pHrodo™ Deep Red TFP Ester

Catalog Numbers P35358 and P35359

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Invitrogen™ pHrodo™ Deep Red TFP Ester, amine reactive dye readily reacts with a biomolecule's primary amines to yield a covalently attached fluorogenic pH probe. pHrodo™ Deep Red TFP Ester enables you to label primary amines on a protein, cell, or virus to create a stable conjugate that only fluoresces in acidic environments. Our low background pHrodo™ Deep Red dye makes it easier than ever to study internalization with higher certainty of results and less optimization as pHrodo™ Deep Red dye only turns on in the late endosome and lysosome. The pHrodo™ Deep Red dye has an excitation and emission maxima of approximately 640 nm and 655 nm respectively and can be detected with standard Cy5 filters.

pHrodo™ Deep Red dye is a low-background pH sensor dye that shows no signal in neutral conditions and only fluoresces in acidic environments. This unique property enables rapid assay development and certainty of results investigating antibody internalization, endocytic and phagocytic pathways. pHrodo™ Deep Red dye enables better discrimination of internalized cargo from outside the cell because it has an approximate pKa of 5 and will not turn on until it enters the late endosome and lysosome. pHrodo™ Deep Red dye can be detected using a Cy5 fluorescent filter set and has been verified for use in a variety of applications, including flow cytometry, fluorescent microscopy, and high content screening (HCS).

Here, we describe a quick and general protocol for using the amine-reactive form of the pHrodo™ Deep Red dye to label purified proteins or antibodies in solution.

Contents and storage

Contents	Cat. No.	Amount	Storage [1]
pHrodo™ Deep Red TFP Ester, amine reactive (MW~1300)	P35358	3 x 100 µg	• ≤–20°C • Dessicate • Protect from light
	P35359	1 mg	

[1] The product is stable for at least 6 months when stored as directed.

Note: The approximate fluorescence excitation and emission maxima of pHrodo™ Deep Red is 640/655 nm.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
1 M sodium bicarbonate, pH 8.5	MLS
(Optional) Gel filtration column or media with a suitable molecular weight cutoff, equilibrated with the buffer of your choice: Zeba™ Dye and Biotin Removal Spin Columns	A44296S or A44298
(Optional) Pierce™ Coomassie Plus (Bradford) Assay Kit	23236
(Optional) 4.3 % (wt%) phosphoric acid for DOL determination	MLS

Guidelines for labeling reaction

Do not prepare the pHrodo™ Deep Red reactive dye stock solution until ready to start the labeling reactions. This reactive dye hydrolyzes readily and therefore should be used immediately.

Guidelines for protein preparation

- The purified protein should be at a concentration of 2.2 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.
- Partially purified protein samples or protein samples containing carriers such as 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 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. For dialysis we recommend using the Slide-A-Lyzer™ Dialysis Cassettes (**thermofisher.com**).
- We recommend PBS pH 7.2–7.5 as a prelabeling dialysis buffer, although 100 mM sodium bicarbonate buffer can also be used.

(Optional) Guidelines for determining the degree of labeling (DOL)

- Several spectrophotometric methods are available for determining the DOL of pHrodo™ Deep Red dye–labeled conjugates. They are based on obtaining the conjugates concentration by absorbance at 280 nm (A_{280}) and 640 nm (A_{640}).
- We recommend using a NanoDrop™ spectrophotometer to analyze the labeled antibody spectrophotometrically. NanoDrop™ instruments (available from **thermofisher.com**) require only 1–2 µL of sample and are full-featured UV/Vis instruments.
- Determination of DOL for the conjugates prepared using the kit are accurate only when they are diluted using a 4.3% (wt %) phosphoric acid solution. We recommend diluting the antibody conjugate samples 1:3 in 4.3% (wt %) phosphoric acid before measuring the absorbance.

  Note: This procedure will likely destroy the conjugate sample and will not make the sample recoverable.
- Excessive dilution of some antibodies with low intrinsic A_{280} may prevent you from deriving accurate A_{280} values for your samples. Use only a portion of your antibody conjugate sample and dilute it only to the minimum volume necessary for your cuvettes and spectrophotometer to avoid readings below the optimal range for your instrument.

Label antibodies with pHrodo™ Deep Red amine-reactive dye

This protocol describes the labeling of 1 mg of whole IgG with a single, 100 µg aliquot of amine-reactive pHrodo™ Deep Red dye. Briefly, the antibody is prepared at 2.2 mg/mL in PBS or similar buffer free of primary amines, and the dye is prepared at ~0.38 mM (0.5 mg/mL) in water.

Antibody labeling reaction

1. Prepare 1 mg of the antibody at 2.2 mg/mL in PBS or a similar neutral buffer free of primary amines.
2. Add 50 µL of a 1 M sodium bicarbonate to the antibody solution.
3. Dissolve the contents of the 100 µg vial of pHrodo™ Deep Red amine-reactive dye in 100 µL of water to prepare a ~0.77 mM (1 mg/mL) labeling solution. Completely dissolve the contents of the vial by pipetting up and down.

