

**Protonex™ Red 600, SE**

 Catalog number: 21208  
 Unit size: 1 mg

Component	Storage	Amount (Cat No. 21208)
Protonex™ Red 600, SE	Freeze (< -15 °C), Minimize light exposure	1 vial (1 mg)

**OVERVIEW**

Protonex™ Red dye demonstrated pH-dependent fluorescence. Unlike most of the existing fluorescent dyes that are more fluorescent at higher pH, acidic conditions enhance the fluorescence of Protonex™ Red dye. The fluorescence of Protonex™ Red dye dramatically increases as pH decreases from neutral to the acidic. The weak fluorescence outside the cell may potentially eliminates the wash steps. Protonex™ Red dye provides a powerful tool to monitor acidic cell compartments such as endosomes and lysosomes. Protonex™ Red dye is weakly fluorescent outside the cells, but its fluorescence is significantly enhanced in acidic compartments (such as phagosomes, lysosomes and endosomes). This Protonex™ Red enables the specific detection of cellular acidic compartments with reduced signal variability and improved accuracy for imaging or flow applications. It can be also used for multiplexing cellular functional analysis with green dyes such as GFP, Fluo-8, calcein, or FITC-labeled antibodies. Protonex™ Red has the spectral properties similar to those of Texas Red, making the common filter set of Texas Red readily available to the assays of Protonex™ Red. This Protonex™ Red SE can be readily used to make a variety of bioconjugates for imaging or flow applications, enabling the specific detection of phagocytosis and endocytosis with reduced signal variability and improved accuracy. These conjugates can be also used for multiplexing cell functional analysis with green dyes such as GFP, Fluo-8, calcein, or FITC-labeled antibodies. Protonex™ Red has the spectral properties similar to those of Texas Red, making the common filter set of Texas Red readily available to the assays of Protonex™ Red.

**PREPARATION OF STOCK SOLUTIONS**

*Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles*

**Protein stock solution (Solution A)**

1. Mix 100 µL of a reaction buffer (e.g., 1 M sodium carbonate solution or 1 M phosphate buffer with pH ~9.0) with 900 µL of the target protein solution (e.g., antibody, protein concentration >2 mg/mL if possible) to give a 1 mL protein labeling stock solution.

**Note:** The pH of the protein solution (Solution A) should be 8.5 ± 0.5. If the pH of the protein solution is lower than 8.0, adjust the pH to the range of 8.0-9.0 using 1 M sodium bicarbonate solution or 1 M pH 9.0 phosphate buffer.

**Note:** The protein should be dissolved in 1X phosphate-buffered saline (PBS), pH 7.2-7.4. If the protein is dissolved in Tris or glycine buffer, it must be dialyzed against 1X PBS, pH 7.2-7.4, to remove free amines or ammonium salts (such as ammonium sulfate and ammonium acetate) that are widely used for protein precipitation.

**Note:** Impure antibodies or antibodies stabilized with bovine serum albumin (BSA) or gelatin will not be labeled well. The presence of sodium azide or thimerosal might also interfere with the conjugation reaction. Sodium azide or thimerosal can be removed by dialysis or spin column for optimal labeling results.

**Note:** The conjugation efficiency is significantly reduced if the

protein concentration is less than 2 mg/mL. For optimal labeling efficiency, the final protein concentration range of 2-10 mg/mL is recommended.

**Protonex™ Green 600, SE stock solution (Solution B)**

1. Add anhydrous DMSO into the vial of Protonex™ Green 600, SE to make a 10 mM stock solution. Mix well by pipetting or vortex.

**Note:** Prepare the dye stock solution (Solution B) before starting the conjugation. Use promptly. Extended storage of the dye stock solution may reduce the dye activity. Solution B can be stored in the freezer for two weeks when kept from light and moisture. Avoid freeze-thaw cycles.

**SAMPLE EXPERIMENTAL PROTOCOL**
**Important Note**

This protocol was developed for conjugating Protonex™ Green 600, SE to goat anti-mouse IgG. Additional optimization may be necessary for your specific protein.

**Note:** Each protein requires a distinct dye/protein ratio, which also depends on the properties of dyes. Over-labeling of a protein could detrimentally affect its binding affinity, while the protein conjugates of low dye/protein ratio give reduced sensitivity.

