INSTRUCTIONS

# Pierce NHS-Rhodamine Antibody Labeling Kit

## 53031

2080.1

| Number | Description |
|---|---|
| **53031** | **Pierce NHS-Rhodamine 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-Rhodamine,** 3 × 35µg vials

Molecular mass: 527.5

Extinction coefficient: 80,000 $M^{-1}$ $cm^{-1}$

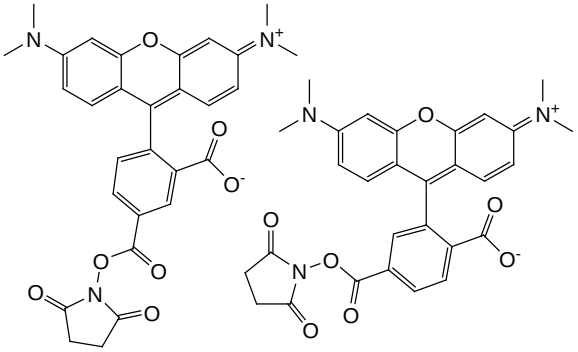
Ex/Em wavelength: 552/575nm

**Borate Buffer (0.67M),** 1mL

**Purification Resin,** 5mL

**Spin Columns,** 6 each

**Microcentrifuge Collection Tubes,** 12 each



NHS-Rhodamine
5-carboxytetramethylrhodamine

NHS-Rhodamine
6-carboxytetramethylrhodamine

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

## Introduction

The Thermo Scientific™ Pierce™ NHS-Rhodamine Antibody Labeling Kit contains all the necessary components for labeling antibodies or other proteins and subsequent excess dye removal. NHS-Rhodamine is an amine-reactive fluorescent labeling reagent that absorbs green visible light (542nm) 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, covalent 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 included in the kit, 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-Rhodamine 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.
- For fluorescent imagers use a spectral line of a green (532) laser for NHS-Rhodamine, Cy3™, Alexa Fluor™ 555, DyLight 549 Dyes.
- 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.
- Buffers that contain primary amines (e.g., Tris or glycine) are not compatible because they react with the NHS-ester.

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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-Rhodamine

**Note:** Labeling with NHS-Rhodamine is more efficient at pH 7-8 especially when using a high molar excess of dye to protein. High pH (> 8.5) and high molar excess can cause antibody and dye precipitation.

### A. Protein Preparation

1. For best results use 1mg of protein at ~2mg/ml. The optimal labeling buffer is phosphate-buffered saline (PBS, 0.1 M sodium phosphate, 0.15 M sodium chloride; pH 7.2-7.4). Alternatively, use 50mM sodium borate (pH 8.5) and prepare the protein as follows:

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

   - **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 (0.67M) 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 0.5 ml 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 be in a buffer free of ammonium ions or primary amines (for example, Tris or glycine), which will interfere with the reaction. If necessary, replace buffer with PBS or 50mM sodium borate (pH 8.5) by dialysis or buffer exchange.

### B. Protein Labeling

1. Equilibrate all reagents to room temperature.
2. Add 0.5mL of the prepared protein to the vial of NHS-Rhodamine Reagent and pipette up-and-down 10 times until all the dye 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 400 µl of the suspension into both spin columns. Centrifuge for 30-45 seconds at ~1000 × *g* to remove the storage solution. Discard the used collection tubes and place the columns in new collection tubes.
3. Add 250µL 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 ~1000 × *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. NHS-Rhodamine-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 552nm (i.e., $A_{max}$ of NHS-Rhodamine).
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 ~210,000 $M^{-1}$ $cm^{-1}$)
- CF = Correction factor $= \frac{A_{280}}{A_{max}} = 0.34$

4. Calculate the degree of labeling as follows:

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

- $\varepsilon_{fluor}$ = 80,000 (NHS-Rhodamine 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 PBS buffer |
| | 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-to-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 Products

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|---|---|
| **46406** | **NHS-Rhodamine,** 25mg |
| **62262** | **DyLight 550 NHS Ester,** 1mg |
| **62263** | **DyLight 550 NHS Ester**, 5 × 50µg |
| **84530** | **DyLight 550 Antibody Labeling Kit** |
| **84531** | **DyLight 550 Microscale Antibody Labeling Kit** |
| **22858** | **Fluorescent Dye Removal Columns** |
| **53027** | **Pierce FITC Antibody Labeling Kit** |
| **53029** | **Pierce NHS-Fluorescein Antibody Labeling Kit** |



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