pHrodo™ Deep Red Antibody Labeling Kits

Catalog Numbers P35355 and P35356

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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 pHrodo™ Deep Red Antibody Labeling Kits provide a method for efficiently labeling varying amounts of IgG with pHrodo™ Deep Red dye. The pHrodo™ Deep Red STP esters contained in these kits readily react with an antibody's amines to yield a covalently attached fluorogenic pH probe.

The spin columns included in the kits are used for purifying the labeled antibody from excess dye with yields of 70–95%. The kits contain sufficient reagents for three labeling reactions of 100 µg or 1 mg of antibody.

This user guide describes a general protocol for using the amine-reactive, TFP ester form of the pHrodo™ Deep Red dye for labeling either 100 µg or 1 mg of IgG.

Contents and storage

Table 1 pHrodo Deep Red Antibody Labeling Kits

Contents	Cat. No.	Amount
pHrodo™ Deep Red Antibody Labeling Kit	P35355	3 x 100 µg
pHrodo™ Deep Red Antibody Labeling Kit	P35356	3 x 1 mg

Table 2 Contents and storage

Component	Amount		Storage[1]
	Cat. No. P35355	Cat. No. P35356	
pHrodo™ Deep Red tetrafluorophenyl (TFP) ester (Component A)	3 vials	3 vials	• 2–8°C • Dessicate • Protect from light • Do not freeze
PBS exchange buffer (Component B)	3 mL	10 mL	
Sodium Bicarbonate (Component C)	84 mg	84 mg	
pHrodo™ Deep Red dye removal column (Component D)[2]	3 each	3 each	
Wash tubes	3 each	—	
Collection tubes	3 each	—	
Wash and collection tubes	—	6 each	

[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 3 Technical specifications of pHrodo™ Deep Red STP ester, amine-reactive dye

Product	Molecular weight (g/mol)	Ex/Em[1]	Molar extinction coefficient (ε dye)[2]
pHrodo™ Deep Red tetrafluorophenyl (TFP) ester	~1,300	640/655	140,000

[1] Excitation/Emission maxima for the dye, in nm conjugated to an antibody
[2] Extinction coefficient at λ_{640} in cm^{-1} M^{-1}

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.

Item	Source
For Cat. No. P35356: Bench top centrifuge capable of 1,000 x *g*	MLS
For Cat. No. P35355: Microcentrifuge capable of 1,000 x *g*	MLS
Antibody desired for labeling, must be free of BSA or any carrier protein	—
(Optional) Pierce™ Coomassie Plus (Bradford) Assay Kit	23236
(Optional) 4.3% (wt%) phosphoric acid for DOL determination	MLS

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.
- 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**). The Zeba™ Dye and Biotin Removal Columns (Cat. Nos. A44296S or A44298) may also be used to re-equilibrate the antibody before labeling in an appropriate buffer.
- 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 the antibody (100 µg scale, Cat. No. P35355)

1. Prepare a 1 M sodium bicarbonate solution by adding 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 bicarbonate solution will have a pH of ~8.3 and can be stored at 2–8°C for up to two weeks. It can be frozen for long-term storage.

2. Prepare the antibody to be labeled in supplied PBS exchange buffer (component B) or a pH neutral buffer at a concentration of 1.1 mg/mL.

3. Add 90 µL of the 1.1 mg/mL antibody solution from the previous step to the pHrodo™ Deep Red tetrafluorophenyl (TFP) ester (Component A).

4. Add 10 µL of the 1 M sodium bicarbonate solution from step 1 to the vial containing the antibody and dye.

5. Mix well to ensure complete dissolution of the dye and antibody by pipetting up and down several times. If any liquid splashes up the side of the container, spin briefly to return the entire reaction volume to the bottom.

6. Incubate the reaction mixture for 2 hours at 20–25°C.

Label the antibody (1 mg scale, Cat. No. P35356)

1. Prepare a 1 M sodium bicarbonate solution by adding 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 bicarbonate solution will have a pH of ~8.3 and can be stored at 2–8°C for up to two weeks. It can be frozen for long-term storage.

