molecular probes®
by life technologies™

# pHrodo™ Red Microscale Labeling Kit

**Catalog number** P35363

**Table 1** Contents and storage

| Material | Amount | Storage | Stability |
|---|---|---|---|
| pHrodo™ Red succinimidyl ester (Component A) | 3 vials | • 2°C–8°C<br>• Desiccate<br>• Protect from light<br>• Do not freeze | When stored as directed this kit is stable for at least 6 months. |
| 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 | | |

## Introduction

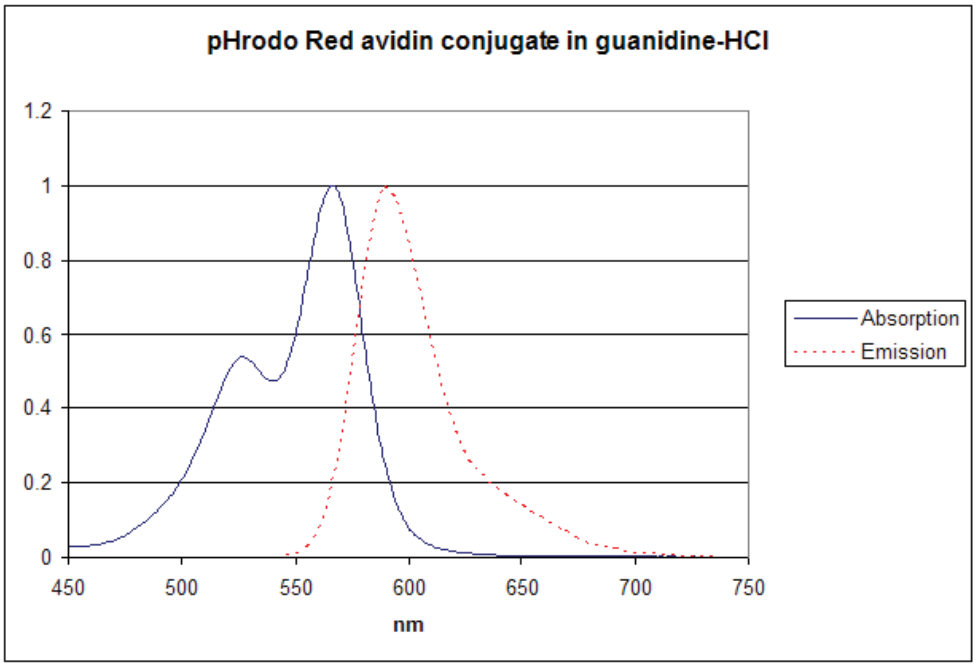
The pHrodo™ Red Microscale Labeling Kit provides a method for efficiently labeling small amounts of protein. The pHrodo™ Red succinimidyl ester contained in this kit readily reacts with a protein's amines to yield a covalently attached fluorogenic pH probe.

The spin columns included in the kit provide a convenient method for purifying the labeled protein from excess dye reagents with yields between 50 and 75%, depending primarily on the molecular weight of the protein to be labeled. 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 pHrodo™ Red Microscale Labeling Kit contains sufficient reagents for three reactions of 20–100 µg each.

The pHrodo™ Red dye-labeled proteins have fluorescence excitation and emission maxima of approximately 566 and 590 nm, respectively (Figure 1, next page). The pHrodo™ Red dye is a fluorogenic dye that dramatically increases in fluorescence as the pH of its surrounding becomes more acidic. This amine-reactive succinimidyl ester form of the dye has a pKa of ~7.3 in solution, which shifts to about ~6.5 upon conjugation to the K-12 strain of *E. coli*. pHrodo™ dye is extremely sensitive to its local environment; therefore, the pH response in your system needs to be determined empirically.

Here, we describe a general protocol for using the amine-reactive, succimidyl ester form of the pHrodo™ dye. Each vial of pHrodo™ Red succinimidyl ester supplied in the kit is sufficient for labeling one sample (20–100 µg at a concentration of 1 mg/mL) of protein with a molecular weight of~20 to ~150 kDa.

