iFluor® 488 maleimide

Catalog number: 1062
Unit size: 1 mg

Component	Storage	Amount (Cat No. 1062)
iFluor™ 488 maleimide	Freeze (< -15 °C), Minimize light exposure	1 vial (1 mg)

OVERVIEW

Although FITC is still the most popular fluorescent labeling dye for preparing green fluorescent bioconjugates, there are certain limitations with FITC, such as severe photobleaching for microscope imaging and pH-sensitive fluorescence. Protein conjugates prepared with iFluor® 488 dyes are far superior to conjugates of fluorescein derivatives such as FITC. iFluor® 488 conjugates are significantly brighter than fluorescein conjugates and are much more photostable. Additionally, the fluorescence of iFluor® 488 is not affected by pH (4-10). This pH insensitivity is a major improvement over fluorescein, which emits its maximum fluorescence only at pH above 9. iFluor® 488 maleimide is reasonably stable and shows good reactivity and selectivity with the thiol group. This iFluor® 488 has spectral properties and reactivity similar to Alexa Fluor® 488 maleimide (Alexa Fluor® is the trademark of Invitrogen).

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

iFluor® 488 maleimide Stock Solution (Solution B)

1. Add anhydrous DMSO into the vial of iFluor® 488 maleimide to make a 10 mM stock solution. Mix well by pipetting or vortex.

   Note: For optimal results, prepare the dye stock solution (Solution B) before starting the conjugation process. Remember to use it promptly, as extended storage of the dye stock solution may reduce its reactivity. Solution B can be stored in the freezer for up to 4 weeks, protected from light and moisture. Avoid freeze-thaw cycles.

Protein Stock Solution (Solution A)

1. 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 a 1 mL protein labeling stock solution.

   Note: The pH of the protein labeling stock solution solution (Solution A) should be 6.5 ± 0.5.

   Note: Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or other proteins will not be labeled well.

   Note: The conjugation efficiency is significantly reduced if the protein concentration is less than 2 mg/mL. For optimal labeling efficiency, it is recommended that the final protein concentration range between 2-10 mg/mL.

2. **Optional.** If your protein does not already contain a free cysteine, it is necessary to treat it with either DTT or TCEP to generate a thiol group. This process is used to convert a disulfide bond into two free thiol groups. If DTT is used, it is important to remove any excess free DTT by dialysis or gel filtration prior to conjugating a dye maleimide to the protein. Below is a sample protocol for generating a free thiol group:

   1. Prepare a fresh solution of 1 M DTT (15.4 mg/100 µL) in distilled water.
   2. To make an IgG solution in 20 mM DTT, add 20 µL of DTT stock per ml of IgG solution while mixing. Let the solution stand at room temperature for 30 minutes without additional mixing (to minimize the reoxidation of cysteines to cystines).
   3. Pass the reduced IgG over a filtration column pre-equilibrated with "Exchange Buffer". Collect 0.25 mL fractions off the column.
   4. Determine the protein concentrations and pool the fractions with the majority of the IgG. This can be done either spectrophotometrically or colorimetrically.
   5. Carry out the conjugation as soon as possible after this step (see Sample Experiment Protocol).

      Note: For the best results, IgG solutions should be >4 mg/mL. If the antibody is less than 2 mg/mL, it should be concentrated. Include an extra 10% for losses on the buffer exchange column.

      Note: The reduction can be carried out in almost any buffer from pH 7 to 7.5, e.g., MES, phosphate, or TRIS buffers.

      Note: Steps 3 and 4 can be replaced by dialysis.

SAMPLE EXPERIMENTAL PROTOCOL

This labeling protocol was developed for the labeling Goat anti-mouse IgG with iFluor® 488 maleimide. Further optimization may be required for your specific proteins.

Note: Each protein requires a distinct dye/protein ratio, which also depends on the properties of dyes. Over-labeling of a protein could detrimentally affect its binding affinity while the protein conjugates of low dye/protein ratio give reduced sensitivity.

Run Conjugation Reaction

1. Use a 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) to the vial of the protein solution (95 µL of Solution A) with effective shaking. The protein concentration is ~0.05 mM, assuming the protein concentration is 10 mg/mL and the protein molecular weight is ~200KD.

   Note: We recommend using a 10:1 molar ratio of Solution B (dye) to Solution A (protein). If the ratio 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 Conjugation

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

1. Prepare the Sephadex G-25 column according to the manufacturer's instructions.

2. Load the reaction mixture (from the "Run Conjugation Reaction" section) 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 should 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.

Optional: Characterize the Desired Dye-Protein Conjugate

Determining the Degree of Substitution (DOS) is crucial in characterizing dye-labeled proteins. Lower DOS proteins tend to have weaker fluorescence, but higher DOS proteins may also have reduced fluorescence. For most antibodies, the optimal DOS is between 2 and 10, depending on the dye and protein properties. For effective labeling, the degree of substitution should be controlled to have 5-8 moles of iFluor® 488 maleimide to one mole of antibody. The following steps are used to determine the DOS of iFluor® 488 maleimide-labeled proteins:

1. Measure absorption—To measure the absorption spectrum of a dye-protein conjugate, the sample concentration should be kept between 1 and 10 μM, depending on the dye's extinction coefficient.

2. Read OD (absorbance) at 280 nm and dye maximum absorption (λmax = 516 nm for iFluor® 488 dyes). For most spectrophotometers, the sample (from the column fractions) must be diluted with de-ionized water so that the OD values range from 0.1 to 0.9. The O.D. (absorbance) at 280 nm is the maximum absorption of protein, while 516 nm is the maximum absorption of iFluor® 488 maleimide. To obtain accurate DOS, ensure the conjugate is free of the non-conjugated dye.

3. Calculate DOS using our DOS calculator:

   https://www.aatbio.com/tools/degree-of-labeling-calculator

EXAMPLE DATA ANALYSIS AND FIGURES

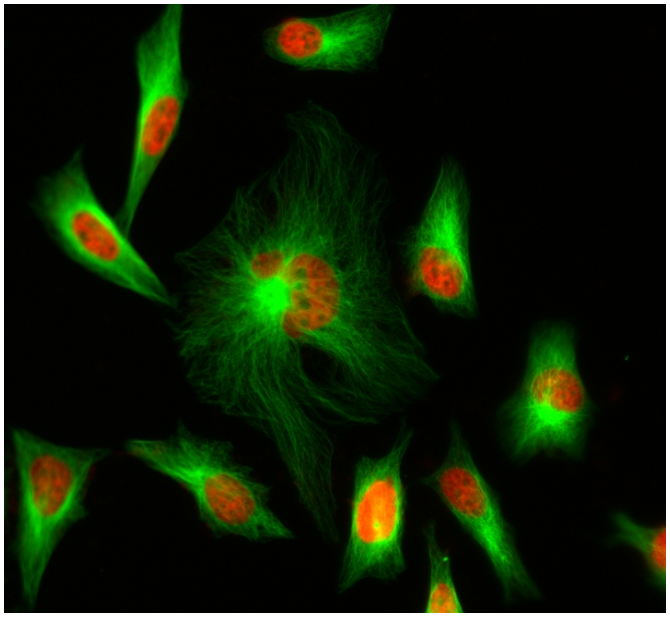

Figure 1. HeLa cells were stained with rabbit anti-tubulin followed by iFluor® 488 goat anti-rabbit IgG (H+L), and nuclei were stained with Nuclear Red™ DCS1 (Cat No. 17552).

DISCLAIMER