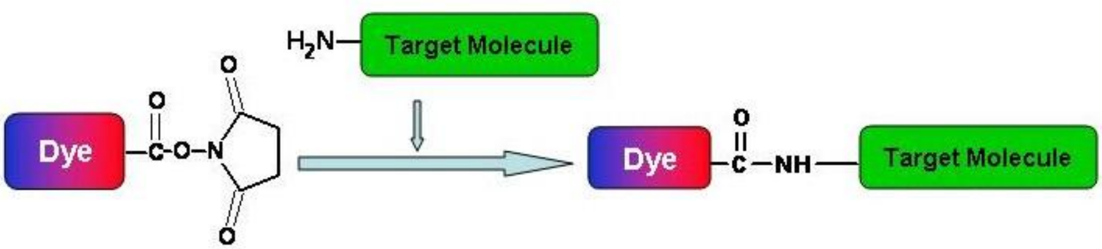
Labeling Proteins with iFluor™ Dye Succinimidyl Esters (SE or NHS Ester)

Introduction

iFluor™ dyes are a series of excellent fluorescent labeling dyes that span the full visible spectrum. All the iFluor™ dyes have excellent water solubility. Their hydrophilic property minimizes the use of organic solvents. The iFluor™ dyes also have much better labeling performance than the classic fluorescent labeling dyes such as FITC, TRITC, Texas Red®, Cy3®, Cy5® and Cy7®. Some of our iFluor™ dyes significantly outperform Alexa Fluor® labeling dyes on certain antibodies. They are the best affordable fluorescent dyes (alternative to Alexa Fluor® dyes) for labeling proteins and nucleic acids without comprised performance. Each iFluor™ dye was developed to match the spectral properties of a particular Alexa Fluor® or other labeling dyes (such as DyLight™ dyes).

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, iFluor™ dyes should be stored at <-15 °C, and kept from light and moisture. The reconstituted DMSO stock solution of iFluor™ dyes 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 iFluor™ 647 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.*
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 iFluor™ dye 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 iFluor™ 647 SE to one mole of antibody. The following steps are used to determine the DOS of iFluor™ 647 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} = 651 nm for iFluor™ 647 dyes):

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 O.D. (absorbance) at 280 nm is the maximum absorption of protein while 651 nm is the maximum absorption of iFluor™ 647 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 $[\text{Protein}] = \frac{\text{A280} - (\text{OD @ Dye Maximum Absoprtion X CF@280nm})}{\text{Protein Extinction Coefficient}} \times \text{dilution factor}$

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] $= [{}^{D}OD_{651} \times {}^{P}\varepsilon_{280}] / [250000 \times (A_{280} - 0.006A_{651})]$

[Dye] is the dye concentration, and can be readily calculated from the Bee-Lambert Law: $A=\varepsilon_{dye}CL$. [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: $A=\varepsilon_{protein}CL$. 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_{651} = 0.006 for iFluor™ 647 dye SE. 250,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of iFluor™ 647 SE.

