pHrodo™, Succinimidyl Ester

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
pHrodo™, succinimidyl ester (MW = ~650)	1 mg lyophilized product	• ≤–20˚C • Desiccate • Protect from light	When stored as directed the product is stable for at least 6 months.
Approximate fluorescence excitation and emission maxima: 560/585 nm.			

Introduction

pHrodo™ dye is a novel, fluorogenic dye that dramatically increases in fluorescence as the pH of its surroundings becomes more acidic. This amine-reactive succinimidyl ester form of the dye has a pKa of ~7.3 in solution, which shifts to about ~6.5 upon conjugation to the K-12 strain of *E. coli*. pHrodo™ dye is extremely sensitive to its local environment, therefore the pH response in your system will need to be determined empirically.

Here, we describe a general protocol for using the amine-reactive, succimidyl ester form of the pHrodo™ dye, to label your own bacteria or amine-surfaced particles. While this is a relatively straightforward procedure, care must be taken to insure that all free dye is removed following the conjugation reaction. We recommend that you use this procedure as a starting point; optimization may be required. Below is a method describing the labeling of a lyophilized sample of *E. coli*, though freshly prepared bacteria will work as long as the user knows the starting concentration of the bacteria in mg/mL.

Before You Begin

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, Invitrogen Cat. no. 14025)
- 150 µL 100% DMSO, anhydrous
- 10 mL 100% methanol
- Distilled, deionized water

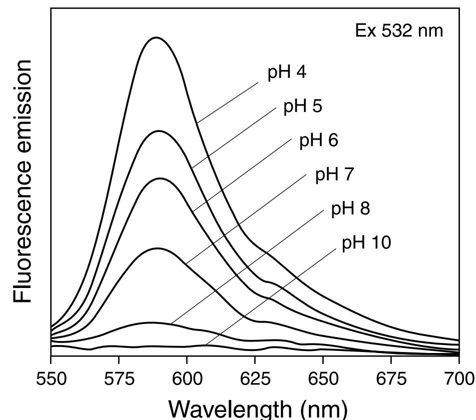

Figure 1. The fluorescence emission spectra of pHrodo™ dye–labeled *E. coli* were measured in a series of 50 mM potassium phosphate buffers ranging in pH from 4 to 10. The *E. coli* were at a concentration of 0.1 mg/mL, and the readings were made on a Hitachi F4500 fluorometer, using an excitation wavelength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

Experimental Protocol

This protocol describes the labeling of 60 mg of *E. coli* bacteria with a single, 1 mg aliquot of amine-reactive pHrodo™ SE dye. Briefly, the bacteria are prepared at 20 mg/mL in the 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, at higher concentrations of dye (>2 mM), we have observed that the pH-dependent fluorescence response can be blunted by overlabeling. 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.

Preparing the Bacteria

1.1 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.

1.2 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

2.1 Resuspend a 1 mg vial of amine-reactive pHrodo™ SE in 150 µL of DMSO for a solution of approximately 10 mM. This stock solution should be used as soon as possible the day it is made.

2.2 Dilute the DMSO stock solution into the *E. coli* for a final concentration of 0.5–1 mM, i.e., add 37.5 µL of dye to each of the four tubes of bacteria for 0.5 mM labeling. Again, for individual use, more or less dye may be necessary, depending on the target.

2.3 Incubate the tubes at room temperature for 45 minutes, protected from light.

Removing Unincorporated Dye from the Conjugate

3.1 After the reaction has run for 45 minutes, add 750 µL of HBSS and centrifuge the tubes at 14,000 RPM in a benchtop centrifuge for 60 seconds. Quickly remove the tubes and carefully aspirate the supernatant.

3.2 Add 1 mL of 100% methanol to each tube, and carefully resuspend the bacteria in this solvent. It is recommended 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.

3.3 Centrifuge the samples for 60 seconds at 14,000 RPM. 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.

3.4 Repeat step 3.3.

3.5 At this point, if you plan to lyophilize the bacteria, they may be resuspended in water at 50 mg/mL, divided into aliquots, and lyophilized (2–5 mg per aliquot is recommended). If they are to be used within 2–3 days, they may be resuspended in the assay buffer described above or in the buffer of your choice.

Determining the pH Response Range of the Conjugate

4.1 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.

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

4.3 Construct average fluorescence values for each pH data point, and plot the pH versus aver age 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
P36600	pHrodo™, Succinimidyl ester	1 mg
P35361	pHrodo™ *E.coli* BioParticles® conjugate for phagocytosis	5 × 2 mg
E2870	*Escherichia coli* BioParticles® opsonizing reagent	1 Unit

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
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Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

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