**Figure 1** Absorbance and emission spectra of pHrodo Red avidin in Guanidine-HCl




## Experimental protocols

### Protein preparation guidelines

- **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. Also, partially purified protein samples or protein samples containing carriers like BSA (e.g., antibodies) will not be labeled well and should not be used. The presence of low concentrations (<0.1% (w/v)) of biocides, including sodium azide and thimerosal, will 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. There are a number of easy-to-use, low-volume dialysis options available, including Tube-O-DIALYZER micro-dialysis cartridges from Genotech (www.gbiosciences.com).
- We suggest PBS, pH 7.2–7.5, as a suitable prelabeling dialysis buffer, although 100 mM sodium bicarbonate buffer can also be used. If bicarbonate buffer is used, you may omit step 1.1 of the labeling reaction as well as the addition of 1/10 volume of bicarbonate in step 1.2.

## Labeling reaction

Table 1 shows the recommended amount in nanomoles of reactive dye that should be added for each nanomole of protein to be labeled. This is the dye: protein molar ratio (MR). The MR values are based on two parameters:

1) the molecular weight of four representative proteins ranging from 20 to 150 kDa, and

2) the optimal pHrodo™ Red degree of labeling (DOL) for these proteins, as determined in our laboratories.

**Table 2** Recommended pHrodo™ Red dye: protein molar ratios (MR) and typical yields for labeling 20–150 kDa proteins

| Protein (MW in kDa) | For Lower DOL | For Optimal DOL | For Higher DOL | % Yield |
|---|---|---|---|---|
| Protein G (20 kDa) | ≤3 | 4 | ≥6 | 50 |
| Streptavidin (53 kDa) | ≤4 | 7 | ≥10 | 60 |
| F(ab')2 (100 kDa) | ≤5 | 8 | ≥12 | 50 |
| IgG (150 kDa) | ≤10 | 15 | ≥20 | 75 |

Because your proteins may differ from those listed, the recommended MR for lower and higher DOL values are also included in Table 1. For your initial labeling attempt, choose the optimal MR for the protein listed in Table 1 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 1 also shows typical % yields for the indicated pHrodo™ Red conjugates. Use Equation 1 to calculate the appropriate volume of reactive dye stock solution to use:

Equation 1

$$\frac{(\mu g \text{ protein}/\text{protein MW}) \times 1{,}000 \times MR}{15.38} = \mu L \text{ reactive dye to add to sample}$$

where µg protein is the mass of protein you want to label, protein MW is the molecular weight of your protein in Da, MR is the dye: protein molar ratio from Table 1, and 15.38 is the concentration of the reactive dye stock solution (see step 1.3 below). For example, to label 60 µg of IgG (MW 150,000):

$$\frac{(60/150{,}000) \times 1{,}000 \times 16}{15.38} = 0.416 \ \mu L \text{ of dye}$$

Do **not** prepare the reactive dye stock solution (step 1.3) until you are ready to start the labeling reactions. This reactive dye hydrolyzes in water and therefore should be used immediately.

**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°C–8°C for up to two weeks. It can also be frozen for long-term storage.

**1.2** Transfer 20–100 µL of a 1 mg/mL solution of protein (20–100 µg) to a reaction tube (Component D). Add 1/10 volume (2–10 µL) of 1 M sodium bicarbonate, and mix by pipetting up and down several times.

**1.3** Add 10 µL DMSO (Component B) to one vial of pHrodo™ Red succinimidyl ester (Component A). Completely dissolve the contents of the vial by pipetting up and down. The concentration of this reactive dye stock solution is 15.38 nmol/µL.

**Note:** This solution should be prepared immediately before use, and any leftover solution should be discarded.

**1.4** Add the appropriate volume of reactive dye solution (based on Equation 1) to the reaction tube containing the pH-adjusted protein and mix thoroughly by pipetting up and down several times.

