pHrodo™ iFL Microscale Protein Labeling Kits

Catalog Nos. P36014, P36015

Pub. No. MAN0017102 **Rev.** A.0

Product description

The pHrodo™ iFL Red and pHrodo™ iFL Green Microscale Protein Labeling Kits provide a method for efficiently labeling small amounts of protein. The pHrodo™ Red and Green iFL STP esters contained in these kits readily react with proteins' amines to yield covalently attached fluorogenic pH probes.

The spin columns included in the kits are used for purifying the labeled protein from excess dye reagents with yields of 50–75%, depending primarily on the molecular weight of the labeled protein. Labeling and purification can be completed in as little as 30 minutes. Alternatively, excess reagents can be removed by dialysis, thereby avoiding further dilution of the labeled protein. The kits contain sufficient reagents for three labeling reactions of 20–100 µg of protein (with MW of ~20–150 kDa) at a concentration of 1 mg/mL.

Table 1 pHrodo™ iFL Microscale Protein Labeling kits

Product*	Amount	Catalog No.
pHrodo™ iFL Red Microscale Protein Labeling Kit	1 kit	P36014
pHrodo™ iFL Green Microscale Protein Labeling Kit	1 kit	P36015
* For kit contents and storage, see Table 2.		

Table 2 Contents and storage

Component	Amount	Storage*
pHrodo™ iFL STP ester, amine-reactive dye (Component A)†	3 vials (100 µg/vial)	• 2–8°C • Desiccate • Protect from light • **Do not freeze**
Dimethylsulfoxide (DMSO), anhydrous (Component B)	500 µL	
Sodium bicarbonate (Component C)	84 mg	
Reaction tubes (Component D)	3 each	
Spin columns (Component E)	4 each	
Purification resin (Bio-Gel™ P-6 fine resin suspended in PBS) (Component F)	3 mL, settled	
* When stored as directed this kit is stable for at least 6 months. † pHrodo™ iFL Red STP ester (Cat. No. P36014) or pHrodo™ iFL Green STP ester (Cat. No. P36015). For the technical specifications of the pHrodo™ iFL Red and Green STP ester, amine-reactive dyes, see Table 3 (page 2).		

Table 3 Technical specifications of pHrodo™ iFL STP ester, amine-reactive dyes

Product	MW*	Ex/Em†	Molar extinction coefficient (ε)‡
pHrodo™ iFL Red STP ester, amine-reactive dye	1,000	560/585	65,000
pHrodo™ iFL Green STP ester, amine-reactive dye	1,000	505/525	74,500

* Molecular weight, in g/mol.

† Excitation/Emission maxima for the dye, in nm.

‡ Molar extinction coefficient at 280 nm, in cm^{-1} M^{-1}.

Important guidelines

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 bicarbonate buffer, you can omit step 1.1 of the labeling reaction and the addition of 1/10 volume of bicarbonate (step 1.3).

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 4 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).

  The MR values are based on two parameters: the molecular weight of representative proteins and the optimal degree of labeling (DOL) with the pHrodo™ iFL dye for these proteins, as determined in our laboratories.

  For your initial labeling attempt, choose the optimal MR for the protein listed in Table 4 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 (see **Notes**, page 7).

Table 4 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 4, 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 a 1:3 dilution into 8 M Guanidine HCl (see **Determine the Degree of Labeling**, page 5).

Label the protein

1.1 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.

Note: 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 also be frozen for long-term storage.

1.2 Prepare a 1 mg/mL protein solution in PBS or a similar buffer free of primary amines, then transfer 100 µL of this solution (100 µg) to a reaction tube (Component D).

1.3 Add 1/10 volume (10 µL) of 1 M sodium bicarbonate to the protein solution, then mix by pipetting up and down several times.

1.4 Dissolve one vial (100 µg) of pHrodo™ iFL Red or Green STP ester, amine-reactive dye (Component A) in 50 µL of DMSO (Component B) to prepare the 2 mg/mL (2 mM) pHrodo™ iFL labeling solution. Completely dissolve the contents of the vial by pipetting up and down.

Note: Prepare this solution immediately before use and discard any leftover solution.

1.5 Add the calculated amount of the 2 mM pHrodo™ iFL labeling solution to the reaction tube containing the protein solution. Mix thoroughly by pipetting up and down several times.

