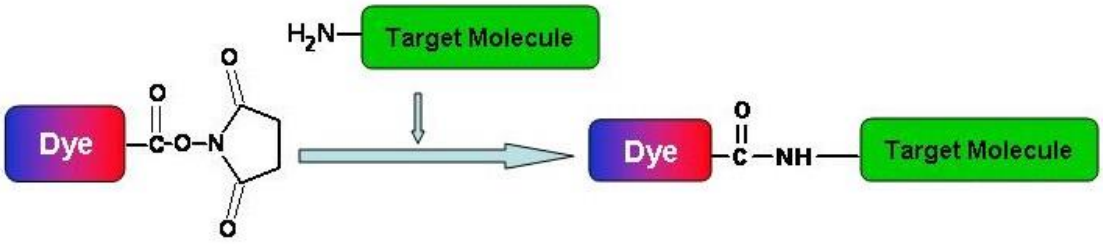
Labeling Proteins with trFluor™ Tb and trFluor™ Eu dyes Succinimidyl Esters

Introduction

Many biological compounds present in cells, serum or other biological fluids are naturally fluorescent, and thus the use of conventional, prompt fluorophores leads to serious limitations in assay sensitivity due to the high background caused by the autofluorescence of the biological molecules to be assayed. The use of long-lived fluorophores combined with time-resolved detection (a delay between excitation and emission detection) minimizes prompt fluorescence interferences. AAT Bioquest's trFluor™ Tb and trFluor™ Eu protein probes enable time-resolved fluorometry (TRF) for the assays that require high sensitivity. These trFluor™ probes have large Stokes shifts and extremely long emission half-lives when compared to more traditional fluorophores such as Alexa Fluor or cyanine dyes. Compared to the other TRF compounds, our trFluor™ Eu and trFluor™ Tb probes have relatively high stability, high emission yield and ability to be linked to biomolecules. Moreover, our trFluor™ Eu and trFluor™ Tb probes are insensitive to fluorescence quenching when conjugated to biological polymers such as antibodies.

Succinimidyl (NHS) esters are proven to be the best reagents for amine modifications because the amide bonds that are formed are essentially identical to, and as stable as the natural peptide bonds. These reagents are generally stable and show good reactivity and selectivity with aliphatic amines. There are few factors that need be considered when succinimidyl esters compounds are used for conjugation reaction: 1). *Solvents:* For the most part, reactive dyes should be dissolved in anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO). 2). *Reaction pH:* The labeling reactions of amines with succinimidyl esters are strongly pH dependent. Amine-reactive reagents react with non-protonated aliphatic amine groups, including the terminal amines of proteins and the ε-amino groups of lysines. Thus amine acylation reactions are usually carried out above pH 7.5. Protein modifications by succinimidyl esters can typically be done at pH 8.5-9.5. 3). *Reaction Buffers:* Buffers that contain free amines such as Tris and glycine and thiol compounds must be avoided when using an amine-reactive reagent. Ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation must also be removed (such as via dialysis) before performing dye conjugations. 4). *Reaction Temperature:* Most conjugations are done at room temperature. However, either elevated or reduced temperature may be required for a particular labeling reaction.



Storage and Handling

Upon receipt, trFluor™ SE should be stored at <-15 °C, and kept from light and moisture. The reconstituted DMSO stock solution of trFluor™ SE can be stored at <-15 °C for less than two weeks. The protein conjugate should be stored at > 0.5 mg/mL in the presence of a carrier protein (e.g., 0.1% bovine serum albumin). The conjugate solution could be stored at 4 °C for two months without significant change when stored in the presence of 2 mM sodium azide and kept from light. For longer storage, the protein conjugates could be lyophilized or divided into single-used aliquots and stored at ≤ –60 °C, and protected from light.

Sample Labeling Protocol

Note: This labeling protocol was developed for the conjugate of Goat anti-mouse IgG with trFluor™ Eu SE. You might need further optimization for your particular proteins.

