INSTRUCTIONS

Pierce NHS-Fluorescein Antibody Labeling Kit

53029

2079.2

Number	Description
53029	**Pierce NHS-Fluorescein Antibody Labeling Kit,** contains sufficient reagents to label and purify 3 × 1mg (2mg/mL) of IgG or similar amounts of other protein.

Kit Contents:

NHS-Fluorescein, 3 × 50µg vials

Molecular mass: 473.4

Extinction coefficient: 70,000M^{-1} cm^{-1}

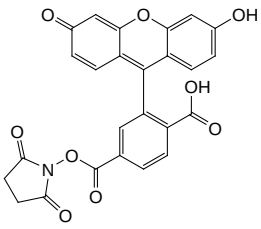
Ex/Em wavelength: 493/516nm

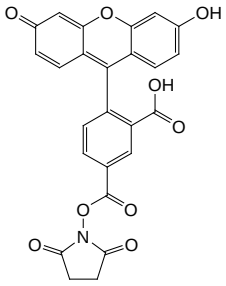
Borate Buffer (0.67M), 1mL

Purification Resin, 5mL

Spin Columns, 6 each

Microcentrifuge Collection Tubes, 12 each





Storage: Upon receipt store the NHS-Fluorescein at -20°C. Store all other components at 4°C. The kit is shipped with an ice pack.

Introduction

The Thermo Scientific™ Pierce™ NHS-Fluorescein Antibody Labeling Kit contains all the necessary components for labeling antibodies or other proteins and subsequent excess dye removal. The *N*-hydroxysuccinimide (NHS)-ester labeling reagents, such as NHS-Fluorescein, are the simplest and most commonly used reagents for labeling proteins. NHS esters react efficiently with primary amino groups (-NH_2) in pH 7-9 buffers to form stable amide bonds. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues that are available as targets for NHS-ester reagents.

The dye purification resin and spin columns eliminate equilibration steps and the need to collect and monitor gravity-flow fractions. This system enables efficient removal of excess fluor and, therefore, accurate determination of the dye-to-protein ratio and exceptional protein recovery.

Important Product Information

- NHS-Fluorescein is moisture-sensitive. To avoid moisture condensation onto the product, vial must be equilibrated to room temperature before opening.
- Hydrolysis of the NHS ester is a competing reaction and increases with increasing pH. Hydrolysis occurs more readily in dilute protein or peptide solutions. In concentrated protein solutions, the acylation reaction is favored.
- Buffers that contain primary amines (e.g., Tris or glycine) are not compatible with NHS-Fluorescein because they react with the NHS-ester moiety and compete with the intended reaction.
- For fluorescent imagers use a spectral line of a green (526) laser.
- Low concentrations of sodium azide (≤ 3mM or 0.02%) or thimerosal (≤ 0.02mM or 0.01%) will not significantly interfere with protein labeling; however, 20-50% glycerol will reduce labeling efficiency.

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Additional Materials Required

- Variable-speed centrifuge
- Phosphate-buffered Saline (PBS; for measuring the fluor-to-protein ratio)

Procedure for Antibody Labeling with NHS-Fluorescein

A. Protein Preparation

Note: If the Borate Buffer has precipitated during storage, solubilize it by warming and vortexing the vial.

1. For best results use 1mg of protein at ~2mg/mL. The optimal labeling buffer is 50mM sodium borate, pH 8.5. Prepare the protein as follows:
   - **Proteins lyophilized in PBS:** Just before use, prepare the labeling buffer by diluting the Borate Buffer (0.67M) to 0.05M in PBS or ultrapure water. Prepare only enough labeling buffer required for the reaction (for example, to prepare 1mL, add 75µL of Borate Buffer (0.67 M) to 925µL of ultrapure water or PBS). Reconstitute 1mg of protein with 0.5mL of labeling buffer.
   - **Proteins in PBS solution:** Add 40µL of the Borate Buffer (0.67M) to 0.5mL of 2mg/mL protein in PBS. If the protein is > 2mg/mL, adjust the concentration to 2mg/mL with labeling buffer (for example, 0.05M sodium borate – see the above bullet point: Proteins lyophilized in PBS).
   - **Proteins in other buffers:** Protein must not be in a buffer containing ammonium ions or primary amines (for example, Tris or glycine). If necessary, replace buffer with 50mM sodium borate (Product No. 28384), pH 8.5 by dialysis or buffer exchange.

