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AFDye 568 Antibody Labeling Kit

* for labeling of 100 µg of antibody *

Product No. 1458

Introduction

The AFDye 568 Antibody Labeling Kit provides all of the necessary reagents to perform labeling of small amounts of antibodies or other proteins (except IgM antibodies) with green-fluorescent AFDye 568 (Alexa Fluor® 568 equivalent). Simply mix your antibody with the reaction additive and pre-measured dye provided, followed by a brief incubation, and the conjugate is ready for simple purification or staining without further purification (see Note 1). The antibody will be covalently labeled with an average of 4-6 dyes per label molecule per antibody molecule. This kit is optimized for the labeling of 100 µg of antibodies per reaction at 1 mg/mL.

This kit utilizes 2,3,5,6-tetrafluorophenyl (TFP) esters instead of succinimidyl ester (SE or NHS) often used in conventional labeling kits. TFP is another type of carboxylic acid derivative that reacts with primary amines to form covalent amide bonds. The amine linkage bond is identical to the one formed by the reaction between primary amines and NHS esters or sulfo-NHS esters. However, in most cases, TFP ester displays much better stability toward hydrolysis in aqueous media resulting in more efficiency and better reproducibility in labeling of biopolymers. As a result of improved efficiency, very little of the non-reactive (hydrolyzed) dye is left in the labeling mixture, which allows staining without further purification.

AFDye 568 produces pH insensitive (pH 3-10), more photostable, and brighter protein conjugates compared to the previous generation of dyes (fluorescein/FITC). AFDye 568 labeled antibodies can be used for different applications, such as flow cytometry, fluorescent microscopy, ELISA, and Western blotting.

Kit Contents

Component	Amount	Storage
AFDye 568 TFP Ester (Component A)	2 vials	-20^{0}C to 4^{0}C, protect from light
Sodium Hydrogen Carbonate (Component B)	1 vial	4^{0}C powder, -20^{0}C solution
Desalting Columns (Component C)	2 columns	4^{0}C

Protein Preparation

For the most effective reaction, the protein should be in a buffer that is free of primary amines and ammonium ions, as they compete with the amine groups of the protein for the reactive dye. The presence of low concentrations of sodium azide (<3 mM or 0.02%) or thimerosal (<1 mM or 0.04%) will not interfere with the reaction. Antibodies stabilized with bovine serum albumin (BSA) or gelatin will not label well. In such cases, for purification of antibodies, one can use commercially available kits such as Abcam Antibody Purification Kit (Protein A) (ab102784), GOLD antibody purification kit (ab204909), Antibody Purification Kit (Protein G) (ab128747), or the BSA Removal Kit (ab173231).

Antibody Labeling

1.1 Add 0.5 mL of deionized water to the vial of sodium hydrogen carbonate to prepare a 1 M solution. Vortex until fully dissolved. This solution can be stored and remain stable at -20°C for a long period of time or at +4°C for two weeks.

1.2 Dilute the antibody to be labeled to 1 mg/mL with a suitable buffer and then add 1/10 v/v of 1 M sodium hydrogen carbonate.

If the antibody is lyophilized from a suitable buffer, prepare a 1 mg/mL solution of it by adding a suitable amount of 0.1 M sodium hydrogen carbonate.

Note: Sodium hydrogen carbonate, pH 8-9, is added to raise the pH of the reaction mixture, as TFP esters react efficiently only at alkaline pH.

1.3 Transfer 100 µl of the protein solution to the tube of reactive dye. Pipet the solution up and down or cap the tube and gently invert it a few times until the dye is fully dissolved. Do not vortex, as vortexing results in protein denaturation.

1.4 Incubate the solution for 1 hour at room temperature. It is recommended that the vial be gently inverted several times every 15-20 minutes to increase the labeling efficiency and to prevent overlabeling.

Purification of the Labeled Antibody

To remove the unbound dye from the dye-conjugated antibody and reduce fluorescent background in further applications, this purification step can be followed:

2.1 Place a desalting spin column in a 2 ml centrifuge tube.

2.2 Centrifuge at 1500 x g for 2 minutes and discard flow-through.

2.3 Wash the column with 200 µl of PBS for 2 minutes at 1500 x g. Discard flow-through.

2.4 Repeat step 2.3 two more times.

2.5 Load the 100 µl reaction volume to the desalting spin column. Allow the resin to adsorb the solution.

2.6 Place the spin column into an empty centrifuge tube and centrifuge for 3 minutes at 1500 x g. Discard the spin column.

2.7 After centrifugation, the collection tube will contain the labeled antibody. If one needs to store the antibody for a long period of time, we suggest stabilizing it with 20-50% v/v Glycerol, 0.05% sodium azide, and 0.05-1% BSA.