1. Prepare protein stock solution (Solution A):

Mix 100 µL of a reaction buffer (e.g., 1 M sodium carbonate solution or 1 M phosphate buffer with pH ~9.0) with 900 µL of the target protein solution (e.g. antibody, protein concentration >2 mg/ml if possible) to give 1 mL protein labeling stock solution.

Note 1*: The pH of the protein solution (Solution A) should be 8.5 ± 0.5. If the pH of the protein solution is lower than 8.0, adjust the pH to the range of 8.0-9.0 using 1 M sodium bicarbonate solution or 1 M pH 9.0 phosphate buffer.*

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Note 2*: The protein should be dissolved in 1X phosphate buffered saline (PBS), pH 7.2-7.4. If the protein is dissolved in Tris or glycine buffer, it must be dialyzed against 1X PBS, pH 7.2-7.4, to remove free amines or ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation.*
Note 3*: Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or gelatin will not be labeled well. The presence of sodium azide or thimerosal might also interfere with the conjugation reaction. Sodium azide or thimerosal can be removed by dialysis or spin column for optimal labeling results.*
Note 4*: The conjugation efficiency is significantly reduced if the protein concentration is less than 2 mg/mL. For optimal labeling efficiency the final protein concentration range of 2-10 mg/mL is recommended.*

2. Prepare dye stock solution (Solution B):

Add anhydrous DMSO into the vial of trFluor™ Eu SE to make a 10-20 mM stock solution. Mix well by pipetting or vortex.

Note: *Prepare the dye stock solution (Solution B) before starting the conjugation. Use promptly. Extended storage of the dye stock solution may reduce the dye activity. Solution B can be stored in freezer for two weeks when kept from light and moisture. Avoid freeze-thaw cycles.*

3. Determine the optimal dye/protein ratio (optional):

Note: *Each protein requires distinct dye/protein ratio, which also depends on the properties of dyes. Over labeling of a protein could detrimentally affects its binding affinity while the protein conjugates of low dye/protein ratio gives reduced sensitivity. We recommend you experimentally determine the best dye/protein ratio by repeating Steps 4 and 5 using a serial different amount of labeling dye solutions. In general 4-6 dyes/protein are recommended for most of dye-protein conjugates.*

3.1 Use 10:1 molar ratio of Solution B (dye)/Solution A (protein) as the starting point: Add 5 µl of the dye stock solution (Solution B, assuming the dye stock solution is 10 mM) into the vial of the protein solution (95 µl of Solution A) with effective shaking. The concentration of the protein is ~0.05 mM assuming the protein concentration is 10 mg/mL and the molecular weight of the protein is ~200KD.

Note: *The concentration of the DMSO in the protein solution should be <10 %.*

3.2 Run conjugation reaction (see Step 4 below).

3.3 Repeat #3.2 with the molar ratios of Solution B/Solution A at 5:1; 15:1 and 20:1 respectively.

3.4 Purify the desired conjugates using premade spin columns.

3.5 Calculate the dye/protein ratio (DOS) for the above 4 conjugates (see next page).

3.6 Run your functional tests of the above 4 conjugates to determine the best dye/protein ratio to scale up your labeling reaction.

4. Run conjugation reaction:

4.1 Add the appropriate amount of dye stock solution (Solution B) into the vial of the protein solution (Solution A) with effective shaking.

Note: *The best molar ratio of Solution B/Solution is determined from Step 3.6. If Step 3 is skipped, we recommend to use 10:1 molar ratio of Solution B (dye)/Solution A (protein).*

4.2 Continue to rotate or shake the reaction mixture at room temperature for 30-60 minutes.

5. Purify the conjugation

The following protocol is an example of dye-protein conjugate purification by using a Sephadex G-25 column.

5.1 Prepare Sephadex G-25 column according to the manufacture instruction.

5.2 Load the reaction mixture (directly from Step 4) to the top of the Sephadex G-25 column.

5.3 Add PBS (pH 7.2-7.4) as soon as the sample runs just below the top resin surface.

5.4 Add more PBS (pH 7.2-7.4) to the desired sample to complete the column purification. Combine the fractions that contain the desired dye-protein conjugate.
Note 1: *For immediate use, the dye-protein conjugate need be diluted with staining buffer, and aliquoted for multiple uses.*
Note 2: *For longer term storage, dye-protein conjugate solution need be concentrated or freeze dried (see below).*

Characterize the Desired 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 too. The optimal DOS for most antibodies is recommended between 2 and 10 depending on the properties of dye and protein. For effective labeling, the degree of substitution should be controlled to have 4-10 moles of trFluor™ Eu SE to one mole of antibody. The following steps are used to determine the DOS of trFluor™ Eu SE labeled proteins.

