pHrodo™ iFL Red and Green Antibody Labeling Kits

Catalog Numbers P36020, P36021, P36022, and P36023

Pub. No. MAN0029967 Rev. A.0

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 pHrodo™ iFL Red and Green Antibody Labeling kits provide an efficient method for labeling varying amounts of IgG with pHrodo™ iFL red or pHrodo™ iFL green dye. pHrodo™ 4-sulfotetrafluorophenyl (STP) esters readily react with the amines of an antibody to yield a covalently-attached fluorogenic pH probe.

The spin columns included in each kit are used for purifying labeled antibodies from excess dye, providing yields of 70–95%.

This user guide describes a general protocol for using the amine-reactive, STP-ester form of the pHrodo™ iFL dye.

Contents and storage

The contents of each kit are sufficient for three labeling reactions of 100 µg or 1 mg of antibody.

Table 1 pHrodo™ iFL Antibody Labeling kits

Contents	Cat. No. (3 × 100 µg)		Cat. No. (3 × 1 mg)		Storage[1]
	P36020 (red)	P36022 (green)	P36021 (red)	P36023 (green)	
pHrodo™ iFL Reactive Dye (red or green) (Component A)	3 vials		3 vials		• 2–8°C • Dessicate • Protect from light • Do not freeze
PBS Exchange Buffer (Component B)	3 mL		12 mL		
Sodium Bicarbonate (Component C)	84 mg		84 mg		
pHrodo™ iFL Dye Removal Column (Component D)[2]	3 each		3 each		
Wash Tubes	3 each		—		
Collection Tubes	3 each		—		
Wash and Collection Tubes	—		6 each		
Dimethyl Sulfoxide (DMSO)	0.5 mL		0.5 mL		

[1] The product is stable for at least 6 months when stored as directed.
[2] The resin is supplied in a 0.1 N NaCl/0.05% sodium azide solution.

Table 2 Technical specifications

Product	Molecular weight	Ex/Em[1]	Molar extinction coefficient (ε dye)[2]
pHrodo™ iFL red 4-sulfotetrafluorophenyl (STP) ester	~1,000 g/mol	560/585 nm	65,000 cm^{-1} M^{-1}
pHrodo™ iFL green 4-sulfotetrafluorophenyl (STP) ester	~1,000 g/mol	505/525 nm	74,500 cm^{-1} M^{-1}

[1] Excitation/emission maxima for the dye (conjugated to an antibody).
[2] Extinction coefficient at the excitation maximum for the dye (conjugated to an antibody).

Required materials not supplied

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

Item	Source[1]
Centrifuge capable of 1,000 × *g*, one of the following:	
For 100-µg kits: Microcentrifuge	MLS
For 1-mg kits: Benchtop centrifuge	MLS
Desired antibody for labeling (free of BSA or any carrier protein)	—
(Optional) Pierce™ Bradford Plus Protein Assay Kit	23236
(Optional) 8 M Guanidine-HCl for degree of labeling determination	MLS

[1] "MLS" indicates that the material is available from fisherscientific.com or another major laboratory supplier.

Guidelines for antibody preparation

- **IMPORTANT!** The purified antibody should be 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.
- Purify protein samples that contain carriers, such as BSA (e.g., antibodies), or any partially-purified protein samples before labeling. The presence of low concentrations (<0.1% (w/v) of biocides, including sodium azide and thimerosal, will not significantly affect the labeling reaction.
- Use dialysis or small-scale gel filtration to remove low molecular weight components from the protein sample (desalting) before labeling. For dialysis we recommend using the Slide-A-Lyzer™ Dialysis Cassettes (available from thermofisher.com). Zeba™ Dye and Biotin Removal Columns (Cat. Nos. A44296S or A44298) can also be used to re-equilibrate the antibody in an appropriate buffer before labeling.
- We recommend PBS, pH 7.2–7.5, as a prelabeling dialysis buffer. Alternatively, a 100 mM sodium bicarbonate buffer can also be used.
- Removal of free dye after the labeling reaction is essential for the accurate determination of dye-to-antibody ratios. For optimal antibody recovery and dye removal, ensure that the appropriate amount of sample and buffer conditions are used.

