

**XFD647 NHS Ester**

Catalog number: 1833, 71909  
Unit size: 1 mg, 5 mg

| Component        | Storage                                    | Amount (Cat No. 1833) | Amount (Cat No. 71909) |
|------------------|--------------------------------------------|-----------------------|------------------------|
| XFD647 NHS Ester | Freeze (< -15 °C), Minimize light exposure | 1 vial (1 mg)         | 1 vial (5 mg)          |

**OVERVIEW**

XFD647, manufactured by AAT Bioquest, is a bright far-red fluorescent dye structurally similar to Alexa Fluor™ 647 (Thermo Fisher). It is efficiently excited by the 594 nm or 633 nm laser lines and is compatible with RFP filters like Cy5, making it well-suited for applications such as fluorescence microscopy and flow cytometry. XFD647 exhibits excellent aqueous solubility and pH stability across a wide range (pH 4–10), ensuring robust signal reproducibility under diverse experimental conditions. The dye enables high-degree conjugation to biomolecules with minimal self-quenching, resulting in enhanced fluorescence intensity and signal stability. Additionally, its high fluorescence quantum yield and superior photostability facilitate the sensitive detection of low-abundance targets, enhancing precision and sensitivity in quantitative fluorescence-based assays.

The N-hydroxysuccinimidyl (NHS) ester of XFD647 is a widely used reagent for the conjugation of this dye to proteins or antibodies. NHS 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 XFD647 NHS 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)**

1. Prepare a 1 mL protein labeling stock solution by mixing 100 µL of reaction buffer (such as 1 M sodium carbonate solution or 1 M phosphate buffer, pH ~9.0) with 900 µL of the target protein solution (e.g., an antibody with a protein concentration of at least 2 mg/mL, if possible).

**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 it to within the 8.0–9.0 range using either 1 M sodium bicarbonate solution or 1 M phosphate buffer at pH 9.0.

**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, dialyze it against 1X PBS, pH 7.2–7.4, to remove any free amines or ammonium salts (such as ammonium sulfate and ammonium acetate) commonly used in protein precipitation.

**Note:** Antibodies that are impure or stabilized with bovine serum albumin (BSA) or gelatin may not label effectively. Additionally, sodium azide or thimerosal can interfere with the conjugation reaction. To achieve optimal labeling results, these preservatives should be removed through dialysis or spin column techniques.

**Note:** For optimal labeling efficiency, it is recommended to maintain a final protein concentration between 2–10 mg/mL. Protein concentrations below 2 mg/mL can significantly reduce

conjugation efficiency.

**XFD647 NHS Ester Stock Solution (Solution B)**

1. To prepare a 10 mM stock solution of XFD647 NHS ester, add anhydrous DMSO directly to the vial of XFD647 NHS ester. Mix well by pipetting or vortexing.

**Note:** Prepare the dye stock solution (Solution B) before starting the conjugation, and use it promptly. Extended storage of the dye stock solution may reduce the dye activity. Solution B can be stored in the freezer for up to two weeks, provided it is protected from light and moisture. Avoid freeze-thaw cycles.

**SAMPLE EXPERIMENTAL PROTOCOL**

This protocol is designed for labeling Goat anti-mouse IgG with XFD647 NHS ester. Additional optimization may be required to adapt the protocol to your specific proteins.

**Note:** Each protein requires a distinct dye/protein ratio, which varies depending on the characteristics of the dye. Over-labeling a protein can negatively impact its binding affinity, whereas using a low dye-to-protein ratio in protein conjugates can result in reduced sensitivity.

**Run Conjugation Reaction**

1. Use a 10:1 molar ratio of Solution B (dye) to 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) to the vial containing 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 using a 10:1 molar ratio of Solution B (dye)/Solution A (protein). If it is too low 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 Conjugate**

The following protocol demonstrates the purification of a dye-protein conjugate using a Sephadex G-25 column.

1. Prepare the Sephadex G-25 column according to the manufacturer's instructions.
2. Carefully load the reaction mixture (from the "Run Conjugation Reaction" step) 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 must be diluted with staining buffer, and aliquoted for multiple uses.

**Note:** For longer-term storage, the dye-protein conjugate solution needs to be concentrated or freeze-dried.

### Characterize the Desired Dye-Protein Conjugate

The Degree of Substitution (DOS) is a critical factor in characterizing dye-labeled proteins. Proteins with a lower DOS generally exhibit weaker fluorescence, while those with a higher DOS (e.g., DOS > 6) may also show reduced fluorescence. The optimal DOS for most antibodies typically ranges between 2 and 10, depending on the specific properties of both the dye and the protein. For effective labeling, it is recommended to achieve a DOS of 6-8 moles of XFD647 NHS ester per mole of antibody. The following steps outline the process for determining the DOS of XFD647 NHS ester-labeled proteins.

### Measure Absorption

For accurate measurement of the absorption spectrum of a dye-protein conjugate, maintain the sample concentration between 1-10  $\mu$ M, adjusting as needed based on the dye's extinction coefficient.

### Read OD (absorbance) at 280 nm and Dye Maximum Absorption ( $\lambda_{max}$ = 650 nm for XFD647 NHS Ester)

For most spectrophotometers, the sample (from the column fractions) needs to be diluted with de-ionized water so that the O.D. 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 650 nm is the maximum absorption of XFD647 NHS ester. To obtain accurate DOS, ensure 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>

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