pHrodo™ iFL STP ester, amine-reactive dyes

Catalog Nos. P36010, P36011, P36012, P36013

Pub. No. MAN0017101 **Rev.** B.0

Product information

The pHrodo™ iFL Green and Red STP ester, amine-reactive dyes are novel, fluorogenic dyes that dramatically increase in fluorescence as the pH of their surroundings becomes more acidic (Figure 1). These amine-reactive forms of pHrodo™ iFL Green and Red dyes have a pKa of ~6.8. The pHrodo™ iFL dyes are extremely sensitive to their local environment; therefore, the pH response in your system needs to be determined empirically.

The pHrodo™ iFL 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™ iFL 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.

Table 1 Contents and storage

Material	Cat. No.	Amount	Storage*
pHrodo™ iFL Red STP ester, amine-reactive dye (MW = ~1,000)	P36010	1 mg	• ≤–20°C • Desiccate • Protect from light
	P36011	3 × 100 µg	
pHrodo™ iFL Green STP ester, amine-reactive dye (MW = ~1,000)	P36012	1 mg	
	P36013	3 × 100 µg	
* When stored as directed the product is stable for at least 6 months.			
Approximate fluorescence excitation and emission maxima: pHrodo™ iFL Green: 505/525 nm; pHrodo™ iFL Red: 560/585 nm.			

I. Label purified proteins with pHrodo™ iFL Green or Red amine-reactive dye

The pHrodo™ iFL 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
- 100% DMSO, anhydrous (Cat. No. D12345)
- Gel filtration column or media with a suitable molecular weight cutoff, equilibrated with the buffer of your choice
- *Optional*: 8 M guanidine-HCl (Cat. No. 24115) for DOL (degree of labeling) determination.

Guidelines for protein preparation

- 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.
- Partially purified protein samples or protein samples containing carriers like BSA (e.g., antibodies) are not labeled well and should not be used. The presence of low concentrations (<0.1% (w/v)) of biocides, including sodium azide and thimerosal, do 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. For dialysis, we recommend using the Thermo Scientific Slide-A-Lyzer™ Dialysis Cassettes (available from **thermofisher.com**).
- We suggest PBS, pH 7.2–7.5, as a suitable pre-labeling dialysis buffer, although 100 mM sodium bicarbonate buffer can also be used. If you use PBS, add 1/10 volume of 1 M sodium bicarbonate to the protein solution before labeling.

Guidelines for labeling reaction

- Use the pHrodo™ iFL STP ester, amine-reactive dye at a concentration of 2 mg/mL (2 mM) in DMSO.
- Refer to Table 2 for the recommended amount of reactive dye (in nanomoles) to add to each nanomole of protein to be labeled. This is the dye:protein molar ratio (MR).

  For your initial labeling attempt, choose the optimal MR for the protein listed in Table 2 that is closest in molecular weight to the one you are labeling. Use the lower and higher MR as a guide for relabeling, if your protein is under- or overlabeled.

Table 2 Recommended pHrodo™ iFL dye:protein molar ratios (MR) for labeling 20–150 kDa proteins

Protein (MW in kDa)	For lower DOL	For optimal DOL	For higher DOL
Streptavidin (53 kDa)	≤4	7	≥10
F(ab')2 (100 kDa)	≤5	8	≥12
IgG (150 kDa)	≤6	10	≥14

- Use the following equation to calculate the amount of 2 mM reactive dye needed to label different amounts of protein:

$$\frac{(\mu g \text{ protein}/\text{protein MW}) \times MR}{2\ \mu g/\mu L} = \mu L \text{ of 2 mM reactive dye to add to sample}$$

  where µg protein is the amount of protein you want to label, protein MW is the molecular weight of your protein in kDa, MR is the dye:protein molar ratio from Table 2, and 2 µg/µL is the concentration of the reactive dye stock solution (2 mM).

  For example, to label 100 µg of 150 kDa IgG at an MR of 10, you will need:

$$\frac{(100\ \mu g/150\ kDa) \times 10}{2\ \mu g/\mu L} = 3.3\ \mu L \text{ of 2 mM reactive dye to add to sample}$$

- Do **not** prepare the pHrodo™ iFL reactive dye stock solution (step 1.4) until you are ready to start the labeling reactions. This reactive dye hydrolyzes in water and therefore should be used immediately.
- Quantify the degree of labeling using an aliquot of your conjugate diluted in 8 M guanidine-HCl (see "*Optional*: Determine the Degree of Labeling (DOL)" on page 4).