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

- Several spectrophotometric methods are available for determining the DOL of pHrodo™ iFL dye-labeled conjugates. Each are based on obtaining the concentration by measuring the absorbance of the protein at 280 nm and the absorbance of the dye at its excitation maximum.
- We recommend using a NanoDrop™ spectrophotometer to analyze the antibody conjugate 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 this kit are accurate only when they are diluted using an 8 M Guanidine-HCl solution (pH 5.0). We recommend diluting the purified antibody conjugates before measuring the absorbance.

  Note: This procedure will likely destroy the conjugate sample, thus making it unrecoverable.
- 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 purified antibody conjugate, then dilute it only to the minimum volume necessary for your cuvettes and spectrophotometer to avoid readings below the optimal range for your instrument.

Perform the labeling and purification procedure

1 Prepare the reagents

- Prepare a 1 M sodium bicarbonate solution—Add 1 mL of deionized water to the vial of Sodium Bicarbonate (Component C). Vortex or pipet up and down until the reagent is fully dissolved.

  The 1M sodium bicarbonate solution will have a pH of ~8.3 and can be stored at 2–8°C for up to two weeks, or frozen for long-term storage.
- Prepare pHrodo™ iFL Reactive Dye immediately before use—Add 80 µL of DMSO to the vial of pHrodo™ iFL Reactive Dye (final concentration = 1.25 mg/mL (1.25 mM)).

  IMPORTANT! Use the pHrodo™ iFL Reactive Dye immediately after dilution with DMSO.

2 Label the antibody

1. Based on the volumes indicated in Table 3, calculate the volume of PBS Exchange Buffer (Component B) that will be required to bring the final volume of the labeling reaction to 50 µL (for 100-µg kits) or 500 µL (for 1-mg kits).

2. Combine the following components (in the order indicated) in an appropriately-sized tube. Vortex after each component is added to the tube.

   Note: The labeling reaction is prepared at a 10:1 molar ratio (dye:antibody). In some cases, however, the optimal molar ratio of dye to antibody may have to be empirically determined.

   Table 3 Labeling reaction

Component	Volume	
	100-µg scale	1-mg scale
PBS Exchange Buffer	Use the volume calculated in step 2.1.	Use the volume calculated in step 2.1.
Antibody[1]	100 µg	1 mg
1 M Sodium bicarbonate solution	5 µL	50 µL
pHrodo™ iFL Reactive Dye (diluted with DMSO)	5.3 µL	53 µL
Final volume	**50 µL**	**500 µL**

   [1] Final antibody concentration = 2 mg/mL.

3. Vortex, then incubate the reaction mixture for 15 minutes at 20°C.

   Proceed to prepare the spin column during the 15-minute incubation (see next section).

3 Prepare the spin column

IMPORTANT! Do not reuse the purification resin.

1. Loosen the cap on a spin column, twist the tab off of the bottom, then place the column into a wash tube.

   Note: For 1-mg kits, the wash tubes and collection tubes are the same. Designate three as wash tubes (lid can be discarded) and three as collection tubes (lid can be saved). For 100-µg kits, the wash tubes do not have caps.

2. Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes.

   Note: When using a fixed angle rotor, place a mark on the side of the column that faces away from the rotor center. For all subsequent centrifugation steps, place the column in the microcentrifuge with the mark facing away from the rotor center.

   IMPORTANT! Improper orientation of the column during centrifugation can result in reduced dye removal.

3. Discard the flow-through, then place the column back into the wash tube.

4. Add the appropriate volume of PBS Exchange Buffer (Component B), then centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes to equilibrate the column.

   - **For 100-µg kits—**Add 400 µL of PBS Exchange Buffer
   - **For 1-mg kits—**Add 1 mL of PBS Exchange Buffer

5. Discard the flow-through.

4 Process the sample

1. Transfer the equilibrated column into a fresh collection tube.

   Note: The collection tubes that are provided in 100-µg kits do not have caps.

2. Carefully drip the entire reaction mixture onto the center of the column. For 100-µg reactions, add the 50-µL reaction mixture, followed by 20 µL of PBS Exchange Buffer.

