Biotin-XX Microscale Protein Labeling Kit (B30010)

Quick Facts

Storage upon receipt:

Component A

- 2–6°C or ≤–20°C, if desired
- Protect from moisture

Component B

- Protect from moisture

Components C and D

- Store at room temperature

Component E

- 2–6°C only; DO NOT FREEZE

Introduction

The Biotin-XX Microscale Protein Labeling Kit provides a convenient means for biotinylating small amounts (20–100 µg) of purified protein. This kit has been optimized for labeling proteins with molecular weights between 12 and 150 kDa, and contains everything needed to perform three labeling reactions and to separate the resulting conjugates from excess reactive biotin. Convenient spin filters are used to purify the labeled protein with yields between 60 and 90%, depending primarily on the molecular weight of the starting material. Labeling and purification can be completed in as little as 30 minutes. For labeling larger amounts of protein, we recommend either the FluoReporter® Biotin-XX Protein Labeling Kit (F2610), which is optimized for 5–20 mg samples, or the FluoReporter® Mini-Biotin-XX Protein Labeling Kit (F6347), which is optimized for 100–3,000 µg samples of >40 kDa proteins.

The water-soluble biotin-XX sulfosuccinimidyl ester (biotin-XX, SSE) provided in this kit reacts efficiently with primary amines of proteins to form stable biotin-protein conjugates. After conjugation the biotin moieties are separated from the protein by a 14-atom spacer consisting of two aminohexanoic acid chains ("XX"). This XX spacer has been shown to enhance the ability of the attached biotin to bind to the relatively deep biotin binding pockets in avidin and streptavidin.[1,2] Each of the vials of reactive biotin-XX supplied in the kit is sufficient for labeling one sample (20–100 µg at a concentration of 1 mg/mL) of protein that has a molecular weight between ~12 and ~150 kDa.

The FluoReporter® Biotin Quantitation Assay Kit for proteins (F30751) is sold separately or in combination with the Biotin-XX Microscale Protein Labeling Kit (B30756). The FluoReporter® Biotin Quantitation Assay Kit for proteins provides a sensitive fluorometric assay for accurately determining the number of biotin labels on a protein. The assay can detect from 4 to 80 pmol of biotin in a sample, providing 50-fold higher sensitivity than the HABA biotin binding assay described by Green.[3] With excitation/emission maxima of 495/519 nm, this assay is compatible with any fluorescence-based microplate reader capable of detecting fluorescein or Alexa Fluor® 488 dye.

Materials

Kit Contents

- **Biotin-XX, sulfosuccinimidyl ester (biotin-XX, SSE), sodium salt** (Component A) 3 vials
- **Sodium bicarbonate** (Component B) 84 mg
- **Reaction tubes** (Component C) 3
- **Spin filters** (Component D) Nanosep MF 0.2 µm centrifugation devices, 3
- **Purification resin** (Component E) Bio-Gel P-6 fine resin suspended in PBS,* ~3 mL settled

Storage and Handling

Upon receipt, all kit components can be stored refrigerated at 2–6°C until required for use. The biotin-XX, SSE (Component A) may be stored at ≤–20°C, if desired. Components A and B should be protected from moisture. DO NOT FREEZE COMPONENT E. When stored appropriately, the kit components should be stable for approximately 6 months.

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, or glutathione) or imidazole. All of these substances will 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 biotinylation reaction.

To aid in removing excess reactive biotin and 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 pre- and postlabeling 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 biotin-XX that should be added for each nanomole of protein to be labeled. This is the biotin: protein molar ratio (MR). The MR values are based on two parameters: 1) the molecular weight of seven representative proteins ranging from 12 to 150 kDa, and 2) the optimal biotin degree of labeling (DOL) for these proteins, as determined in our laboratories. Because your proteins may differ from those listed, the recommended MR for both a lower and a higher DOL 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). Table 1 also shows typical % yields for the indicated biotin-XX conjugates.