General protein labeling reaction

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

Note: If your protein is in PBS or a similar buffer, add 1/10 volume of 1 M sodium bicarbonate to the protein solution before proceeding with the labeling reaction.

1.2 Dissolve the contents of the 100 µg vial of the pHrodo™ iFL amine-reactive dye in 50 µL of DMSO or 1 mg vial in 500 µL of DMSO to prepare a ~2 mM (2 mg/mL) pHrodo™ iFL dye stock 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.

1.3 Based on the amount of protein you wish to label, determine the amount of reactive dye to use that will give you the desired MR (see Table 2, page 2).

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.

Purify 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, purify the conjugate by extensive dialysis to avoid further dilution.

Note: You can obtain microdialysis apparatus for small protein volumes (10–500 µL)from Pierce Chemical Company (**piercenet.com**) and Spectrum Laboratories (**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: 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 (λ_{max}).

Determination of DOL for the protein conjugates prepared using the kit are accurate only when they are diluted in 8 M guanidine-HCl (Cat. No. 24115), a chaotrope or denaturant, that disrupts hydrophobic or intramolecular non-covalent 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™ iFL conjugate at 280 nm (A_{280}) and at the λ_{max} for the pHrodo™ dye. Table 3 provides the absorbance maxima, the extinction coefficient, and the correction factor for the pHrodo™ iFL dyes.

Table 3 Physical characteristics of pHrodo™ iFL dye

pHrodo™ iFL dye	Absorbance maximum (λ_{max})	Extinction coefficient (ε_{dye})	CF_{280}*	CF_{260}†
Green	505 nm	74,500	0.2	0.44
Red	560 nm	65,000	0.12	0.36

* Correction factor for absorption readings (A_{280}) at 280 nm
Example: $A_{280,actual} = A_{280,observed} - (CF_{280} \times \lambda_{max})$
Measurements taken in 8 M guanidine-HCl

† Correction factor for absorption readings (A_{260}) at 260 nm
Example: $A_{260,actual} = A_{260,observed} - (CF_{260} \times \lambda_{max})$
Measurements taken in potassium phosphate, pH 4

3.3 Calculate the concentration of protein in the sample:

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

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

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: Determine the pH response range of the conjugate

See "Determine the pH response range of the conjugate" on page 7.

II. Label antibodies with pHrodo™ Green or Red amine-reactive dye (quick protocol)

This protocol describes the labeling of 100 µg of whole IgG with a single, 100 mg aliquot of amine-reactive pHrodo™ iFL Green or Red dye. Briefly, the antibody is prepared at 1 mg/mL in PBS or similar buffer free of primary amines, and the dye is prepared at 2 mM (2 mg/mL) in DMSO.

Materials required but not provided

- 100 µg of the antibody at 1 mg/mL in PBS or a similar buffer free of primary amines
- 1 M sodium bicarbonate, pH 8.5
- 100% DMSO, anhydrous (Cat. No. D12345)
- Gel filtration column or media with a suitable molecular weight cutoff, equilibrated with the buffer of your choice
- *Optional*: Spin column and gel resin such as Bio-Gel™ P-30 for the purification of the labeled antibody

Antibody labeling reaction

4.1 Prepare 100 µg of the antibody at 1 mg/mL in PBS or a similar buffer free of primary amines.

4.2 Add 1/10 volume (10 µL) of 1 M sodium bicarbonate to the antibody solution.

4.3 Dissolve the contents of the 100 µg vial of pHrodo™ iFL amine-reactive dye in 50 µL of DMSO to prepare a ~2 mM (2 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.

