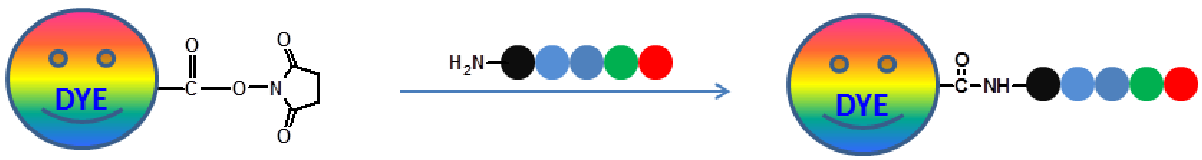
Labeling Proteins with iFluor™ Dye Succinimidyl Esters

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.*

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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).*

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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} = 649 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 649 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] $= [{}^{D}OD_{649} \times {}^{P}\varepsilon_{280}] / [250000 \times (A_{280} - 0.03A_{649})]$

[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_{649} = 0.03 for iFluor™ 647 dye SE. 250,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of iFluor™ 647 SE.

References

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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.

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

iFluor™ Dye	Ex(nm)	Em (nm)	Features and Benefits	Ordering Information
***i*Fluor™ 350**	345 nm	450 nm	*Alternative to Alexa Fluor® 350 and DyLight™ 350* • Much stronger absorption • Much stronger fluorescence • Less environment-sensitive	#1020 (SE, NH_2-reactive) #1220 (labeling kit) #1050 (maleimide, SH-reactive)
***i*Fluor™ 405**	404 nm	428 nm	*Alternative to Cascade Blue®, Alexa Fluor® 405 and DyLight™ 405* • pH-insensitive fluorescence • Photostable	#1021 (SE, NH_2-reactive) #1051 (maleimide, SH-reactive)
***i*Fluor™ 430**	427 nm	496 nm	*Alternative to Alexa Fluor® 430* • pH-insensitive fluorescence • Photostable	#1052 (SE, NH_2-reactive) #1054 (maleimide, SH-reactive)
***i*Fluor™ 450**	478 nm	510 nm	• pH-insensitive fluorescence • Photostable	#1026 (SE, NH_2-reactive)
***i*Fluor™ 488**	492 nm	516 nm	*Alternative to Alexa Fluor® 488 and DyLight™ 488* • pH-insensitive fluorescence • High labeling efficiency • Photostable	#1023 (SE, NH_2-reactive) #1052 (maleimide, SH-reactive) #1255 (labeling kit)
***i*Fluor™ 514**	511 nm	527 nm	*Alternative to Alexa Fluor® 514* • Strong fluorescence • Photostable	#1024 (SE, NH_2-reactive)
***i*Fluor™ 532**	537 nm	560 nm	*Alternative to Alexa Fluor® 532* • Strong fluorescence • Photostable	#1025 (SE, NH_2-reactive) #1061 (maleimide, SH-reactive)
***i*Fluor™ 546**	541 nm	557 nm	*Alternative to Alexa Fluor® 546* • Strong fluorescence • Photostable	#1048 (SE, NH_2-reactive)
***i*Fluor™ 555**	552nm	567 nm	*Alternative to Cy3®, Alexa Fluor® 555 and DyLight™ 550* • Strong fluorescence • More photostable than Cy3®	#1028 (SE, NH_2-reactive) #1053 (maleimide, SH-reactive) #1227 (labeling kit)
***i*Fluor™ 568**	568 nm	587 nm	*Alternative to Alexa Fluor® 568* • Strong fluorescence • Photostable	#1049 (SE, NH_2-reactive) #1055 (maleimide, SH-reactive)
***i*Fluor™ 594**	588 nm	604 nm	*Alternative to Texas Red®, Texas Red-X, Alexa Fluor® 594 and DyLight™ 594* • Strong fluorescence • Photostable	#1029 (SE, NH_2-reactive) #1054 (maleimide, SH-reactive) #1230 (labeling kit)
***i*Fluor™ 610**	604 nm	623 nm	*Alternative to Alexa Fluor® 610* • Strong fluorescence • Photostable	#1038 (SE, NH_2-reactive)
***i*Fluor™ 633**	638 nm	653 nm	*Alternative to Alexa Fluor® 633* • Strong fluorescence • Photostable	#1030 (SE, NH_2-reactive) #1056 (maleimide, SH-reactive) #1260 (labeling kit)
***i*Fluor™ 647**	649 nm	664 nm	*Alternative to Cy5®, Alexa Fluor® 647 and DyLight™ 650* • Strong fluorescence • More photostable than Cy5®	#1031 (SE, NH_2-reactive) #1055 (maleimide, SH-reactive) #1235 (labeling kit)
***i*Fluor™ 660**	663 nm	678 nm	*Alternative to Alexa Fluor® 660 and* • Strong fluorescence	#1032 (SE, NH_2-reactive)
***i*Fluor™ 680**	674 nm	692 nm	*Alternative to Cy5.5®, IRDye® 700, Alexa Fluor® 680 and DyLight™ 680* • Strong fluorescence • More photostable than Cy5.5®	#1035 (SE, NH_2-reactive) #1056 (maleimide, SH-reactive) #1240 (labeling kit)
***i*Fluor™ 700**	686 nm	705 nm	*Alternative to Alexa Fluor® 700* • Strong fluorescence • Good photostability	#1036 (SE, NH_2-reactive) #1067 (maleimide, SH-reactive) #1245 (labeling kit)
***i*Fluor™ 710**	716 nm	755 nm	• Strong fluorescence • Good photostability	#1045 (SE, NH_2-reactive)
***i*Fluor™ 750**	754 nm	779 nm	*Alternative to Alexa Fluor® 750 and DyLight™ 750*	#1037 (SE, NH_2-reactive)

