molecular probes®
by life technologies™

pHrodo™ Green and Red Amine-Reactive Labels

Table 1 Contents and storage

Material	Cat. no.	Amount	Storage*
pHrodo™ Green STP ester (MW = ~750)	P35369	500 µg lyophilized product	• ≤–20˚C • Desiccate • Protect from light
pHrodo™ Red succinimidyl (NHS) ester (MW = ~650)	P36600	1 mg lyophilized product	
* When stored as directed the product is stable for at least 6 months.			
Approximate fluorescence excitation and emission maxima: pHrodo™ Green: 505/525 nm; pHrodo™ Red: 560/585 nm.			

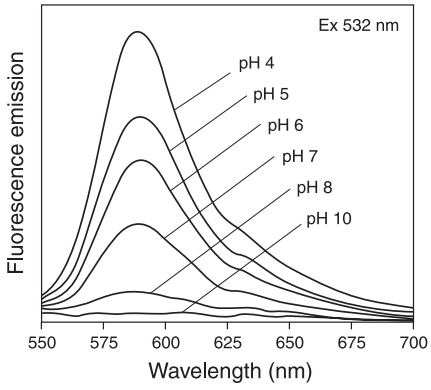
Introduction

The pHrodo™ Green and Red dyes are novel, fluorogenic dyes that dramatically increase in fluorescence as the pH of their surroundings becomes more acidic (Figure 1). The amine-reactive forms of pHrodo™ Green and Red have a pKa of ~7.3 in solution, which shifts to about ~6.5 upon conjugation. The pHrodo™ dyes are extremely sensitive to their local environment; therefore the pH response in your system will need to be determined empirically.

The pHrodo™ Green dye has excitation and emission maxima of approximately 505 nm and 525 nm, respectively, and can be detected with standard FITC (fluorescein) or Alexa Fluor® 488 filters. The pHrodo™ red dye has excitation and emission maxima of approximately 566 nm and 590 nm and can be detected with standard TRITC (tetramethylrhodamine) or Alexa Fluor® 555 filters.

Here, we describe a general protocol for using the amine-reactive forms of the pHrodo™ dyes to label purified proteins or antibodies in solution, as well as bacteria or other amine-surfaced particles for use in phagocytosis, endocytosis and internalization studies.

Figure 1 The fluorescence emission spectra of pHrodo™ dye–labeled *E. coli* were measured in a series of 50 mM potassium phosphate buffers ranging in pH from 4 to 10. The *E. coli* were at a concentration of 0.1 mg/mL, and the readings were made on a Hitachi F4500 fluorometer, using an excitation wavelength of 532 nm.



I. Labeling Purified Proteins with pHrodo™ Green and Red Amine-reactive Dyes

The pHrodo™ Green and Red amine-reactive dyes readily react with a protein's primary amines to yield a covalently attached fluorogenic pH probe. Here, we describe a general protocol for using the amine-reactive forms of the pHrodo™ dyes to label purified proteins or antibodies in solution.

Materials Required but Not Provided

- 100 mM sodium bicarbonate, pH 8.5
- 150 µL 100% DMSO, anhydrous
- Gel filtration column or media with a suitable molecular weight cutoff, equilibrated with the buffer of your choice
- *Optional*: 8 M guanidine-HCl for DOL (degree of labeling) determination.

Guidelines for Preparing Proteins

- **IMPORTANT!** The purified protein should be at a concentration of 1 mg/mL in a buffer that does not contain primary amines (e.g., ammonium ions, Tris, glycine, ethanolamine, triethylamine, glutathione), or imidazole. All of these substances significantly inhibit protein labeling. Also, partially purified protein samples or protein samples containing carriers like BSA (e.g., antibodies) will not be labeled well and should be purified prior to labeling. The presence of low concentrations (<0.1% (w/v)) of biocides, including sodium azide and thimerosal, will not significantly affect the labeling reaction.
- To aid in removing low molecular weight components from the protein sample (desalting) prior to labeling, it is possible to use dialysis or small-scale gel filtration. There are a number of easy-to-use, low-volume dialysis options available, including Tube-O-DIALYZER micro-dialysis cartridges from Genotech (www.gbiosciences.com).
- We suggest PBS, pH 7.2–7.5, as a suitable prelabeling dialysis buffer, although 100 mM sodium bicarbonate buffer can also be used.

