iFluor® 560 succinimidyl ester

Catalog number: 1040, 71040, 71518, 71568
Unit size: 1 mg, 100 ug, 5 mg, 10 mg

Component	Storage	Amount (Cat No. 1040)	Amount (Cat No. 71040)	Amount (Cat No. 71518)	Amount (Cat No. 71568)
iFluor® 560 succinimidyl ester	Freeze (< -15 °C), Minimize light exposure	1 mg	100 µg	5 mg	10 mg

OVERVIEW

Cy3 is recognized as the dimmest fluorophore in the Cy dye family. To overcome this limitation, Cy3B was developed as an enhanced derivative, exhibiting a substantial increase in fluorescence quantum yield and photostability. Despite these improvements, Cy3B's limited aqueous solubility presents significant challenges for its efficient conjugation to biomolecules. In response, iFluor® 560 has been introduced, offering enhanced water solubility while retaining spectral properties closely aligned with both Cy3B and Cy3, positioning it as a robust alternative for the development of bioconjugates with bright orange fluorescence. It is optimally excited by 532 nm and 561 nm laser lines and is fully compatible with TRITC filter sets, facilitating its integration into existing experimental workflows. iFluor® 560 is suitable for labeling a wide range of antigens, including cell surface, intracellular, and intranuclear targets. It performs exceptionally well in complex multicolor panels, bridging the gap between fluorophores like FITC and PE. In simpler panels, iFluor® 560 can effectively replace PE to minimize emission spreading into PE tandem dyes. This dye allows for high molar ratio conjugation to proteins with minimal self-quenching, resulting in brighter conjugates. Its robust fluorescence quantum yield and photostability make iFluor® 560 ideal for detecting low-abundance biological targets, delivering greater precision and sensitivity in quantitative fluorescence assays. The succinimidyl ester of iFluor® 560 is a widely used reagent for the conjugation of this dye to proteins or antibodies. Succinimidyl esters react selectively and efficiently with primary amines (such as the side chains of lysine residues or aminosilane-coated surfaces) at pH 7–9, forming stable covalent amide bonds. This property makes iFluor® 560 succinimidyl ester an excellent choice for labeling proteins, amine-modified oligonucleotides, and other amine-containing molecules.

PREPARATION OF STOCK SOLUTIONS

Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles

Protein stock solution (Solution A)

Mix 100 µL of a reaction buffer (e.g., 1 M sodium bicarbonate 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: 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: 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: 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: 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.

iFluor® 560 SE stock solution (Solution B)

Add anhydrous DMSO into the vial of iFluor® 560 SE to make a 10 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.

SAMPLE EXPERIMENTAL PROTOCOL

This labeling protocol was developed for the conjugate of Goat anti-mouse IgG with iFluor® 560 SE. You might need further optimization for your particular proteins.

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.

Run conjugation reaction

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: We recommend to use 10:1 molar ratio of Solution B (dye)/Solution A (protein). If it is too less or too high, determine the optimal dye/protein ratio at 5:1, 15:1 and 20:1 respectively.

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

Purify the conjugation

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

1. Prepare Sephadex G-25 column according to the manufacture instruction.
2. Load the reaction mixture (From "Run conjugation reaction") to the top of the Sephadex G-25 column.
3. Add PBS (pH 7.2-7.4) as soon as the sample runs just below the top

resin surface.

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: For immediate use, the dye-protein conjugate need be diluted with staining buffer, and aliquoted for multiple uses.

   Note: For longer term storage, dye-protein conjugate solution need be concentrated or freeze dried.

EXAMPLE DATA ANALYSIS AND FIGURES

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 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 5-8 moles of iFluor® 560 SE to one mole of antibody. The following steps are used to determine the DOS of iFluor® 560 SE labeled proteins.

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.

Read OD (absorbance) at 280 nm and dye maximum absorption (λmax = 571 nm for iFluor® 560 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 571 nm is the maximum absorption of iFluor® 560 SE. To obtain accurate DOS, make sure that the conjugate is free of the non-conjugated dye.

Calculate DOS

You can calculate DOS using our tool by following this link: https://www.aatbio.com/tools/degree-of-labeling-calculator

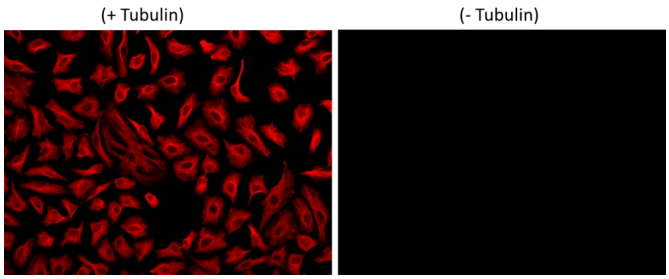

Figure 1. HeLa cells were incubated with (+ Tubulin) or without (- Tubulin) mouse anti-tubulin followed by iFluor® 560 goat anti-mouse IgG conjugate stain and visualized with Cy3 Filter.

DISCLAIMER

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