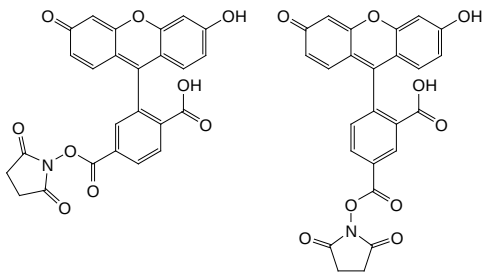
INSTRUCTIONS

NHS-Fluorescein

46409 46410

2082.1

Number	Description
46409	**NHS-Fluorescein,** 1g
46410	**NHS-Fluorescein,** 100mg



Chemical name: 5-(and 6-)carboxyfluorescein, succinimidyl ester

Molecular weight: 473.4

Ex/Em wavelength: 494/518nm

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

CAS # 76608-16-7

CAS Name: benzoic acid, 4-[{(2,5-dioxo-1-pyrrolidinyl)oxy}carbonyl](6-hydroxy-3-oxo-3H-xanthen-9-yl)

Storage: Upon receipt store product at -20°C in the foil pouch with desiccant to protect from light and moisture. Product is shipped at ambient temperature.

Introduction

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. The reaction results in the release of *N*-hydroxysuccinimide. 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.

Important Product Information

- NHS-Fluorescein is moisture-sensitive. To avoid moisture condensation onto the product, the vial must be equilibrated to room temperature before opening.
- Prepare this reagent immediately before use. The NHS-ester moiety readily hydrolyzes and becomes non-reactive; therefore, do not prepare stock solutions for storage. Discard any unused reconstituted crosslinker.
- 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.

Example Protocol for Antibody Labeling with NHS-Fluorescein

Materials Required

- Dimethylformamide (DMF; Product No. 20673) or Dimethylsulfoxide (DMSO; Product No. 20688)
- Conjugation Buffer: The optimal labeling buffer is 50mM borate, pH 8.5 (BupH™ Borate Buffer Packs, Product No. 28384). Buffers that contain primary amines (e.g., Tris or glycine) are not compatible because they will react with the NHS-ester moiety. Other non-amine-containing buffer at pH 7-9, such as 20mM sodium phosphate, 0.15M NaCl (Product No. 28372); 20mM HEPES; and 100mM carbonate/bicarbonate may also be used.
- Device to remove excess dye, such as the Fluorescent Dye Removal Columns (Product No. 22858), a Zeba™ Desalt Spin Column (Product No. 89891) or a Slide-A-Lyzer® Dialysis Cassette 10,000 MWCO (Product No. 66380)

Procedure for Antibody Labeling

A. Antibody Preparation

- If the antibody is lyophilized and salt-free, dissolve it in Conjugation Buffer. For each labeling reaction, use 50µL-1mL of purified antibody at 1-10mg/mL. After antibody reconstitution, proceed to the Calculations section. Similar quantities of other proteins also may be used.
- If the antibody is in amine-containing buffer, perform buffer exchange (by dialysis or desalting) into Conjugation Buffer.

B. Calculations

Perform the following calculations before beginning the Labeling Reaction. The amount of NHS-Fluorescein to use for each reaction depends on the amount of the protein to be labeled. By using the appropriate molar ratio of labeling reagent to protein, the extent of conjugation can be controlled. When conjugating antibody with NHS-Fluorescein, a 15- to 20-fold molar excess of the fluorescent dye is optimal; however, this ratio may be varied to alter the degree of labeling.

1. Calculate millimoles of NHS-Fluorescein labeling reagent to add to the reaction:

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{15\,\text{mmol NHS - Fluorescein}}{\text{mmol protein}} = \text{mmol NHS - Fluorescein}$$

2. Calculate microliters of NHS-Fluorescein solution to add to the reaction:

$$\text{mmol NHS - Fluorescein} \times \frac{473.4\text{mg}}{\text{mmol NHS - Fluorescein}} \times \frac{100\,\mu\text{L}}{1\text{mg}} = \mu\text{L NHS - Fluorescein}$$

- 15 = Molar excess of NHS-Fluorescein to protein
- 473.4 = Molecular weight of NHS-Fluorescein
- 100 = Microliters of solvent in which the 1mg of NHS-Fluorescein is dissolved

EXAMPLE:
For 1mL of a 1mg/mL solution of IgG (150,000 Da), 4.7µL NHS-Fluorescein will be used.