			• Stronger fluorescence • More photostable than Cy7®	#1058 (maleimide, SH-reactive) #1250 (labeling kit)
iFluor™ A7	760 nm	780 nm	*Alternative to BD H7 and HiLyte Fluor™ 750* • Optimized for preparing APC tandem conjugate • More photostable than Cy7®	#1039 (SE, NH_2-reactive)
***i*Fluor™ 790**	782 nm	803 nm	*Alternative to IRDye® 800, Alexa Fluor® 790 and DyLight™ 800* • Stronger fluorescence • Higher Photostability	#1059 (maleimide, SH-reactive) #1265 (labeling kit) #1368 (SE, NH_2-reactive)
***i*Fluor™ 800**	801 nm	814 nm	• Stronger fluorescence • Higher Photostability	#1378 (maleimide, SH-reactive) #1379 (SE, NH_2-reactive)
***i*Fluor™ 810**	812 nm	826 nm	• Stronger fluorescence • Higher Photostability	#1388 (maleimide, SH-reactive) #1389 (SE, NH_2-reactive)
***i*Fluor™ 820**	820 nm	849 nm	• Stronger fluorescence • Higher Photostability	#1398 (maleimide, SH-reactive) #1399 (SE, NH_2-reactive)
***i*Fluor™ 860**	812 nm	826 nm	• Stronger fluorescence • Higher Photostability	#1408 (maleimide, SH-reactive) #1409 (SE, NH_2-reactive)

Appendix 2. Spectral Properties of iFluor™ Fluorescent Labeling Dyes

Labeling Dye	Extinction Coefficient[1] ($cm^{-1}M^{-1}$)	Abs(nm)	Em(nm)	FQY[2]	CF at 260 nm[3]	CF at 280 nm[4]
iFluor 350	20000	348	450	0.95	0.83	0.23
iFluor 405	37000	403	428	0.91	0.48	0.77
iFluor 430	40000	427	496	0.78	0.68	0.3
iFluor 450	40000	449	504	0.82	0.45	0.27
iFluor 488	75000	492	516	0.9	0.21	0.11
iFluor 514	80000	511	527	0.83	0.25	0.11
iFluor 532	90000	536	560	0.68	0.26	0.16
iFluor 546	100000	543	557	0.67	0.25	0.15
iFluor 555	100000	552	567	0.64	0.23	0.14
iFluor 568	100000	570	587	0.57	0.34	0.15
iFluor 594	180000	587	604	0.53	0.05	0.04
iFluor 610	110000	605	623	0.85	0.32	0.49
iFluor 633	250000	638	653	0.29	0.062	0.044
iFluor 647	250000	648	664	0.25	0.03	0.03
iFluor 660	250000	662	678	0.26	0.07	0.08
iFluor 680	220000	675	692	0.23	0.097	0.094
iFluor 700	220000	684	705	0.23	0.09	0.04
iFluor 710	190000	713	735	0.14	0.12	0.07
iFluor 750	275000	754	779	0.12	0.044	0.039
iFluor A7	275000	759	780	0.1	0.03	0.03
iFluor 790	250000	784	803	0.13	0.1	0.09
iFluor 800	250000	801	814	0.11	0.03	0.08
iFluor 810	250000	812	826	0.05	0.09	0.15
iFluor 820	250000	820	849	N/A [5]	0.11	0.16
iFluor 860	250000	852	877	N/A [5]	0.1	0.14

Note:
1. Extinction Coefficient at its maximum absorption wavelength in aqueous buffer (pH 7.2);
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);
4. 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. FQY = fluorescence quantum yield is not determined at date when this form is released.

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