RatioWorks™ PDMPO, SE

Catalog number: 21210
Unit size: 1 mg

Component	Storage	Amount
RatioWorks™ PDMPO, SE	Freeze (< -15 °C), Minimize light exposure	1 vial (1 mg)

OVERVIEW

The existing pH probes are ill-adapted to study acidic organelles such as lysosomes, endosomes, phagosomes, spermatozoa and acrosomes because their fluorescence is significantly reduced at lower pH. The growing potential of ratio imaging is significantly limited by the lack of appropriate fluorescent probes for acidic organelles although ratio imaging has received intensive attention in the past few decades. RatioWorks™ PDMPO is characterized as acidotropic dual-excitation and dual-emission pH probe. It emits intense yellow fluorescence at lower pH and gives intense blue fluorescence at higher pH. This unique pH-dependent fluorescence makes RatioWorks™ PDMPO an ideal pH probe for acidic organelles with pKa = 4.47. Additionally, the very large Stokes shift and excellent photostability of RatioWorks™ PDMPO make it an excellent fluorescent acidotropic reagent for fluorescence imaing and flow cytometry applications. The unique fluorescence properties of RatioWorks™ PDMPO might give researchers a new tool with which to study endocytosis, phagocytosis and acidic organelles of live cells. RatioWorks™ PDMPO can be well excited by the violet laser at 405 nm for flow cytometric applications. This RatioWorks™ PDMPO 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. The short emission band of RatioWorks™ PDMPO is ~450 nm while the longer emission is ~550 nm, making the common filter sets of Pacific Blue and Pacific Orange readily available to the assays of RatioWorks™ PDMPO.

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.

1. Protein stock solution (Solution A)
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 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.

2. RatioWorks™ PDMPO, SE stock solution (Solution B)
Add anhydrous DMSO into the vial of RatioWorks™ PDMPO, 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 freezer for two weeks when kept from light and moisture. Avoid freeze-thaw cycles.

SAMPLE EXPERIMENTAL PROTOCOL

This labeling protocol was developed for the conjugate of Goat anti-mouse IgG with RatioWorks™ PDMPO, SE. You might need further optimization for your particular proteins. **Note:** Each protein requires distinct dye/protein ratio, which also depends on the properties of dyes. Over labeling of a protein could detrimentally affects its binding affinity while the protein conjugates of low dye/protein ratio gives reduced sensitivity.

Run conjugation reaction

1. Use 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 ~200KD. **Note:** We recommend to use 10:1 molar ratio of Solution B (dye)/Solution A (protein). If it is too less 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 Sephadex G-25 column according to the manufacture instruction.

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 need be diluted with staining buffer, and aliquoted for multiple uses. **Note:** For longer term storage, dye-protein conjugate solution need 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 RatioWorks™ PDMPO, SE to one mole of antibody. The following steps are used to determine the DOS of RatioWorks™ PDMPO, 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 µM depending on the extinction coefficient of the dye.

Read OD (absorbance) at 280 nm and dye maximum absorption (λmax = 333 nm for RatioWorks™ PDMPO, 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 333 nm is the maximum absorption of RatioWorks™ PDMPO, 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

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