Labeling Proteins with FITC-xtra

Introduction

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 FITC-xtra are far superior compared to the corresponding FITC conjugates. FITC-xtra conjugates are significantly brighter than FITC conjugates and are much more photostable. Additionally, the fluorescence of FITC-xtra is not affected by pH (4-10). This pH insensitivity is a major improvement over FITC, which emits its maximum fluorescence only at pH above 9. FITC-xtra has spectral properties almost identical FITC. In addition, FITC-xtra give much higher conjugation yield under mild conjugation conditions than FITC. Like 5-FITC, FITC-xtra antibody conjugates have excitation ideally suited to the 488 nm laser line, making them alternatives to the corresponding FITC-labeled antibody conjugates. Under the same conditions tested, FITC-xtra antibody conjugates give much higher signal/background ratios than the corresponding FITC-labeled conjugates.

Sample Labeling Protocol

Note: This labeling protocol was developed for the conjugate of Goat anti-mouse IgG with FITC-xtra. 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.*
***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 FITC-xtra 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. Prepare the desired protein conjugate:

***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. Add the appropriate amount of dye stock solution (Solution B) into the vial of the protein solution (Solution A) with effective shaking. Continue to rotate or shake the reaction mixture at room temperature for 30-60 minutes.

3.3 Repeat #3.2 with the molar ratios of Solution B/Solution A at 5:1; 15:1 and 20:1 respectively if desired.

3.4 Purify the desired conjugates using premade spin columns or other techniques.

3.5 Calculate the dye/protein ratio (DOS) for the above 4 conjugates (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 4-10 moles of FITC-xtra to one mole of antibody. The following steps are used to determine the DOS of FITC-xtra 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 @496 nm:

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 496 nm is the maximum absorption of FITC-xtra. 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 $[\text{Protein}] = \frac{\text{A280} - (\text{OD @ 496 nm X 0.12})}{\text{Protein Extinction Coefficient}} \times \text{dilution factor}$

3.2 Calculate dye concentration $[\text{Dye}] = \frac{\text{OD @ 496 nm}}{80{,}000} \times \text{dilution factor}$

3.3 Calculate the degree of labeling DOS = [Dye]/[Protein] $= [OD_{496} \times {}^{P}\varepsilon_{280}] / [200000 \times (OD_{280} - 0.12 \times OD_{496})]$

[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 200,000 $cm^{-1}M^{-1}$. ${}^{P}\varepsilon_{280}$ = protein molar extinction coefficient at 280 nm (e. g. the molar extinction coefficient of IgG is 200,000 $cm^{-1}M^{-1}$). CF (dye absorption correction factor at 280 nm) = OD_{280}/OD_{496} = 0.12 for FITC-xtra. 80,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of FITC-xtra.

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.