B. Protein Labeling

1. Equilibrate all the reagents to room temperature.
2. Add 0.5mL of the prepared protein to the vial of NHS-Fluorescein Reagent and pipette up-and-down 10 times until all reagent is dissolved. Vortex briefly if required.

   Note: The reagent must be completely dissolved for effective labeling.
3. Briefly centrifuge the vial to collect the sample in the bottom of the tube.
4. Incubate the reaction mixture for 60 minutes at room temperature protected from light.

C. Protein Purification

1. Place two spin columns in separate microcentrifuge collection tubes.
2. Mix the Purification Resin to ensure uniform suspension and add 400µL of the suspension into both spin columns. Centrifuge for 30-45 seconds at ~1,000 × *g* to remove the storage solution. Discard the used collection tubes and place the columns in new collection tubes.
3. Add 250-270µL of the labeling reaction to each spin column and mix the sample with the resin by pipetting up and down or briefly vortexing.
4. Centrifuge columns for 30-45 seconds at ~1,000 × *g* to collect the purified proteins. Combine the samples from both columns (~0.5mL total). Discard the used columns.
5. Store the labeled protein protected from light at 4°C for up to one month. Alternatively, store labeled protein in single-use aliquots at -20°C. Avoid repeated freeze/thaw cycles. If the final concentration of conjugate is < 1mg/ml, add a stabilizing agent, such as bovine serum albumin at 1-10mg/mL.

D. Fluorophore-to-Protein Ratio Estimation

1. Dilute a small amount of labeled purified protein in PBS.
2. Use a 1cm path length cuvette to measure absorbance at 280nm and 495nm (i.e., A_{max} of NHS-Fluorescein).

3. Calculate protein concentration as follows:

$$\text{Protein concentration (M)} = \frac{A_{280} - (A_{max} \times CF)}{\varepsilon_{protein}} \times \text{dilution factor}$$

- $\varepsilon_{protein}$ = protein molar extinction coefficient (e.g., the molar extinction coefficient of IgG is ~210,000M^{-1} cm^{-1})
- CF = Correction factor $= \frac{A_{280}}{A_{max}} = 0.3$

4. Calculate the degree of labeling as follows:

$$\text{Moles fluor per mole protein} = \frac{A_{max} \text{ of the labeled protein}}{\varepsilon_{fluor} \times \text{protein concentration (M)}} \times \text{dilution factor}$$

- ε_{fluor} = 70,000 (NHS-Fluorescein molar extinction coefficient)

Troubleshooting

Problem	Possible Cause	Solution
Protein was not labeled	Protein buffer contained amines that interfered with labeling	Perform buffer exchange via dialysis or other method into 50mM sodium borate
	The NHS ester is hydrolyzed and non-reactive	Prepare labeling reagent immediately before use – do not store reagent in aqueous solution
The downstream application was unsuccessful	Protein was not labeled	Determine if the protein was labeled by calculating the fluorophore:protein ratio
Sample or buffer does not flow through resin	Centrifugation problem	Ensure that centrifuge is in proper working condition
Low yield	Improper centrifugation	Make sure to use the indicated centrifugation speed
		Add 40µL of suitable buffer to the top of the resin and repeat centrifugation step
	Unstable protein	Equilibrate the column with PBS or other suitable buffer before adding the labeled protein

Additional Information

Please visit our website for additional information including the following items:

- Tech Tip #43: Protein Stability and Storage
- Tech Tip #6: Extinction Coefficients Guide
- Tech Tip #31: Calculate Dye:Protein (F/P) Molar Ratios
- Tech Tip #40: Convert Between Times Gravity (× *g*) and Centrifuge Rotor Speed (RPM)

Related Thermo Scientific Products

22858	**Fluorescent Dye Removal Columns**
46402	**DyLight 488 NHS Ester,** 1mg
46403	**DyLight 488 NHS Ester,** 5 × 50µg
53024	**DyLight 488 Antibody Labeling Kit**
53025	**DyLight 488 Microscale Antibody Labeling Kit**
46409	**NHS-Fluorescein,** 1g
46410	**NHS-Fluorescein,** 100mg

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