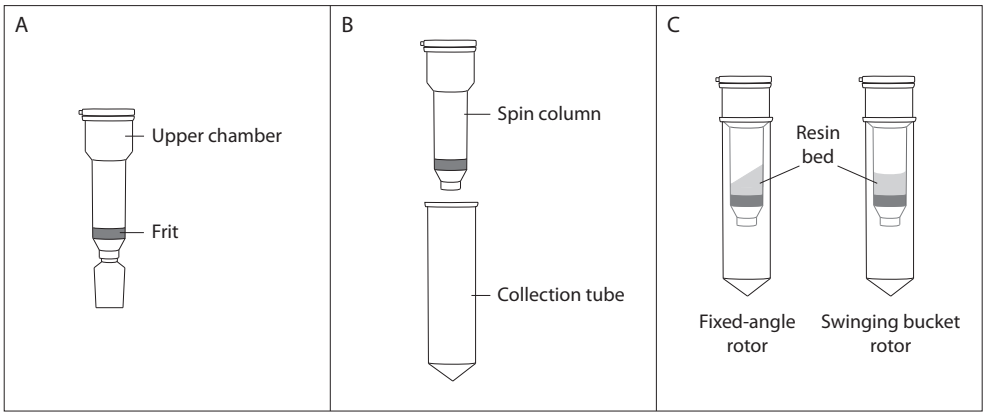
1.5 Incubate the reaction mixture for 15 minutes at room temperature.

## Purify the conjugates

**2.1** To prepare for separating the labeled protein from unreacted dye, take the container of gel resin (Component F) and one spin column (Component E; Figure 2) out of the kit. Fully resuspend the gel resin by gently rocking the container; do not vortex or use a magnetic stir bar to agitate the material. Fill the upper chamber of the spin column up to the lip with the suspended gel resin; approximately 800 µL of resin will be needed, 500 µL if purifying less than 50 µg of protein. Snap off the tip from the spin column and place the column with the cap off into a collection tube. 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 will cause the resin to pack down with a low side and a high side. After spinning, the resin should be about 10–20 mm above theabove the frit, depending on the amount of protein being purified.

Figure 2 illustrates the spin column and collection tube, and what the filled spin column should look like after centrifugation. If the 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, and centrifuge again to repack the bed.

**Figure 2** 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** Occasionally, some resin will get 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.

If you wish to purify the conjugate 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 10.

**2.3** After the spin column is prepared, pipet the conjugate reaction mixture onto the center of the resin bed surface. Place the spin column in the centrifuge and spin at 1,000 × *g* for a total of 5 minutes.

**2.4** Each collection tube now 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 red color (see **Notes**, page 7). The procedure described in steps 1.1–2.3 can be performed in as little as 30 minutes.

## Determine the Degree of Labeling (DOL)

Several spectrophotometric methods are available for determining the DOL of the pHrodo™ Red dye–labeled protein conjugate. They are based on obtaining the protein concentration by absorbance at 280 nm ($A_{280}$) and at 566 nm ($A_{566}$).

**Note:** Determination of degree of substitution for the conjugates prepared using the kit were determined to be accurate only when using 8 M guanidine-HCl, a chaotrope or denaturant, that disrupts hydropbobic or intramolecular noncovalent interactions.

**3.1** The easiest way to analyze the conjugates spectrophotometrically is using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Delaware, USA). This instrument requires only 2 µL of sample and is a full-featured UV/Vis instrument. A variety of cuvettes are available for use with small sample volumes if you would prefer to not dilute your labeled protein in order to use standard 0.5 or 1.5 mL cuvettes.

**Note:** Quartz cuvettes from Starna Cells, Inc. (Atascadero, California, USA) that hold 15–115 µL of sample are ideal for this purpose.

**3.2** The conjugate samples (appropriately diluted in 8 M guanidine-HCl) can also be placed in wells of a microplate and read at 280 and 566 nm in a microplate reader that permits the user to specify the desired detection wavelengths, such as the Tecan® Safire™ (Tecan US, Research Triangle Park, North Carolina, USA).

**3.3** The conjugate samples can be diluted as necessary prior to measurement of $A_{280}$ and $A_{566}$ using cuvettes and spectrophotometers of your choice. However, excessive dilution of some proteins with low intrinsic $A_{280}$ may prevent you from deriving accurate $A_{280}$ values for your samples. The entire conjugate sample, or a portion of it, should be diluted only to the minimum volume necessary for your cuvettes and spectrophotometer to avoid relying on readings below the optimal range for your instrument.

**3.4** Calculate the concentration of the protein in the sample:

**Equation 2**

$$\text{Protein concentration (mg/mL)} = \frac{[A_{280} - 0.20\,(A_{566})] \times \text{dilution factor}}{A_{280} \text{ of protein at 1 mg/mL}}$$

In this equation, 0.20 is a correction factor for the fluorophore's contribution to the $A_{280}$.