2. Prepare the antibody to be labeled in supplied PBS exchange buffer (component B) or a pH neutral buffer at a concentration of 2.2 mg/mL.

3. Add 450 µL of the 2.2 mg/mL antibody solution from the previous step to the pHrodo™ Deep Red tetrafluorophenyl (TFP) ester (Component A).

4. Add 50 µL of the 1 M sodium bicarbonate solution from step 1 to the vial containing the antibody and dye.

5. Mix well to ensure complete dissolution of the dye and antibody by pipetting up and down several times. If any liquid splashes up the side of the container, spin briefly to return the entire reaction volume to the bottom.

6. Incubate the reaction mixture for 2 hours at 20–25°C.

Purifying the labeled antibody

pHrodo™ Deep Red dye removal columns contain a ready to use resin that is designed for rapid removal of pHrodo™ Deep Red dye with exceptional antibody recovery. Removal of free dye after a 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.

Prepare the spin column

IMPORTANT! Do not reuse the purification resin.

1. Ten minutes before the end of the 2 hour incubation, loosen the cap on a spin column, twist the tab off of the bottom of the column, and place into a wash vial.

   Note: For Cat. No. P35356, the wash vials and collection vials are the same. Designate three as wash vials (lid can be discarded) and three as collection vials (lid can be saved). For Cat. No. P35355, the wash vials have no cap.

2. Centrifuge the column tube assembly at 1,000 × *g* for 2 minutes to remove the storage buffer and pack the column.

   Note: When using a fixed angle rotor, place a mark on the side of the column facing 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 and set the column back into the wash vial.

4. Add 400 µL (for Cat. No. P35355) or 500 µL (for Cat. No. P35356) of supplied PBS exchange buffer (Component B), or desired buffer to equilibrate the column by centrifuging the column tube assembly at 1,000 × *g* for 2 minutes.

5. Discard the flow-through.

Process the sample

1. Transfer the packed and equilibrated column into a fresh collection vial.

   Note: For Cat. No. P35355, the collection vials have caps.

2. Carefully drip the entire reaction mixture onto the column.

3. Centrifuge the column tube assembly at 1,000 × *g* for 2 minutes to collect the sample. Discard the column.

The antibody pHrodo™ Deep Red conjugate is in the collection tube.

(Optional) Make note of the volume collected for yield determination.

(Optional) Determining antibody yield

1. Set aside 10 µL of conjugated antibody solution.

2. The antibody yield can be determined using the Pierce™ Coomassie Plus (Bradford) Assay Kit (Cat. No. 23236).

   Note: For Cat. No. P35356 we recommend a 1:1 dilution of antibody conjugate prior to determining yield.

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

   The absorbance maxima, extinction coefficient, and correction factor (for the fluorophore's contribution to A_{280}) for pHrodo™ Deep Red dye are shown in the following table.

Dye	Absorbance maximum (λ max)	Extinction coefficient (ε dye)	Correction factor (CF)
pHrodo Deep Red	640 nm	140,000	0.33

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

3. Calculate the concentration of protein in the sample using the following 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:

$$DOL = \frac{\text{moles dye}}{\text{moles protein}} = \frac{A_{640} \times \text{dilution factor}}{140{,}000 \times \text{protein concentration (M)}}$$

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

Related products

Cat. No.	Product name	Amount
P36014	pHrodo™ iFL Red Microscale Labeling Kit	1 Kit
P36015	pHrodo™ iFL Green Microscale 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 STP Ester	3 x 100 µg
P35359	pHrodo™ Deep Red STP Ester	1 mg

Notes

Many protein- and dye-specific properties determine how efficiently a protein can be labeled with an amine-reactive dye. Important factors include the number of solvent-accessible primary amines in the protein, the protein's pI, and its solubility and stability at pH 8–8.3. Reactive labels vary in amine reactivity, often in a protein-specific way, and their behavior can be predicted with confidence for only a few proteins such as antibodies and streptavidin.