Labeling Reaction

1.1 Resuspend the 500 µg vial of amine-reactive pHrodo™ Green STP ester in 75 µL of DMSO or the 1 mg vial of amine-reactive pHrodo™ Red NHS ester in 150 µL of DMSO for a stock solution of approximately 8.9 mM (pHrodo™ Green) or 10.2 mM (pHrodo™ Red). This stock solution should be used as soon as possible on the day it is prepared.

1.2 Prepare the protein to be labeled in 0.1 M sodium bicarbonate buffer, pH 8.3 to a concentration of at least 1 mg/mL.

1.3 Based on the amount of protein you wish to label, determine the amount of reactive dye to use that will give you a dye to protein molar ratio (MR) of 5–20 moles of dye per mole of protein.

1.4 Add the appropriate amount of reactive dye to the protein solution in sodium bicarbonate buffer and mix by pipetting up and down several times.

1.5 Incubate the reaction for 15–60 minutes at room temperature, protected from light.

Purifying the Protein Conjugates

We recommend using Sephadex® G, BioGel® P, or equivalent gel filtration media with an appropriate molecular weight cutoff

2.1 Equilibrate a 10 × 300-mm column with PBS or buffer of choice. The excluded fraction, which corresponds to the first fluorescent band to elute, is the conjugate. If you are conjugating a dilute antibody, you may want to purify the conjugate by extensive dialysis to avoid further dilution.

Note: You can obtain microdialysis apparatus for small volumes of proteins, e.g., 10–500 µL, from Pierce Chemical Company (www.piercenet.com) and Spectrum Laboratories (www.spectrapor.com).

If you prefer to purify your conjugate by column chromatography, after elution, add bovine serum albumin (BS) or any other stabilizer of choice to a final concentration of 1-10 mg/mL to prevent denaturation.

Optional: Determining the Degree of Labeling (DOL)

You may need to optimize the labeling efficiency to achieve the desired results of the conjugate in your application. You can determine the relative efficiency of a labeling reaction by measuring the absorbance of the protein at 280 nm and the absorbance of the dye at its excitation maximum.

Determination of degree of substitution for pHrodo™ protein conjugates are accurate only when using 8 M guanidine-HCl, a chaotrope or denaturant, that disrupts any hydrophobic or intramolecular noncovalent interactions.

3.1 Dilute the conjugate sample to approximately 0.1 mg/mL in 8 M guanidine-HCl.

3.2 Measure the absorbance of the pHrodo™ conjugate at 280 nm (A_{280}) and at the λ_{max} for the pHrodo™ dye. The table below provides the absorbance maxima, the extinction coefficient, and the correction factor for the pHrodo™ dyes.

Table 2 pHrodo™ dye properties

pHrodo™ dye	Absorbance maximum (λ_{max})	Extinction coefficient (ε_{dye})	Correction factor (CF)
Green	505 nm	75,000	0.2
Red	560 nm	65,000	0.12

3.3 Calculate the concentration of protein in the sample:

$$\text{Protein concentration (M)} = \frac{[A_{280} - (\text{pHrodo™ } \lambda_{max} \times \text{pHrodo™ CF})] \times \text{dilution factor}}{\text{protein extinction coefficient}}$$

Note: The molar extinction coefficient of a typical IgG is 203,000 $cm^{-1}M^{-1}$ is

3.4 Calculate the degree of labeling (DOL):

$$\text{moles dye per mole protein} = \frac{\text{pHrodo™ } \lambda_{max} \times \text{dilution factor}}{\varepsilon_{dye} \times \text{protein concentration}}$$

Optional: Determining the pH Response Range of the Conjugate

See Section 7 at the end of "Labeling bacteria with pHrodo™ Green and Red amine-reactive dyes", page 5

I. Labeling Bacteria with pHrodo™ Green and Red Amine-reactive Dyes

This protocol describes the labeling of 60 mg of *E. coli* bacteria with a single, 1 mg aliquot of amine-reactive pHrodo™ Green or Red dye. Briefly, the bacteria are prepared at 20 mg/mL in sodium bicarbonate, and the dye is prepared at 10 mM in DMSO. The dye is then diluted into the bacterial suspension for a final dye concentration of 0.5 mM in the labeling reaction. This can be adjusted up or down for your particular needs, but we have found this concentration to be optimal for *E. coli*, as the pH dependent fluorescence response can be blunted by over labeling at higher concentrations of dye (>2 mM). The amine-labeling reaction then proceeds for 45 minutes at room temperature, and then the bacteria are washed with a series of centrifugations through saline and methanol, and finally resuspended in either water for lyophilization, or the buffer of your choice for use within 3–5 days.