Use equation 1 to calculate the appropriate volume of reactive biotin stock solution to use:

Equation 1

$$\frac{[(\mu g \text{ protein}/\text{protein MW}) \times 1{,}000] \times MR}{14.93} = \mu L \text{ reactive biotin-XX to add to sample}$$

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 biotin: protein molar ratio from Table 1, and 14.93 is the concentration of the biotin-XX stock solution (see step 1.3 below). For example, to label 60 µg of IgG (MW 150,000):

$$\frac{[(60/150{,}000) \times 1{,}000] \times 18}{14.93} = 0.5 \text{ µL of biotin-XX}$$

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

1.1 Prepare a 1 M sodium bicarbonate solution by adding 1 mL deionized water to the vial of sodium bicarbonate (Component B). 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 2–6°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 C). Add 1/10 volume (2–10 µL) of 1 M sodium bicarbonate, and mix by pipetting up and down several times.

Table 1. Recommended biotin: protein molar ratios (MR) and typical yields for labeling 12–150 kDa proteins.

Protein (MW in kDa)	For Lower DOL	For Optimal DOL	For Higher DOL	% Yield
parvalbumin (12)	≤4	5	≥10	60
soybean trypsin inhibitor (20)	≤5	10	≥18	72–86
thrombin (40)	≤5	12	≥15	78–90
streptavidin (53)	≤5	12	≥15	81–93
transferrin (80)	≤8	15	≥20	79–95
$F(ab)_2$ (100)	≤8	13	≥18	82–94
IgG (150)	≤10	18	≥25	82–96

1.3 Add 10 µL deionized water to one vial of biotin-XX, SSE (Component A; referred to also as "reactive biotin"). Completely dissolve the contents of the vial by pipetting up and down. The concentration of this reactive biotin stock solution is 14.93 nmol/µL. As noted above, this solution should be prepared immediately before use, and any leftover solution should be discarded.

1.4 Add the appropriate volume of biotin-XX 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.

Conjugate Purification

2.1 To prepare for separating the biotinylated protein from unreacted biotin, take the container of gel resin (Component E) and one spin filter (Component D; 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 filter up to the lip with the suspended gel resin; approximately 800 µL of resin will be needed. Centrifuge the spin filter at 16,000 × g in a microcentrifuge for a total of 15 seconds (including run-up time). Using a fixed-angle rotor will cause the resin in the spin filter to pack down with a low side and a high side. The edge of the resin bed on the low side should be about 2–3 mm above the green collar, and the edge of the high side should not be above the top lip of the spin filter. If a swinging bucket rotor is used, the resin bed should fill about half of the upper chamber (~5 mm). Figure 1 illustrates the two parts of the spin filter and what the filled spin filter 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.

2.2 Occasionally, some resin will get into the collection tube during filter preparation. When the resin bed is at the correct level, rinse out the collection tube under the spin filter several times with buffer to remove any resin particles that may be found there. 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. While the resin in the bottle is completely settled, you can decant or aspirate the buffer provided and

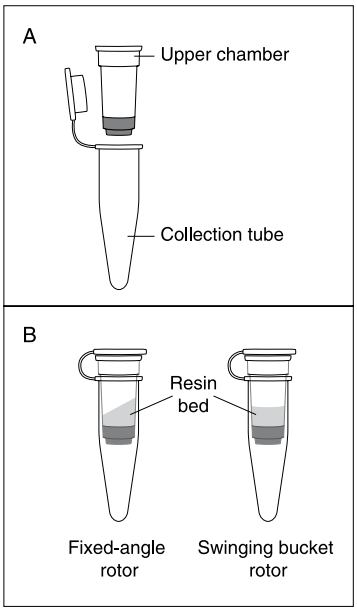

Figure 2. Spin filter. (A) An empty filter showing the separate parts. (B) Appearance of the resin bed after centrifugation in a fixed-angle rotor or swinging bucket rotor.