   IMPORTANT! Prepare this solution immediately before use and discard any leftover solution.
4. Add all 100 µL of the ~0.77 mM labeling solution from to the antibody solution and mix by pipetting up and down several times.
5. Incubate the reaction for 2 hours at room temperature, protected from light.
6. If desired, purify the conjugated antibody using a spin column such as Zeba™ Dye and Biotin Removal Spin Columns.
7. Antibody is ready to use.

Label purified proteins with pHrodo™ Deep Red amine-reactive dye

The pHrodo™ Deep 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™ dye to label purified proteins or antibodies in solution.

General protein labeling reaction

1. Prepare 1 mg of the protein at 2.2 mg/mL in PBS or a similar buffer free of primary amines.
2. Add 50 µL of a 1 M sodium bicarbonate to the antibody solution.
3. Dissolve the contents of the 100 µg vial of pHrodo™ Deep Red amine-reactive dye in 100 µL of water to prepare a ~0.77 mM (1 mg/mL) labeling solution. Completely dissolve the contents of the vial by pipetting up and down.

   IMPORTANT! Prepare this solution immediately before use and discard any leftover solution.

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 5–20 moles of dye per mole of protein.

   For IgG we recommend a starting molar ratio of 10.

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

6. Incubate the reaction for 2 hours at room temperature, protected from light.

7. If desired, purify the conjugated protein using a spin column such as Zeba™ Dye and Biotin Removal Spin Columns.

Purify the protein conjugates

We recommend using Zeba™ Dye and Biotin Removal Spin Columns or equivalent gel filtration media with an appropriate molecular weight cutoff.

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

(Optional) Determine the conjugation yield

1. Set aside 10 µL of conjugated antibody solution and note total volume of recovered conjugate.

2. The protein yield can be determined using the Pierce™ Coomassie Plus (Bradford) Assay Kit.

   Note: Depending on the concentration of the conjugation reaction the sample may need to be diluted.

3. Calculate mg/mL of the conjugate from the sample and index this result to the volume of recovered sample to determine mg of antibody recovered for percent yield.

(Optional) Determine 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 (640 nm).

1. Remove a small volume of labeled conjugate and dilute in a 1:3 ratio using 4.3% (wt %) phosphoric acid.

   Note: This procedure will likely destroy the conjugate sample and will not make the sample recoverable.

   Note: 4.3% phosphoric acid can be made by diluting 5 µL 85% (wt%) phosphoric acid into 95 µL DI H_2O.

2. Dilute the antibody conjugate samples 1:3 in 4.3 % (wt%) phosphoric acid solution and measure the absorbance of the antibody conjugate at 280 nm (A_{280}) and at the 640 nm for the pHrodo™ Deep Red dye.

   For the absorbance maxima, extinction coefficient, and correction factor (for the fluorophore's contribution to A_{280}) for pHrodo™ Deep Red dye, see "Physical characteristics of pHrodo™ Deep Red dye" on page 4.

   Note: If yield was determined using the Coomassie test proceed to step 4.

3. Calculate the concentration of protein in the sample using the folloiwng formula:

$$\text{Protein concentration (M)} = \frac{[A_{280} - 0.33\ (A_{640})] \times \text{dilution factor}}{\text{Protein extinction coefficient}}$$

   Note: 203,000 is the molar extinction coefficient (ε) in $cm^{-1}M^{-1}$of a typical IgG at 280 nm and is also suitable for IgA, IgD, and IgE. In this equation, 0.33 is a correction factor for the fluorophore's contribution to A_{280}.

4. Calculate the degree of labeling (DOL) using the following formula:

$$\text{DOL} = \frac{\text{moles of dye}}{\text{moles of protein}} = \frac{A_{640} \times \text{dilution factor}}{140{,}000 \times \text{protein extinction coefficient}}$$

   Note: Where 140,000 $cm^{-1}M^{-1}$ is the approximate molar extinction coefficient of pHrodo™ Deep Red dye.

Guidelines for protein conjugate storage

- Store the labeled protein at 2–8°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 2–8°C for several months.
- Your proteins may have special storage requirements.
- If it is appropriate to do so with your antibody, divide the conjugate into small aliquots and freeze them at ≤–20°C for longer storage.
- Avoid repeated freezing and thawing, and protect from light.
- We recommend to centrifuge solutions of conjugates in a microcentrifuge before use, and to only use the supernatant in the experiment. This step will remove any aggregates that may have formed during storage.

Physical characteristics of pHrodo™ Deep Red dye

Dye	Absorbance maximum (λ max)	Extinction coefficient (ε dye)	CF_{280}[1]	CF_{260}[2]
pHrodo™ Deep Red dye	640 nm	140,000	0.33	0.14

[1] Correction factor for absorption readings (A_{280}) at 280 nm; e.g., $A_{280,actual} = A_{280,observed} - (CF_{280} \times \lambda max)$.

Note: Measurements taken in 4.3% (wt %) phosphoric acid.

[2] Correction factor for absorption readings (A_{260}) at 260 nm; e.g., $A_{260,actual} = A_{260,observed} - (CF_{260} \times \lambda max)$.

Note: Measurements taken in 4.3% (wt %) phosphoric acid.

Related products

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
pHrodo™ iFL Red Microscale Protein Labeling Kit	P36014
pHrodo™ iFL Green Microscale Protein Labeling Kit	P36015
pHrodo™ Deep Red Antibody Labeling Kit	P35355, P35356

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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