The number of reactive fluorescent dyes that can be attached to a protein before fluorescence quenching or protein inactivation or precipitation occurs is roughly proportional to the protein's molecular weight. For example, the optimal DOL with this dye would usually be ~1 for a ~20 kDa protein, while the optimal DOL for a ~150 kDa protein, e.g., an IgG, would usually be 2–4. The DOL that you obtain with a protein using the pHrodo™ iFL Protein Labeling Kit may be higher or lower than the generally accepted optimum. We recommend that you evaluate your protein conjugate in its intended application before you conclude that it is under- or overlabeled.

Underlabeling

- Even trace amounts of primary amine-containing components (e.g., Tris, glycine, ammonium ions, ethanolamine, triethylamine, or glutathione) or imidazole in the starting protein sample decreases labeling efficiency.
- Efficient labeling will probably not occur if the concentration of protein starting material is <1 mg/mL.
- The addition of sodium bicarbonate is designed to raise the pH of the reaction mixture to ~8, as STP esters react most efficiently with primary amines at slightly alkaline pH. If the protein solution is strongly buffered at a lower pH, the addition of 1/10 volume of bicarbonate solution will not raise the pH to the optimal level. Either more bicarbonate can be added, or the buffer can be exchanged with PBS, pH 7.2 (and bicarbonate solution added again), or with 100 mM sodium bicarbonate buffer, pH 8.3, by dialysis or another method prior to starting the labeling reaction.
- Because proteins react with fluorophores at different rates and retain biological activity at different degrees of dye labeling, using a dye:protein molar ratio (MR) of 10 may not always result in optimal labeling. To increase the DOL, the same protein sample can be relabeled, or a new protein sample can be labeled using more reactive dye. Three vials of reactive dye are provided to allow three labeling reactions. Although this kit was designed for optimal labeling in 15 minutes at room temperature, higher DOL may be obtained with longer incubation times. We have not evaluated incubation times >15 minutes.
- Underlabeling may be the reason for the fluorescent signal being lower than expected in your application. Should this occur, relabel the sample, or label another sample with more reactive dye.
- We have observed that dye-labeling of some proteins to any degree can destroy their biological activity.

Overlabeling

- Overlabeling may be indicated by the formation of a red or green precipitate in the reaction mixture or deposition of red or green particles on the upper surface of the resin bed after centrifugation of the conjugate. Precipitation usually results in a decreased yield of labeled protein. If your % yield is <50%, it is likely that the protein is overlabeled. Repeat the labeling reaction with less reactive dye. Some proteins cannot be labeled with amine-reactive dyes under any circumstances and may irreversibly precipitate.
- If no visible precipitate forms during labeling but the fluorescent signal in your application is lower than expected, the fluorescence of the protein conjugate may be quenched due to overlabeling. To reduce the DOL, use a smaller amount of reactive dye, or try labeling the protein at a concentration of >1 mg/mL. We have not evaluated labeling efficiency with this kit on proteins at concentrations >1 mg/mL.
- One cause of apparent overlabeling is inefficient removal of unreacted dye. Although using the spin columns in this kit exactly as described removed all traces of free dye from all of the proteins we tested, it is possible that some free dye may be present in your sample after the purification step. The presence of free dye, which can be determined by thin layer chromatography, results in erroneously high calculated DOL values. Free dye remaining after use of the spin column can be removed by applying the conjugate to another spin filter or by extensive dialysis. Applying no more than 50 µg of conjugate to 500 µL resin and no more than 100 µg of conjugate to 800 µL resin packed in each spin column is the best way to avoid contamination with free dye.
- We have observed that dye-labeling of some proteins to any degree can destroy their biological activity.

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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Revision	Date	Description
B.0	16 February 2022	The formula for degree of labeling (DOL) was replaced.
A.0	05 November 2020	New document.

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