molecular probes®
by life technologies™

pHrodo™ Green and Red Thiol-Reactive Labels

Table 1 Contents and storage

Material	Cat. no.	Amount	Storage*
pHrodo™ Green C_2-maleimide (MW = ~900)	P35370	1 mg lyophilized product	• ≤–20°C • Desiccate • Protect from light
pHrodo™ Red C_2-maleimide (MW = ~700)	P35371		
* 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.			

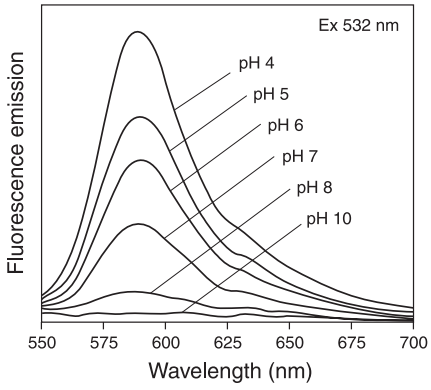
Introduction

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, respectively, and can be detected with standard TRITC (tetramethylrhodamine) or Alexa Fluor® 555 filters.

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

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.



Labeling purified proteins with pHrodo™ Green and Red Amine-reactive dyes

Materials Required but Not Provided

- 10–100 mM phosphate (such as phosphate-buffered saline (PBS)), Tris, or HEPES buffer with pH between 7.0–7.5.
- Dimethylformamide (DMF) or dimethylsulfoxide (DMSO)
- Gel filtration column (Sephadex® G-25 or equivalent), equilibrated in buffer of choice.
- *Optional*: Glutathione, mercaptoethanol, or other low molecular weight thiol to stop the reaction.
- *Optional*: DTT (dithiothreitol, Cat. no. D1532) or TCEP (tris-(2-carboxyethyl)phosphine, Cat. no. T2556). These reagents can be used to reduce disulfide bonds in proteins to liberate free thiols.

Labeling Reaction

1.1 Dissolve the protein at 50–100 µM in a suitable buffer at pH 7.0–7.5 (10–100 mM phosphate, Tris, HEPES) at room temperature. In this pH range, the protein thiol groups are sufficiently nucleophilic so that they react almost exclusively with the reagent in the presence of the more numerous protein amines, which are protonated and relatively unreactive.

1.2 Reduction of disulfide bonds in the protein is best carried out at this stage. A 10-fold molar excess of a reducing agent such as DTT (Cat. no. D1532) or TCEP (Cat. no. T2556) is usually sufficient. If DTT is used, then dialysis is required to remove the excess DTT prior to introducing the reactive dye. It is not necessary to remove excess TCEP during conjugation with maleimides.

Note: It may be advisable to carry out thiol modifications in an oxygen-free environment because thiols can oxidize to disulfides. This precaution is particularly important if the protein has been treated with a reducing reagent such as DTT prior to thiol modification. In this case, all buffers should be deoxygenated and the reactions carried out under an inert atmosphere to prevent the re-formation of disulfides.

1.3 Prepare a 1–10 mM stock solution of the pHrodo™ reactive dye in DMF or DMSO immediately prior to use. We do not recommend storing the dyes in solution for more than 24 hours.

1.4 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 3–20 moles of dye per mole of protein.

1.5 Add the pHrodo™ reagent dropwise to the protein solution as it is stirring.

1.6 Allow the reaction to proceed for 2 hours at room temperature or overnight at 4°C, protected from light.

1.7 Upon completion of the reaction with the protein, an excess of glutathione, mercaptoethanol, or other soluble low molecular weight thiol can be added to consume the excess thiol-reactive reagent, thus ensuring that no reactive species are present during the purification step.

1.8 Separate the conjugate on a gel filtration column, such as a Sephadex® G-25 column or an equivalent matrix, or by extensive dialysis at 4°C in an appropriate buffer.

Storing the Conjugates

We typically store labeled protein at 2–6°C, protected from light. It may be necessary to add a stabilizer such as BSA (1–10 mg/mL) or glycerol to your conjugate to improve its stability. In the presence of 2 mM sodium azide or other biocides, a typical antibody conjugate should be stable at 2–6°C for several months. Your proteins may have special storage requirements. If it is appropriate to do so with your proteins, you can divide the conjugate into small aliquots and freeze them at ≤–20°C for longer storage. Avoid repeated freezing and thawing, and protect from light..

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 hydropbobic or intramolecular noncovalent interactions.

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

2.2 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

2.3 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

2.4 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

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
P35370	pHrodo™ Green C_2-maleimide	1 mg
P35371	pHrodo™ Red C_2-maleimide	1 mg

Purchaser Notification

Corporate Headquarters
5791 Van Allen Way
Carlsbad, CA 92008
USA
Phone: +1 760 603 7200
Fax: +1 760 602 6500
Email: techsupport@lifetech.com

European Headquarters
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF
UK
Phone: +44 141 814 6100
Toll-Free Phone: 0800 269 210
Toll-Free Tech: 0800 838 380
Fax: +44 141 814 6260
Tech Fax: +44 141 814 6117
Email: euroinfo@invitrogen.com
Email Tech: eurotech@invitrogen.com

Japanese Headquarters
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Japan
Phone: +81 3 5730 6509
Fax: +81 3 5730 6519
Email: jpinfo@invitrogen.com

Additional international offices are listed at
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