Note: To label different amounts of protein or to label the protein using a different MR, use Table 4 and the equation in **Guidelines for labeling reaction** to calculate the amount of 2 mM reactive dye to add to the reaction mix.

1.6 Incubate the reaction mixture for at least 15 minutes at room temperature.

Purify the labeled protein

2.1 To prepare the purification spin column, fully resuspend the gel resin (Component F) by gently rocking the container (do **not** vortex or use a magnetic stir bar), then fill the upper chamber of the spin column (Component E) up to the lip with the suspended gel resin (Figure 1).

Note: You need approximately 800 µL of gel resin to purify 100 µg of labeled protein. If purifying less than 50 µg of protein, you can use 500 µL of gel resin.

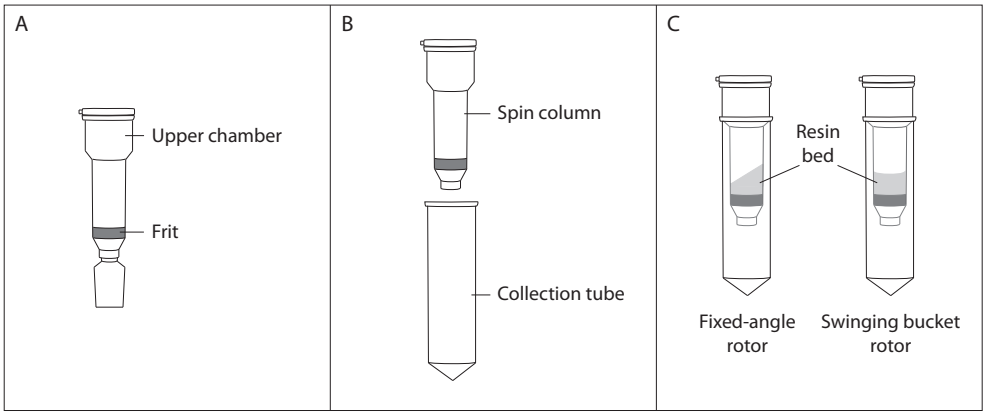

Figure 1 Spin column and collection tube. (A) An empty spin column with the tip attached showing the separate parts. (B) An empty spin column with the tip snapped off and a collection tube. (C) Appearance of the resin bed after centrifugation in a fixed-angle rotor or a swinging bucket rotor.

2.2 Remove the yellow cap from inside the column (not shown in Figure 1), snap off the tip from the bottom of the spin column, then place the column with the cap off into a collection tube.

Note: The yellow cap is only needed if the column is set up in advance of step 2.3 to prevent it from drying out.

2.3 Centrifuge the spin column at 1,000 × *g* in a microcentrifuge or swinging bucket rotor for a total of 3 minutes (including run-up time). Using a fixed-angle rotor causes the resin to pack down with a low side and a high side. After centrifugation, the resin should be about 10–20 mm above the frit, depending on the volume of gel necessary to purify the amount of protein.

Figure 1 illustrates the spin column and collection tube, and what the filled spin column should look like after centrifugation. If the resin bed is too small, add more suspended resin and centrifuge again. If there is too much resin, resuspend the resin in the upper chamber in buffer, remove the necessary amount, then centrifuge again to repack the resin bed.

Note: Occasionally, some resin gets into the collection tube during column preparation, especially if the column has been centrifuged at too high a speed. When the resin bed is at the correct level, rinse out the collection tube under the spin column several times with buffer to remove any resin particles that may be found there, and replace the resin-containing insert.

2.4 *Optional*: If you wish to purify the labeled protein in a buffer other than the PBS, pH 7.2, in which the resin is suspended, there are two ways to exchange the buffer:

- When the resin in the bottle is completely settled, you can decant or aspirate the buffer provided and replace it with another buffer of your choice. Add your buffer to the bottle, mix gently to resuspend the resin, and let it settle completely. Carefully remove the buffer again, and repeat this washing process several times.
- You can also exchange the buffer after the resin bed is prepared in the spin column by washing your chosen buffer through the bed several times by brief low-speed centrifugation. The Bio-Gel P-6 fine resin provided is stable between pH 2 and pH 10.

2.5 After you have prepared the spin column, pipet the reaction mixture containing the labeled protein onto the center of the resin bed surface, then centrifuge at 1,000 × *g* for a total of 5 minutes.