1. Measure absorption:

To measure the absorption spectrum of a dye-protein conjugate, it is recommended to keep the sample concentration in the range of 1-10 μM depending on the extinction coefficient of the dye.

2. Read OD (absorbance) at 280 nm and dye maximum absorption (λ_{max} = 346 nm for trFluor™ Eu dye):

For most spectrophotometers, the sample (from the column fractions) need be diluted with de-ionized water so that the OD values are in the range of 0.1 to 0.9. The OD (absorbance) at 280 nm is the maximum absorption of protein while 346 nm is the maximum absorption of trFluor™ Eu SE. To obtain accurate DOS, make sure that the conjugate is free of the non-conjugated dye.

3. Calculate DOS using the following equations:

3.1 Calculate protein concentration $[\text{Protein}] = \frac{\text{OD280} - (\text{OD @ Dye Maximum Absoprtion X CF@280nm})}{\text{Protein Extinction Coefficient}} \times \text{dilution factor}$

3.2 Calculate dye concentration $[\text{Dye}] = \frac{\text{OD @ Maximum Absoprtion}}{\text{Dye Extinction Coefficient}} \times \text{dilution factor}$

3.3 Calculate the degree of labeling DOS = [Dye]/[Protein] =$[OD_{346} \times \varepsilon_{280}\,(\text{protein})] / [22000 \times (OD_{280} - 0.777\ OD_{346})]$

[Dye] is the dye concentration, and can be readily calculated from the Bee-Lambert Law: OD =ε_{dye}CL. [Protein] is the protein concentration. This value can be either estimated by the weight (added to the reaction) if the conjugation efficiency is high enough (preferably > 70%) or more accurately calculated by the Beer-Lambert Law: OD =$\varepsilon_{protein}$CL. For example, IgG has the ε value to be 203,000 $cm^{-1}M^{-1}$. ${}^{P}\varepsilon_{280}$ = protein molar extinction coefficient at 280 nm (e. g. the molar extinction coefficient of IgG is 203,000 $cm^{-1}M^{-1}$). CF (dye absorption correction factor at 280 nm) = OD_{280}/OD_{346} = 0.777 for trFluor™ Eu dye SE. 22,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of trFluor™ Eu SE.

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Appendix. trFluor™ Fluorescence-Labeling Dye Selection Guide

trFluor™ Doners	Ex(nm)	Em (nm)	Extinction Coefficient[1]	CF at 260 nm[2]	CF at 280 nm[3]	Recommended Acceptors	Ordering Information
triFluor™ Eu	346 nm	617 nm	~22,000 $cm^{-1}M^{-1}$	0.777	0.797	iFluor™ 647, TF5, APC, Alexa Fluor® 647	#1433 (SE, NH_2-reactive) #1434 (maleimide, SH-reactive) #1300 (labeling kit)
trFluor™ Tb	401 nm	420 nm	~20,000 $cm^{-1}M^{-1}$	0.911	0.942	iFluor™ 488, TF2, FITC, Alexa Fluor® 488	#1443 (SE, NH_2-reactive) #1444 (maleimide, SH-reactive) #1305 (labeling kit)

Notes: 1. Extinction Coefficient at their maximum absorption wavelength; 2. CF at 260 nm is the correction factor used for eliminating the dye contribution to the absorbance at 260 nm (for oligo and nucleic acid labeling); 3. CF at 280 nm is the correction factor used for eliminating the dye contribution to the absorbance at 280 nm (for peptide and protein labeling); 4. Alexa Fluor® is the trademarks of Molecular Probes. iFluor™ is the trademark of AAT Bioquest.

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