INSTRUCTIONS

Thermo SCIENTIFIC

NHS-Rhodamine

46406
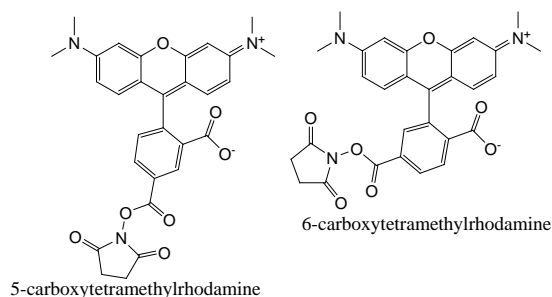
Number	Description
46406	**NHS-Rhodamine**, 25mg

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

Molecular weight: 528

Ex/Em wavelength: 552/575nm

Extinction coefficient: $80,000 M^{-1} cm^{-1}$ (in aqueous buffer at pH 9.0)

5-carboxytetramethylrhodamine

6-carboxytetramethylrhodamine

Introduction

Thermo Scientific™ NHS-Rhodamine is an amine-reactive fluorescent labeling reagent that absorbs green visible light (552nm) and emits orange-red visible light (575nm). Rhodamine displays lower sensitivity to pH than fluorescein and is more photostable; however, rhodamine conjugates have a significantly lower quantum yield than fluorescein conjugates.

N-Hydroxysuccinimide (NHS)-ester labeling reagents 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-Rhodamine 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 labeling reagent.

- 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.

Example Protocol for Antibody Labeling with NHS- Rhodamine

A. Materials Required

- Dimethylformamide (DMF; Product No. 20673) or Dimethylsulfoxide (DMSO; Product No. 20688)

- Conjugation Buffer: 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 (PBS, Product No. 28372). Other non-amine-containing buffers at pH 7-9, such as Thermo Scientific™ BupH™ Borate Buffer Packs, Product No. 28384) (Product No. 28372); 20mM HEPES; and 100mM carbonate/bicarbonate may also be used. Do not use buffers that contain primary amines (e.g., Tris or glycine) because they will react with the NHS-ester moiety and compete with the intended reaction.

- Device to remove excess dye, such as the Thermo Scientific™ Fluorescent Dye Removal Columns (Product No. 22858), Zeba™ Desalt Spin Column (Product No. 89891) or a Slide-A-Lyzer™ Dialysis Cassette, 10K MWCO (Product No. 66380)

B. Antibody Preparation

- If the antibody is lyophilized and salt-free, dissolve it in Conjugation Buffer. For each labeling reaction, use 50μL to 1mL of purified antibody sample at a concentration from 1-10mg/mL. After reconstitution, proceed to Calculations section.

- If the antibody is already in solution, exchange the buffer with Conjugation Buffer by dialysis or desalting.

C. Calculations

Perform the following calculations before beginning the Labeling Reaction. The amount of NHS-Rhodamine 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 proteins with NHS-Rhodamine, a 10- to 15-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-Rhodamine labeling reagent to add to the reaction:

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{10 \text{ mmol NHS-Rhodamine}}{\text{mmol protein}} = \text{mmol NHS-Rhodamine}$$

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

$$\text{mmol NHS-Rhodamine} \times \frac{528 \text{ mg}}{\text{mmol NHS-Rhodamine}} \times \frac{100 \, \mu\text{L}}{1 \text{ mg}} = \mu\text{L NHS-Rhodamine}$$

- 10 = Recommended molar ratio of NHS-Rhodamine to protein
- 528 = Molecular weight of NHS-Rhodamine
- 100 = Microliters of solvent in which the 1mg of NHS-Rhodamine is dissolved

EXAMPLE:
For 1mL of a 1mg/mL solution of IgG (150,000Da), 3.5μL NHS-Rhodamine will be used.

$$1 \text{ mL IgG} \times \frac{1 \text{ mg IgG}}{1 \text{ mL IgG}} \times \frac{\text{mmol IgG}}{150,000 \text{ mg IgG}} \times \frac{10 \text{ mmol NHS-Rhodamine}}{\text{mmol IgG}} = 0.00007 \text{ mmol NHS-Rhodamine}$$

$$0.00007 \text{ mmol NHS-Rhodamine} \times \frac{528 \text{ mg}}{\text{mmol NHS-Rhodamine}} \times \frac{100 \, \mu\text{L}}{1 \text{ mg}} = 3.5 \, \mu\text{L}$$

D. Labeling Reaction

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

Note: Using a high molar excess with antibody in borate or carbonate buffer (pH 8.5 or pH 9.0, respectively) could cause antibody or dye precipitation. For initial reaction use PBS for conjugation.

1. Transfer the antibody solution to a reaction tube.
2. Dissolve NHS-Rhodamine at 10mg/mL in DMF or DMSO. Mix thoroughly.
3. Transfer the appropriate amount of dye (from **Calculations** Section) to the antibody solution and mix well.
4. Incubate the reaction at room temperature for 1 hour or on ice for 2 hours.
5. Remove non-reacted NHS-Rhodamine by dialysis or gel filtration, or with a Dye Removal Column. Use the Thermo Scientific™ BCA Protein Assay Reagent Kit (Product No. 23225) to estimate protein concentration.
6. Store Rhodamine-labeled protein at 4°C protected from light until ready to use. To prevent microbial contamination, add a preservative such as sodium azide to a final concentration of 0.1%.

E. Calculate the Degree of Labeling

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

1. Use a 1cm path length cuvette to measure the absorbance of the labeled protein at 280nm and 555nm. It may be necessary to dilute a small amount of the desalted/dialyzed sample.

2. Calculate the concentration of the protein in the sample:

- $\varepsilon_{protein}$ = protein molar extinction coefficient (e.g., the molar extinction coefficient of IgG is ~210,000M^{-1} cm^{-1})
- $A_{max} = A_{555}$
- CF = Correction factor $= \dfrac{A_{280}}{A_{max}}$; CF for Rhodamine = 0.3400

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

3. Calculate the degree of labeling:

- ε' = Rhodamine molar extinction coefficient = 80,000 M^{-1} cm^{-1}

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

Additional Information Available on Our Website

- 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 Cassette**, **10K MWCO**

89890   **Zeba Desalt Spin Columns, 2 ml,** 25 each, for 200-700 µl samples

53031   **Pierce NHS-Rhodamine Antibody Labeling Kit**

53027   **Pierce FITC Antibody Labeling Kit**

53029   **Pierce NHS-Fluorescein Antibody Labeling Kit**

General References

Harlow, E. and Lane, D. (1988). Antibodies; A laboratory manual. Cold Spring Harbor Laboratory, New York, **409**.

Fluorescent Antibody Techniques and their Application. Ed. by A. Kawamura Jr., 1977, Univ. of Tokyo Press, Baltimore.

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