4.4 Add 3.3 µL of the 2 mM labeling solution to the antibody solution and mix by pipetting up and down several times.

4.5 Incubate the reaction for at least 15 minutes at room temperature, protected from light.

4.6 If desired, purify the conjugated antibody using a spin column and gel resin such as Bio-Gel™ P-30.

III. Label bacteria with pHrodo™ iFL Green or Red amine-reactive dye

This protocol describes the labeling of 60 mg of *E. coli* bacteria with a single, 1 mg aliquot of amine-reactive pHrodo™ iFL 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 the dye (>2 mM). The amine-labeling reaction then proceeds for 45 minutes at room temperature, the bacteria are washed with a series of centrifugations through saline and methanol, and finally resuspended in water for lyophilization or the buffer of your choice for use within 3–5 days.

Although the following procedure 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 (Cat. No. D12345)
- 10 mL 100% methanol
- Distilled, deionized water

Prepare the bacteria

4.1 Weigh out 60 mg of lyophilized *E. coli* into a 15-mL screw-cap tube. Resuspend the bacteria 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.

Prepare the dye and label the bacteria

5.1 Dissolve the contents of the 1 mg vial of pHrodo™ iFL amine-reactive dye in 100 µL of DMSO to prepare a ~10 mM (10 mg/mL) pHrodo™ iFL dye stock 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.

5.2 Dilute the pHrodo™ iFL dye stock solution in DMSO 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.

Remove 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. Remove the tubes and aspirate the supernatant.

6.2 Add 1 mL of 100% methanol to each tube and gently resuspend the bacteria. 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, then vortex the samples for 30 seconds.

6.3 Centrifuge the samples at 14,000 RPM (>15,000 × *g*) for 60 seconds. Aspirate the supernatant from each tube and replace with 1 mL of HBSS. Carefully resuspend the sample in each tube, add 0.5 mL of HBSS, then 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 can resuspend them in water at 50 mg/mL, divide into aliquots, then lyophilize (we recommend 2–5 mg per aliquot). If they are to be used within 2–3 days, you can resuspend them in the assay buffer or in the buffer of your choice.

Determine the pH response range of the conjugate

7.1 Resuspend aliquots of your conjugate at a final concentration of 1 mg/mL in 500 µL each of a series of buffers with the 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. Increases of 8-fold or higher are favorable for cellular experiments.

Ordering information

Cat. No.	Product name	Unit size
P36010	pHrodo™ iFL Red STP ester, amine reactive dye	1 mg
P36011	pHrodo™ iFL Red STP ester, amine reactive dye	3 × 100 µg
P36012	pHrodo™ iFL Green STP ester, amine reactive dye	1 mg
P36013	pHrodo™ iFL Green STP ester, amine reactive dye	3 × 100 µg

Related products

P36014	pHrodo™ iFL Red Microscale Labeling Kit	1 kit
P36015	pHrodo™ iFL Green Microscale Labeling Kit	1 kit
24115	8 M Guanidine-HCl solution	200 mL
14025092	Hanks' Balanced Salt Solution (HBSS)	500 mL
D12345	DMSO, Anhydrous	3 × 10 mL

Purchaser notification

These high-quality reagents and materials must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Read the Safety Data Sheet provided for each product; other regulatory considerations may apply.

Obtaining support
For the latest services and support information for all locations, go to **thermofisher.com/support**.

At the website, you can:
- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**thermofisher.com/support**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

SDS
Safety Data Sheets (SDSs) are available at **thermofisher.com/support**.

Certificate of Analysis
The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **thermofisher.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the product packaging (tube, pouch, or box).

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 found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **thermofisher.com/support**.

For Research Use Only. Not for use in diagnostic procedures.

Disclaimer
TO THE EXTENT ALLOWED BY LAW, LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. MAN0017101

Revision	Date	Description
B.0	22 October 2021	Edits to Table 3 regarding dye physical characteristics.
A.0	21 June 2017	New User Guide

Important Licensing Information
These products may be covered by one or more Limited Use Label Licenses. By use of these products, you accept the terms and conditions of all applicable Limited Use Label Licenses.

Manufacturer: Life Technologies Corporation | 29851 Willow Creek Road | Eugene, OR 97402

Trademarks
All trademarks are the property of Thermo Fisher Scientific and its subsidiaries, unless otherwise specified.
NanoDrop is a trademark of NanoDrop Technologies, Inc. Tecan and Safire are trademarks of Tecan Group AG.

ThermoFisher SCIENTIFIC