4 Process the sample *(continued)*

3. Centrifuge the column-tube assembly at 1,000 × *g* for 2 minutes.

   The purified antibody conjugate is in the collection tube.

4. *(Optional)* If precipitates are visible in the collected sample, centrifuge at 16,000 × *g* for 5 minutes to remove the precipitated material.

(Optional) Record the volume of purified antibody conjugate for yield determination.

(Optional) Determine the protein concentration and DOL

Labeling efficiency may require optimization 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.

1. Dilute a small volume of the purified antibody conjugate 1:3 in 8 M Guanidine-HCl (pH 5.0), then measure OD_{280} and OD_{560} for pHrodo™ iFL red dye, or OD_{280} and OD_{505} for pHrodo™ iFL green dye.

   Note: This procedure will likely destroy the conjugate sample, thus making it unrecoverable.

   The absorbance maxima, extinction coefficient, and correction factor (for the contribution of the fluorophore to A_{280}) for pHrodo™ iFL red and green dyes are shown in the following table.

Dye	Absorbance maximum (λ max)	Extinction coefficient (ε dye)	Correction factor (CF)
pHrodo™ iFL red	560 nm	65,000	0.12
pHrodo™ iFL green	505 nm	74,500	0.20

2. Calculate the concentration of protein in the sample using the following formula.

$$\text{Protein concentration}_{\text{Red}}\ (\text{M}) = \frac{[A_{280} - 0.33\ (A_{560})] \times \text{dilution factor}}{\text{Protein extinction coefficient}}$$

$$\text{Protein concentration}_{\text{Green}}\ (\text{M}) = \frac{[A_{280} - 0.33\ (A_{505})] \times \text{dilution factor}}{\text{Protein extinction coefficient}}$$

   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 contribution of the fluorophore to A_{280}.

3. Calculate the DOL using the following formula.

   Note: Alternatively, calculate the DOL using the **Degree of Labeling Calculator for Antibody Labeling**. Go to **thermofisher.com/us/en/home/life-science/antibodies/antibody-labeling/dol-calculator**

$$\text{DOL}_{\text{Red}} = \frac{\text{moles dye}}{\text{moles protein}} = \frac{A_{560} \times \text{dilution factor}}{65{,}000 \times \text{protein concentration (M)}}$$

$$\text{DOL}_{\text{Green}} = \frac{\text{moles dye}}{\text{moles protein}} = \frac{A_{505} \times \text{dilution factor}}{74{,}500 \times \text{protein concentration (M)}}$$

   Note: Where 65,000 $cm^{-1}M^{-1}$ is the approximate molar extinction coefficient of pHrodo™ iFL red dye and 74,500 $cm^{-1}M^{-1}$ is the approximate molar extinction coefficient of pHrodo™ iFL green dye.

(Optional) Determine antibody yield

Note: For 1-mg kits, we recommend diluting the purified conjugate 1:1 before determining the antibody yield.

1. Set aside 10 µL of the purified antibody conjugate.
2. Measure protein concentration using the Pierce™ Bradford Plus Protein Assay Kit (Cat. No. 23236).
3. Calculate the amount (mg) of antibody recovered—Multiply the antibody concentration (mg/mL) that was determined in step 2 by the volume of the recovered sample.

Related products

Cat. No.	Product name	Amount
P36014	pHrodo™ iFL Red Microscale Protein Labeling Kit	1 kit
P36015	pHrodo™ iFL Green Microscale Protein Labeling Kit	1 kit
A44296S	Zeba™ Dye and Biotin Removal Columns, 0.5 mL	5 columns
A44298	Zeba™ Dye and Biotin Removal Columns, 2 mL	5 columns
P35358	pHrodo™ Deep Red dye (TFP ester)	3 x 100 µg
P35359	pHrodo™ Deep Red dye (TFP ester)	1 mg

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**.

Life Technologies Corporation | 29851 Willow Creek Road | Eugene, Oregon 97402 USA
For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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Revision	Date	Description
A.0	12 September 2023	New document for pHrodo™ iFL Red and Green Antibody Labeling kits.