IgG, add 6.5 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 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 F) 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 H) 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 G) 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 I). 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 fluorescent dye-proteins conjugates. It provides amount of dye molecules bound with protein.

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_{280nm}).
The maximal absorption of Europium (A_{max}) is at 325nm.
The maximal absorption of HiLyte™ Fluor647 (A_{max}) is approximately at 652 nm.

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

Molar concentration of Europium:

[Europium] = (A_{325} x dilution factor) /$\varepsilon_{Europium}$ $\varepsilon_{Eu} = 21{,}600\ cm^{-1}M^{-1}$

ε is the extinction coefficient.

Molar concentration of HiLyte™ Fluor647:

[HF647] = (A_{652} x dilution factor) /$\varepsilon_{HiLyte™\ Fluor\ 647}$ $\varepsilon_{HiLyte™\ Fluor647} = 250{,}000\ cm^{-1}M^{-1}$

ε is the extinction coefficient.

Molar concentration of protein conjugate:

[Protein-Europium]= ((A_{280} – 0.42* x A_{325}) x dilution factor) /$\varepsilon_{protein}$

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

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

[Protein- HiLyte™ Fluor647]= ((A_{280} – 0.05* x A_{652}) x dilution factor)

*0.05 = 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

Storage of Dye - Protein Conjugates

The dye-labeled protein can be stored at > 0.5 mg/mL or in the presence of a carrier protein (e.g., 0.1% Bovine Serum Albumin) at 4°C for two months without significant changes if kept away from light. We recommend adding preservative (e.g. 0.01% sodium azide). 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. Glickman,FJ. et al, *J Biomol Screen* **7**, 1 (2002)