Degree of Labeling (DOL) Measuring

3.1 Dilute a small amount of the purified conjugate in PBS or other suitable buffer and measure the absorbance in a cuvette with a 1-cm pathlength at 280 nm (A280) and at 577 nm (A577).

Calculate the concentration of protein in the sample:

$$Protein\ concentration\ (M) = \frac{[A280\ -\ (A577\ x\ 0.46)]\ x\ dilution\ factor}{203{,}000}$$

where 203,000 is the molar extinction coefficient (ε) in $cm^{-1}\ M^{-1}$ of a typical IgG, IgA, IgD, and IgE at 280 nm.

3.2 Calculate the degree of labeling:

$$DOL = \frac{A577\ x\ dilution\ factor}{91{,}300\ x\ protein\ concentration\ (M)}$$

where 91,300 is the molar extinction coefficient (ε) in $cm^{-1}\ M^{-1}$ of AFDye 568 at 577 nm.

3.3 If using a NanoDrop®, the nominal pathlength is 1 mm. For the DOL calculation, use 2,030,000 and 913,000 instead of 203,000 and 91,300.

3.4 If using a cuvette of a pathlength smaller than 1 cm, multiply the 203,000 and 91,300 by the ratio of the cuvette pathlength per 1 cm (10 mm). For example, if using a cuvette with a 2 mm pathlength, (10 mm / 2 mm) = 5. Multiply the numbers by 5.

An optimal degree of labeling for whole IgG is 3-6.

Storage of prepared conjugates

Store the labeled antibody at +4°C, protected from light. If it is necessary to store the antibody for a long period of time, we suggest stabilizing it with 20-50% v/v glycerol, 0.05% Sodium azide, and 0.05-1% BSA. Then, aliquot and store at -20°C**. Avoid repeated freezing and thawing. Protect from light.**

Troubleshooting

Underlabeling

If calculations point out that the protein is labeled with significantly lower than optimal DOL, repeat the labeling using second tube of provided reactive dye. Don't forget to add another 1/10 v/v of 1 M sodium hydrogen carbonate to the solution of protein.

Underlabeling might be caused by a number of conditions:

- *Trace amounts of primary amines in the buffer.* If the antibody has been dissolved in primary amine-containing buffers (Tris, glycine, BSA), purify it by dialysis versus PBS before labeling or use commercially available antibody purification kits.
- *Low concentration of antibody.* If the concentration of antibody solution is less than 0.5 mg/mL, the reaction of labeling will not proceed efficiently.
- *Low pH.* If the antibody is strongly buffered at a low pH, the addition of sodium hydrogen carbonate will not raise the pH to the optimal level. If so, more sodium hydrogen carbonate can be added or the buffer can be exchanged either to PBS, or to 0.1 M sodium bicarbonate, pH 8.3.
- Due to the unique properties of each protein, the standard protocol may not always result in optimal labeling. To increase the DOL, the labeling procedure can be repeated using a second vial of the reactive dye, another 1/10 v/v of 1 M sodium hydrogen carbonate, and underlabeled sample following the same protocol. For some proteins, better labeling can be achieved with overnight incubations at +4°C after an initial incubation of one hour at room temperature.

Overlabeling

If calculations point out that the antibody-dye conjugate is significantly higher than the optimal DOL, the protein is most likely overlabeled. For some applications, conjugates with high DOL may be acceptable for use, however the overlabeling can cause aggregation of the antibodies and reduced specificity for the antigen, both of which can lead to nonspecific staining. In addition, overlabeling often results in fluorescence quenching of the conjugate. To reduce the DOL, add more protein to the reaction, use smaller amount of reactive dye (for example, make a solution of reactive dye in 20 µL of water and add 10 µL to the solution of a protein), or allow the reaction to proceed for a shorter period of time (15-30 minutes).

Notes

We have tested several secondary labeled antibodies prospered with and without purification by desalting for immunocytochemical staining. For most antibody-dye conjugates the standard protocol with column purification produced slightly higher signal/noise ratios. We encourage researchers to consider whether the column purification step would significantly alter the outcome of their experiments. In addition, addition of signal enhancers, such as Image-iT® FX (available from Thermo Fisher Scientific, cat# I36933) to the cells prior to staining with the labeled protein conjugate reduced the slight background fluorescence due to the presence of free dye, producing results that were nearly indistinguishable from those obtained with a column-purified conjugate.

Importantly, we found that even without the column purification step, labeling kits that utilize TFP ester produced fluorescent conjugates that were far superior to those of other one-step labeling kits tested, in terms of signal strength and background fluorescence. Thus, with improved labeling efficiency and simplified workflow, our labeling kits provide one-step labeling convenience with high yields and bright results.