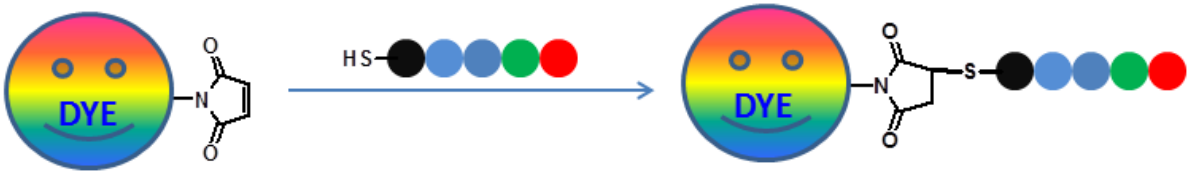
Labeling Proteins with iFluor™ Dye Maleimides

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).

Because free thiol (SH) groups, also called mercapto groups, are not present as abundantly as amino groups in most peptides, thiol-reactive reagents often provide a means of selectively modifying a peptide at a defined site. Thiol-reactive dyes are often used to prepare fluorescent peptides for probing biological structures, functions and interactions. There are many types of thiol-reactive dyes reported in the literature, including iodoacetamides, disulfides, maleimides, vinyl sulfones and various electron-deficient aryl halides and sulfonates. Maleimide is by far the most popular thiol-reactive moiety. Maleimides readily react with thiol moieties of biopolymers to form thioether conjugates even under neutral conditions. The thioether bond formed is quite stable. Maleimides require conjugation conditions less stringent than those of iodoacetamides, and do not react with histidine and methionine under physiological conditions. For example, most conjugations can be done at room temperature at neutral pH.



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™ dye maleimides can be stored at <-15 °C for up to 4 weeks. The desired 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 maleimide. You might need further optimization for your particular proteins.

1. Prepare protein stock solution (Solution A):

1.1. (Optional) if your protein does not contain a free cysteine, you must treat your protein with DTT or TCEP to generate a thiol group. 10 molar equivalents of DTT or TCEP are sufficient for converting a disulfide bond to two free thiol groups. If DTT is used you must remove free DTT by dialysis or gel filtration before conjugating a dye maleimide to your protein. Following is a sample protocol for generating a free thiol group:

a) Prepare a fresh solution of 1 M DTT (15.4 mg/100 µl) in distilled water.
b) Make IgG solution in 20 mM DTT: add 20 µl of DTT stock per ml of IgG solution while mixing. Let stand at room temp for 30 minutes without additional mixing (to minimize reoxidation of cysteines to cystines).
c) Pass the reduced IgG over a filtration column pre-equilibrated with "Exchange Buffer". Collect 0.25 ml fractions off the column.
d) Determine the protein concentrations and pool the fractions with the majority of the IgG. This can be done either spectrophotometrically or colorimetrically.
e) Carry out the conjugation as soon as possible after this step (see below).

Note 1: *IgG solutions should be >4 mg/ml for the best results. The antibody should be concentrated if less than 2 mg/ml. Include an extra 10% for losses on the buffer exchange column.*
Note 2: *The reduction can be carried out in almost any buffers from pH 6 to 7, e.g., MES, phosphate or TRIS buffers.*
Note 3: *Steps c and d can be replaced by dialysis.*

1.2. Mix 100 µL of a reaction buffer (e.g., 100 mM MES buffer with pH ~6.0) 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 6.5 ± 0.5.*
Note 2*: Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or other proteins will not be labeled well.*
Note 3*: 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 maleimide to make a 10-20 mM stock solution. Mix well by pipetting or vortex under subdued light (if possible).

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 up to 4 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 2-6 moles of iFluor™ 647 dye to one mole of antibody. The following steps are used to determine the DOS of iFluor™ 647 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 dye. 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 $\text{DOS} = [\text{Dye}]/[\text{Protein}] = [{}^{D}OD_{649} \times {}^{P}\varepsilon_{280}] / [250000 \times (A_{280} - 0.03A_{649})]$

[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 dye.

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™ Dye	Ex(nm)	Em (nm)	Features and Benefits	Ordering Information
***i*Fluor™ 350**	345 nm	442 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**	401 nm	420 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™ 488**	491 nm	514 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**	518 nm	542 nm	*Alternative to Alexa Fluor® 514* • Strong fluorescence • Photostable	#1024 (SE, NH_2-reactive)
***i*Fluor™ 532**	542 nm	558 nm	*Alternative to Alexa Fluor® 532* • Strong fluorescence • Photostable	#1025 (SE, NH_2-reactive)
***i*Fluor™ 555**	555 nm	565 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™ 594**	594 nm	614 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**	605 nm	627 nm	*Alternative to Alexa Fluor® 610* • Strong fluorescence • Photostable	#1038 (SE, NH_2-reactive)
***i*Fluor™ 633**	638 nm	655 nm	*Alternative to Alexa Fluor® 633* • Strong fluorescence • Photostable	#1030 (SE, NH_2-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™ 680**	676 nm	695 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**	685 nm	710 nm	*Alternative to Alexa Fluor® 700* • Strong fluorescence • Good photostability	#1036 (SE, NH_2-reactive) #1067 (maleimide, SH-reactive) #1245 (labeling kit)
***i*Fluor™ 750**	749 nm	776 nm	*Alternative to Alexa Fluor® 750 and DyLight™ 750* • Stronger fluorescence • More photostable than Cy7®	#1037 (SE, NH_2-reactive) #1058 (maleimide, SH-reactive) #1250 (labeling kit)
i*Fluor™ *A7	758 nm	784 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	811 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)

Ordering: sales@aatbio.com; Tel: 800-990-8053 or 408-733-1055; Fax: 408-733-1304
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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	20,000	345	442	0.95	0.246	0.187
iFluor™ 405	29,000	401	420	0.91	0.229	0.697
iFluor™ 488	75,000	491	514	0.90	0.444	0.139
iFluor™ 514	80,000	518	542	0.95	0.316	0.182
iFluor™ 532	81,000	542	558	0.90	0.354	0.192
iFluor™ 555	150,000	555	565	0.10[5]	0.042	0.073
iFluor™ 594	110,000	594	614	0.15[5]	0.116	0.070
iFluor™ 610	90,000	605	627	0.85	0.468	0.441
iFluor™ 633	250,000	638	655	0.24	0.062	0.045
iFluor™ 647	250,000	649	665	0.25	0.026	0.030
iFluor™ 680	220,000	676	695	0.18	0.094	0.101
iFluor™ 700	220,000	685	710	0.20	0.040	0.036
iFluor™ 750	275,000	749	775	0.13	0.025	0.036
iFluor™ 790	250,000	782	811	0.09	0.098	0.091
iFluor™ A7	275,000	758	784	0.15	0.012	0.022

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.

Note 2. Texas Red® and Alexa Fluor® are the trademarks of Molecular Probes. CyDye, Cy3®, Cy5®, Cy5.5® and Cy7® are the trademarks of GE Health Care. HiLyte Fluor™ is tthe trademark of AnaSpec. IRDye® 700 and IRDye® 800 are the trademarks of Li-COR. iFluor™ is the trademark of AAT Bioquest.