**Equation 3**

$$\text{Protein concentration (M)} = \frac{\text{answer from Equation 2}}{\text{protein molecular weight (Da)}}$$

If you know the molar extinction coefficient (ε, in $cm^{-1}M^{-1}$) of your protein at 280 nm, use this value as the divisor in Equation 2 to directly calculate the molarity of the protein.

**Example:**

You have labeled IgG (MW 150,000; $A_{280}$ at 1 mg/mL = 1.4; ε = 210,000 $cm^{-1}M^{-1}$) and diluted the sample 1:10 in guanidine-HCl to make the measurements.
The readings obtained are $A_{280}$ = 0.10 and $A_{566}$ = 0.12.

$$\text{Protein concentration (mg/mL)} = \frac{[0.10 - 0.20\,(0.12)] \times 10}{1.4} = 0.54 \text{ mg/mL}$$

$$\text{Protein concentration (M)} = \frac{0.54}{150{,}000} = 3.6 \times 10^{-6} \text{ M}$$

or

$$\text{Protein concentration (M)} = \frac{[0.10 - 0.20\,(0.12)] \times 10}{210{,}000} = 3.6 \times 10^{-6} \text{ M}$$

**3.5** Calculate the degree of labeling (DOL):

**Equation 4**

$$\text{DOL} = \frac{\text{moles dye}}{\text{mole protein}} = \frac{A_{566} \times \text{dilution factor}}{65{,}000 \times \text{protein concentration (M)}}$$

where 65,000 $cm^{-1}\,M^{-1}$ is the approximate molar extinction coefficient of the pHrodo™ Red dye.

**Example:**

$$\text{DOL} = \frac{0.12 \times 10}{65{,}000 \times (3.6 \times 10^{-6}\text{ M})} = 5.1$$

**3.6** 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 conjugate. The % yield from the spin columns is related to the molecular weight of the protein, and typical yields are provided in Table 2 (page 3). Once you have a value for % yield, the approximate protein concentration can be calculated using Equation 5:

Equation 5

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

**3.7** 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 $A_{566}$ of the conjugate. The $A_{566}$ is used in Equation 4 (step 3.5) to determine the DOL.

### Store conjugates

We typically store labeled protein at 2°C–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 2–6°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. Thus, the recommended dye: protein molar ratios in Table 2 (page 3) are given as guidelines only, and we cannot guarantee that they will yield optimal labeling with your particular protein(s).

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™ Red Protein Labeling Kit may be higher or lower than the generally accepted optimum. We highly recommend that you evaluate your protein conjugate in its intended application before you conclude that it is under- or overlabeled. A number of conditions can cause under- or overlabeling.

### 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 will decrease 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 1.2) is designed to raise the pH of the reaction mixture to ~8, as succinimidyl 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, the recommendations shown in Table 1 (page 3) 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 precipitate in the reaction mixture or deposition of red particles on the upper surface of the resin bed after centrifugation of the conjugate. Precipitation will usually result in a decreased yield of conjugate. 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 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. (Note: 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, will result 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.

## Product list Current prices may be obtained from our website or from our Customer Service Department.

| Cat. no. | Product name | Unit size |
|---|---|---|
| P35363 | pHrodo™ Red Microscale Labeling Kit *for 20 to 100 µgs* *3 labelings* | 1 kit |
| | ***pHRodo™ reagents*** | |
| A10010 | pHrodo™ *S. aureus* BioParticles® conjugate for phagocytosis | 5 x 2 mg |
| A10025 | pHrodo™ E. coli BioParticles® Phagocytosis Kit *for flow cytometry* *100 tests* | 1 kit |
| A10026 | pHrodo™ Phagocytosis Particle Labeling Kit *for flow cytometry* *100 tests* | 1 kit |
| P10361 | pHrodo™ dextran, 10,000 MW *for endocytosis* | 0.5 mg |
| P35361 | pHrodo™ E. coli BioParticles® conjugate for phagocytosis | 5 x 2 mg |
| P35362 | pHrodo™ Red Avidin *Fluorogenic pH sensor* | 1 mg |
| P36600 | pHrodo™, succinimidyl ester (pHrodo™, SE) | 1 mg |

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11 April 2014


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