2.6 After centrifugation, each collection tube contains purified dye-labeled protein in approximately 30–130 µL of buffer. The unreacted dye is retained in the filter and the resin will have a reddish or greenish color, depending on the pHrodo™ iFL dye used (see **Notes**, page 7).

Determine the Degree of Labeling (DOL)

Guidelines for determining the DOL

- Several spectrophotometric methods are available for determining the DOL of pHrodo™ iFL dye–labeled protein conjugates. They are based on obtaining the protein concentration by absorbance at 280 nm (A_{280}) and the absorbance of the dye at its excitation maximum (λ_{max}).
- We recommend using a NanoDrop™ spectrophotometer to analyze the labeled protein spectrophotometrically. NanoDrop™ instruments (available from **thermofisher.com**) require only 1–2 µL of sample and are full-featured UV/Vis instruments. A variety of cuvettes are available for use with small sample volumes, if you would prefer not to dilute your labeled protein to use in standard 0.5-mL or 1.5-mL cuvettes.

  Note: You can also use a microplate reader that allows you to specify the desired detection wavelengths, such as the Tecan™ Safire™ (Tecan US, Research Triangle Park, North Carolina, USA).
- 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. We recommend diluting the protein conjugate samples 1:3 in 8 M guanidine-HCl before measuring the absorbance.
- Excessive dilution of some proteins with low intrinsic A_{280} may prevent you from deriving accurate A_{280} values for your samples. Use only a portion of your protein 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.

Determine the DOL

3.1 Dilute the protein conjugate samples 1:3 in 8 M guanidine-HCl and Measure the absorbance of the protein conjugate at 280 nm (A_{280}) and at the λ_{max} for the pHrodo™ iFL dye. Table 5 provides the absorbance maxima, the extinction coefficient, and the correction factor (for the fluorophore's contribution to A_{280}) for the pHrodo™ iFL dyes.

Table 5 pHrodo™ iFL dye properties

pHrodo™ iFL dye	Absorbance maximum (λ_{max})	Extinction coefficient (ε_{dye})	Correction factor (CF)
Red	560 nm	65,000	0.12
Green	505 nm	74,500	0.2

3.2 Calculate the concentration of the protein in the sample:

$$\text{Protein concentration (mg/mL)} = \frac{[A_{280} - (CF \times \lambda_{max})] \times \text{dilution factor}}{A_{280} \text{ of protein at 1 mg/mL}}$$

3.3 Calculate the molarity of the protein sample:

$$\text{Protein concentration (M)} = \frac{\text{Protein concentration (mg/mL; from Step 3.2)}}{\text{Protein molecular weight (Da)}}$$

Note: If you know the molar extinction coefficient (ε, in $cm^{-1}\ M^{-1}$) of your protein at 280 nm, you can use this value as the divisor in Step 3.2 to directly calculate the molarity of the protein sample.

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

3.4 Calculate the degree of labeling (DOL):

$$DOL = \frac{\text{moles dye}}{\text{mole protein}} = \frac{\lambda_{max} \times \text{dilution factor}}{\varepsilon_{dye} \times \text{protein concentration (M)}}$$

3.5 If the A_{280} of your protein is too low to measure accurately, you can get an approximate protein concentration by estimating the % yield of the protein conjugate. The % yield from the spin columns is related to the molecular weight of the protein. Once you have a value for % yield, you can calculate the approximate protein concentration using the following equation:

$$\text{Concentration of labeled protein (mg/mL)} = \frac{\text{mass of starting protein (mg)} \times \%\text{ yield}}{\text{volume recovered (mL)}}$$

3.6 Divide the concentration (in mg/mL) by the protein's molecular weight (in Da) to calculate the approximate molar concentration of the protein. To determine the approximate DOL, you still must spectrophotometrically determine the λ_{max} of the conjugate, which is used in step 3.4 to determine the DOL.

Store the protein conjugate

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 protein 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 proteins, you can divide the conjugate into small aliquots and freeze them at ≤–20°C for longer storage. **Avoid repeated freezing and thawing, and protect from light.**

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 (step 1.3) 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.

Ordering information

Cat. No.	Product name	Unit size
P36014	pHrodo™ iFL Red Microscale Labeling Kit	1 kit
P36015	pHrodo™ iFL Green Microscale Labeling Kit	1 kit
Related products		
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
24115	8 M Guanidine-HCl solution	200 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.

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Revision	Date	Description
A.0	21 June 2017	New User Guide

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