**Run Conjugation Reaction**

1. Use a 10:1 molar ratio of Solution B (dye)/Solution A (protein) as the starting point: Add 5 µL of the dye stock solution (Solution B, assuming the dye stock solution is 10 mM) into the vial of the protein solution (95 µL of Solution A) with effective shaking. The concentration of the protein is ~0.05 mM assuming the protein concentration is 10 mg/mL and the molecular weight of the protein is ~200 KD.

**Note:** We recommend using a 10:1 molar ratio of Solution B (dye)/Solution A (protein). If it is too low or too high, determine the optimal dye/protein ratio at 5:1, 15:1, and 20:1, respectively.

2. Continue to rotate or shake the reaction mixture at room temperature for 30-60 minutes.

**Purify the Conjugation**

The following protocol is an example of dye-protein conjugate purification by using a Sephadex G-25 column.

1. Prepare the Sephadex G-25 column according to the manufacturer's instructions.
2. Load the reaction mixture (From "Run conjugation reaction") to the top of the Sephadex G-25 column.
3. Add PBS (pH 7.2-7.4) as soon as the sample runs just below the top

resin surface.

4. Add more PBS (pH 7.2-7.4) to the desired sample to complete the column purification. Combine the fractions that contain the desired dye-protein conjugate.

**Note:** For immediate use, the dye-protein conjugate needs to be diluted with staining buffer, and aliquoted for multiple uses.

**Note:** For longer-term storage, the dye-protein conjugate solution needs to be concentrated or freeze-dried.

## EXAMPLE DATA ANALYSIS AND FIGURES

### Characterize the Desired Dye-Protein Conjugate

The Degree of Substitution (DOS) is the most important factor for characterizing dye-labeled protein. Proteins of lower DOS usually have weaker fluorescence intensity, but proteins of higher DOS (e.g. DOS > 6) tend to have reduced fluorescence too. The optimal DOS for most antibodies is recommended between 2 and 10 depending on the properties of dye and protein. For effective labeling, the degree of substitution should be controlled to have 6-8 moles of Protonex™ Green 600, SE to one mole of antibody. The following steps are used to determine the DOS of Protonex™ Green 600, SE labeled proteins.

#### Measure absorption

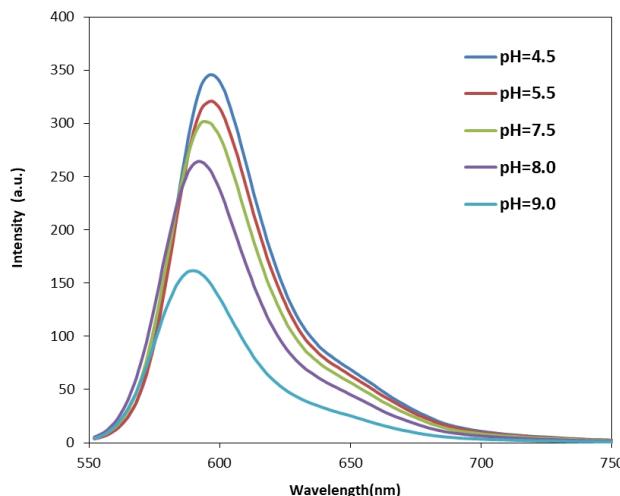
To measure the absorption spectrum of a dye-protein conjugate, it is recommended to keep the sample concentration in the range of 1-10  $\mu\text{M}$  depending on the extinction coefficient of the dye.

#### Read OD (absorbance) at 280 nm and dye maximum absorption ( $\lambda_{\text{max}} = 597 \text{ nm}$ for Protonex™ Green 600, SE dyes)

For most spectrophotometers, the sample (from the column fractions) need be diluted with de-ionized water so that the OD values are in the range of 0.1 to 0.9. The O.D. (absorbance) at 280 nm is the maximum absorption of protein while 597 nm is the maximum absorption of Protonex™ Green 600, SE. To obtain accurate DOS, make sure that the conjugate is free of the non-conjugated dye.

#### Calculate DOS

You can calculate DOS using our tool by following this link: <https://www.aatbio.com/tools/degree-of-labeling-calculator>



**Figure 1.** The pH dependent Emission spectra of Protonex™ Red 600.

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