$$1\text{mL IgG} \times \frac{1\text{mg IgG}}{1\text{mL IgG}} \times \frac{\text{mmol IgG}}{150{,}000\text{mg IgG}} \times \frac{15\,\text{mmol NHS - Fluorescein}}{\text{mmol IgG}} = 0.00010\text{mmol NHS - Fluorescein}$$

$$0.00010\,\text{mmol NHS - Fluorescein} \times \frac{473.4\text{mg}}{\text{mmol NHS - Fluorescein}} \times \frac{100\,\mu\text{L}}{1\,\text{mg}} = 4.7\mu\text{L of NHS - Fluorescein}$$

C. Labeling Reaction

Note: To protect the reagent from moisture, allow NHS-Fluorescein to equilibrate to room temperature before opening.

1. Transfer the antibody solution to a reaction tube.
2. Reconstitute 1mg of NHS-Fluorescein with 100µL of DMF or DMSO.
3. Transfer the appropriate amount of NHS-Fluorescein (from **Calculations** section) to the tube containing the antibody solution.
4. Mix well and incubate at room temperature for 1 hour or on ice for 2 hours.
5. Remove non-reacted NHS-Fluorescein by dialysis or gel filtration or with a Dye Removal Column. Use the Pierce® BCA Protein Assay Kit (Product No. 23225) to estimate protein concentration.

6. Store fluorescein-labeled protein at 4°C until ready to use. To prevent microbial contamination, add a preservative, such as sodium azide to a final concentration of 0.1%.

D. Calculate the Degree of Labeling

The non-reacted dye must be completely removed for optimal results and accurate determination of the fluor-to-protein ratio.

1. Use a 1cm path length cuvette to measure the absorbance of the labeled protein at 280nm and 493nm (i.e., A_{max} of NHS-Fluorescein). It may be necessary to dilute a small amount of the desalted/dialyzed sample.
2. Calculate the protein concentration:
   - $\varepsilon_{protein}$ = protein molar extinction coefficient (e.g., the molar extinction coefficient of IgG is ~210,000 M^{-1} cm^{-1})
   - $A_{max} = A_{493}$
   - CF = Correction factor $= \frac{A_{280}}{A_{max}} = 0.3$

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

3. Calculate the degree of labeling:
   - ε_{fluor} = 70,000 (NHS-Fluorescein molar extinction coefficient)

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

Additional Information

Please visit our web site for additional information relating to this product including the following:

- Tech Tip #43: Protein Stability and Storage
- Tech Tip #6: Extinction Coefficients Guide
- Tech Tip #31: Calculate Dye:Protein (F/P) Molar Ratios

Related Thermo Scientific Products

28384	**BupH Borate Buffer Packs**, 40 packs
28372	**BupH Phosphate Buffered Saline Packs**, 40 packs
22858	**Fluorescent Dye Removal Columns**
66380	**Slide-A-Lyzer Dialysis Cassettes,** 10,000 MWCO
89889	**Zeba Desalt Spin Columns, 2 ml,** 5 each, for 200-700µL samples
89890	**Zeba Desalt Spin Columns, 2 ml,** 25 each, for 200-700µL samples
53029	**Pierce NHS-Fluorescein Antibody Labeling Kit**
53027	**Pierce FITC Antibody Labeling Kit**
53024	**DyLight 488 Antibody Labeling Kit**
53025	**DyLight 488 Microscale Antibody Labeling Kit**

General References

Harlow, E. and Lane, D. (1988). Antibodies: A laboratory manual. Cold Spring Harbor Laboratory, New York, p. 562.Khanna, P.L. and Ullman, E.F. (1980). 4´,5´-Dimethoxy-6-carboxyfluorescein: A novel dipole-dipole coupled fluorescence energy transfer acceptor useful for fluorescence immunoassays. *Anal. Biochem*. **108:**156.

Sheehan, D.C. and Hrapchak, B.B. (1980). Theory and Practice of Histotechnology. C. V. Mosby Comp. 2nd edition.

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Vigers, G.P.A., *et al*. (1988). Fluorescent microtubules break up under illumination. *J. Cell Biol*. **107:**1011.

Zuk, R.F., *et al.* (1979). Fluorescence protection immunoassay: A new homogeneous assay technique. *Clinical. Chem*. **25:**1554.

Current product instructions are available at *www.thermoscientific.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.