References

1. Hermanson GT (1996). *Biocojugate 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.

Appendix 1. iFluor™ Fluorescence-Labeling Dye Selection Guide Chart

iFluor™	Ex(nm)	Em (nm)	Features and Benefits	Ordering Information
iFluor™ 350	345 nm	442 nm	*Alternative to Alexa Fluor® 350 and DyLight™ 350* • Much stronger absorption • Much stronger fluorescence • Less environment-sensitive	#1050 (maleimide, SH-reactive) #1020 (SE, NH_2-reactive) #1220 (labeling kit)
iFluor™ 405	401 nm	420 nm	*Alternative to Cascade Blue®, Alexa Fluor® 405 and DyLight™ 405* • pH-insensitive fluorescence • Photostable	#1051 (maleimide, SH-reactive) #1021 (SE, NH_2-reactive)
iFluor™ 488	491 nm	520 nm	*Alternative to Alexa Fluor® 488 and DyLight™ 488* • pH-insensitive fluorescence • High labeling efficiency • Photostable	#1052 (maleimide, SH-reactive) #1023 (SE, NH_2-reactive) #1255 (labeling kit)
iFluor™ 514	518 nm	542 nm	*Alternative to Alexa Fluor® 514* • Strong fluorescence • Photostable	#1024 (SE, NH_2-reactive)
iFluor™ 532	542 nm	558 nm	*Alternative to Alexa Fluor® 532* • Strong fluorescence • Photostable	#1025 (SE, NH_2-reactive)
iFluor™ 555	559 nm	569 nm	*Alternative to Cy3®, Alexa Fluor® 555 and DyLight™ 550* • Strong fluorescence • More photostable than Cy3®	#1053 (maleimide, SH-reactive) #1028 (SE, NH_2-reactive) #1227 (labeling kit)
iFluor™ 594	592 nm	614 nm	*Alternative to Texas Red®, Texas Red-X, Alexa Fluor® 594 and DyLight™ 594* • Strong fluorescence • Photostable	#1054 (maleimide, SH-reactive) #1029 (SE, NH_2-reactive) #1230 (labeling kit)
iFluor™ 610	605 nm	627 nm	*Alternative to Alexa Fluor® 610* • Strong fluorescence • Photostable	#1038 (SE, NH_2-reactive)
iFluor™ 633	638 nm	655 nm	*Alternative to Alexa Fluor® 633* • Strong fluorescence • Photostable	#1030 (SE, NH_2-reactive) #1260 (labeling kit)
iFluor™ 647	654 nm	674 nm	*Alternative to Cy5®, Alexa Fluor® 647 and DyLight™ 650* • Strong fluorescence • More photostable than Cy5®	#1055 (maleimide, SH-reactive) #1031 (SE, NH_2-reactive) #1235 (labeling kit)
iFluor™ 680	682 nm	701 nm	*Alternative to Cy5.5®, IRDye® 700, Alexa Fluor® 680 and DyLight™ 680* • Strong fluorescence • More photostable than Cy5.5®	#1056 (maleimide, SH-reactive) #1035 (SE, NH_2-reactive) #1240 (labeling kit)
iFluor™ 700	693 nm	713 nm	*Alternative to Alexa Fluor® 700* • Strong fluorescence • Good photostability	#1057 (maleimide, SH-reactive) #1036 (SE, NH_2-reactive) #1245 (labeling kit)
iFluor™ 750	753 nm	779 nm	*Alternative to Alexa Fluor® 750 and DyLight™ 750* • Stronger fluorescence • More photostable than Cy7®	#1058 (maleimide, SH-reactive) #1037 (SE, NH_2-reactive) #1250 (labeling kit)
iFluor™ 790	782 nm	811 nm	*Alternative to IRDye® 80, Alexa Fluor® 790 and DyLight™ 800* • Stronger fluorescence • Higher Photostability	#1059 (maleimide, SH-reactive) #1368 (SE, NH_2-reactive) #1265 (labeling kit)

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Appendix 2. Spectral Properties of iFluor™ Fluorescent Labeling Dyes

Labeling Dye	Extinction Coefficient[1]	Abs (nm)	Em (nm)	FQY[2]	CF at 260 nm[3]	CF at 280 nm[4]
iFluor™ 350	20,000 $cm^{-1}M^{-1}$	345	442	0.95	0.246	0.187
iFluor™ 405	29,000 $cm^{-1}M^{-1}$	401	420	0.91	0.229	0.697
iFluor™ 488	75,000 $cm^{-1}M^{-1}$	491	514	0.90	0.444	0.139
iFluor™ 514	80,000 $cm^{-1}M^{-1}$	518	542	0.95	0.316	0.182
iFluor™ 532	81,000 $cm^{-1}M^{-1}$	542	558	0.90	0.354	0.192
iFluor™ 555	150,000 $cm^{-1}M^{-1}$	559	569	0.10[5]	0.038	0.082
iFluor™ 594	90,000 $cm^{-1}M^{-1}$	592	614	0.91	0.234	0.187
iFluor™ 610	90,000 $cm^{-1}M^{-1}$	605	627	0.85	0.468	0.441
iFluor™ 633	250,000 $cm^{-1}M^{-1}$	638	655	0.24	0.062	0.045
iFluor™ 647	250,000 $cm^{-1}M^{-1}$	654	674	0.25	0.046	0.039
iFluor™ 680	220,000 $cm^{-1}M^{-1}$	682	701	0.18	0.121	0.111
iFluor™ 700	220,000 $cm^{-1}M^{-1}$	693	713	0.20	0.188	0.164
iFluor™ 750	275,000 $cm^{-1}M^{-1}$	753	779	0.12	0.114	0.114
iFluor™ 790	250,000 $cm^{-1}M^{-1}$	782	811	0.09	0.250	0.224

Note 1. Extinction Coefficient at their maximum absorption wavelength; 2. FQY = fluorescence quantum yield in aqueous buffer (pH 7.2); 3. 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); 5. Fluorescence intensity is significantly increased upon coupled to proteins.

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