Although the procedure below describes the labeling of a lyophilized sample of *E. coli*, the procedure can also be applied to freshly prepared bacteria as long as the starting concentration of the bacteria in mg/mL is known.

Materials Required but Not Provided

- *E. coli* lyophilized powder, or freshly prepared *E. coli* at a known concentration in mg/mL
- 10 mL of freshly prepared 100 mM sodium bicarbonate, pH 8.5
- Hanks' Balanced Salt Solution (HBSS, Cat. no. 14025)
- 150 µL 100% DMSO, anhydrous
- 10 mL 100% methanol
- Distilled, deionized water

Preparing the Bacteria

4.1 Weigh out 60 mg of lyophilized *E. coli* bacteria into a 15-mL screw-cap tube. Resuspend at 20 mg/mL using 3 mL of the freshly prepared 100 mM sodium bicarbonate solution, and split into four 1.5-mL snap-top tubes, at 750 µL each.

4.2 Centrifuge the bacteria at 14,000 RPM in a benchtop microfuge (>15,000 × *g*) for 60 seconds. Resuspend the sample in each tube in fresh sodium bicarbonate at 20 mg/mL, or 750 µL for each tube. The bacteria are now ready for the dye, and should not be stored for more than 24 hours before use.

Preparing the Dye and Labeling the Bacteria

5.1 Resuspend a 1 mg vial of amine-reactive pHrodo™ STP or NHS ester in 150 µL of DMSO for a stock solution of approximately 8.9 mM (pHrodo™ Green) or 10.2 mM (pHrodo™ Red). This stock solution should be used as soon as possible the day it is made.

5.2 Dilute the DMSO stock solution into E. coli for a final concentration of 0.5–1 mM. For individual use, more or less dye may be necessary depending on the target.

5.3 Incubate the tubes at room temperature for 45–60 minutes, protected from light.

Removing Unincorporated Dye from the Conjugate

6.1 After the reaction has run for 45–60 minutes, add 750 µL of HBSS and centrifuge the tubes at 14,000 RPM (>15,000 × *g*) in a benchtop centrifuge for 60 seconds. Quickly remove the tubes and carefully aspirate the supernatant.

6.2 Add 1 mL of 100% methanol to each tube, and carefully resuspend the bacteria in this solvent. We recommend that you cut the tip off of a P1000 pipette tip for this procedure, as the aggregates that normally form in this solution can clog the opening of an unmodified pipette tip. Try to minimize losses of the particulate matter during this and subsequent steps, as losses have an impact on your final yield. When each sample has been resuspended, add an additional 0.5 mL of methanol to each tube, and vortex the samples for 30 seconds.

6.3 Centrifuge the samples for 60 seconds at 14,000 RPM (>15,000 × *g*). Carefully aspirate the supernatant from each and replace with 1 mL of HBSS. Carefully resuspend the sample in each tube, add 0.5 mL of HBSS, and vortex for 30 seconds to evenly disperse the bacteria.

6.4 Repeat step 6.3.

6.5 At this point, if you plan to lyophilize the bacteria, you may resuspend them in water at 50 mg/mL, divide into aliquots, and lyophilize (we recommend 2–5 mg per aliquot). If they are to be used within 2–3 days, you may resuspend them in the assay buffer described above or in the buffer of your choice.

Determining the pH Response Range of the Conjugate

7.1 Resuspend some of your conjugate at a final concentration of 1 mg/mL in 500 µL each of a series of buffers with pH adjusted between pH 4 and pH 8.

7.2 Pipette 100 µL of each in quadruplicate into a 96-well plate and measure the fluorescence in a plate reader.

7.3 Construct average fluorescence values for each pH data point, and plot the pH versus average fluorescence. You can also calculate the ratio of the average fluorescence measured at pH 4 versus the average fluorescence measured at pH 8. Fold increases of 8 or higher are favorable for cellular experiments.

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
P35369	pHrodo™ Green STP ester	500 µg
P36600	pHrodo™ Red succinimidyl ester	1 mg

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