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 filter, 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 filter is prepared, pipet no more than 50 µL of the conjugate reaction mixture onto the center of the resin bed surface. If the volume of conjugate reaction is 51–100 µL, divide it into two aliquots and purify them on separate spin filters. Place the spin filter(s) in the microcentrifuge with the high side of the resin bed on the outside. Centrifuge at 16,000 × g for a total of 1 minute.

Note: We have not carefully evaluated reusing a spin filter for two aliquots of a larger (≥50 µL) sample, and we do not recommend it.

2.4 Each collection tube now contains purified biotinylated protein in approximately 60–100 µL of buffer. The unreacted biotin is retained in the resin bed. The procedure described in steps 1.1–2.3 can be performed in as little as 30 minutes.

Determination of Conjugate Concentration

Several methods are available for determining the concentration of the biotinylated conjugate:

3.1 One can assume a % yield based on the molecular weight of the protein (see Table 1) and estimate the approximate protein concentration using equation 2:

Equation 2

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

3.2 A more accurate protein concentration (in mg/mL) can be obtained by determining the A_{280} of the conjugate solution and dividing this value by the A_{280} of a 1 mg/mL solution of the starting protein. Biotin does not absorb at 280 nm. The easiest way to analyze the conjugates at 280 nm 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. Quartz cuvettes from Starna Cells, Inc. (Atascadero, California, USA) that hold 15–115 µL of sample are ideal for this purpose.

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

3.4 The conjugate protein concentration can also be determined fluorometrically with the Quant-iT™ Protein Assay Kit (Q33210). This kit requires as little as 5 µL of protein sample and is ideal for conjugates biotinylated with the Biotin-XX Microscale Protein Labeling Kit.

3.5 Divide the protein concentration (in mg/mL, determined as described above in 3.1) by the protein molecular weight to determine the molar concentration, which is needed to calculate the biotin DOL.

Notes

Many protein- and reactive biotin–specific properties determine how efficiently a protein can be labeled with an amine-reactive biotin. 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. Amine-reactive biotins like biotin-XX, SSE can vary in their ability to biotinylate proteins, and their behavior can be predicted with confidence for only a few proteins like antibodies and enzymes like horseradish peroxidase and alkaline phosphatase. Thus, the recommended biotin: protein molar ratios in Table 1 are given as guidelines only, and we cannot guarantee that they will yield optimal labeling with your particular protein(s). As long as a single accessible biotin is present, subsequent detection with streptavidin or avidin is almost always possible. On the other hand, too many attached biotins may cause an increase in background signal or even loss of signal.

Because biotin is a small, neutral molecule, there is wide latitude in the number of biotins that can be attached before a protein's structure and function are affected adversely. The optimal

biotin DOL would usually be 1–2 for a ~20 kDa protein, while the optimal DOL for a ~150 kDa protein, e.g., an IgG, would usually be 5–8. The DOL that you obtain with a protein using the Biotin-XX Microscale 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. The ElutaTube microdialysis vials provided can be used to remove these low molecular weight substances from the protein sample prior to labeling.

• 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 biotin-XX, SSE at different rates, the recommendations shown in Table 1 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 biotin. Three vials of biotin-XX, SSE 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 detection signal being lower than expected in your application. Should this occur, relabel the protein, or label another sample with more reactive biotin.

• In rare cases, biotinylating a protein to any degree may destroy its biological activity.

Overlabeling

• Overlabeling may be indicated by the formation of a faintly visible white precipitate in the reaction mixture or deposition of precipitate 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 biotin. Some proteins may not be labeled with amine-reactive biotin under any circumstances and may irreversibly precipitate.

• If no visible precipitate forms during labeling but the detection signal in your application is lower than expected or the background is increased, the protein may be overlabeled. To reduce the DOL, use a smaller amount of reactive biotin, 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 biotin. Although using the spin filters in this kit exactly as described removed all traces of free biotin from all of the proteins we tested, it is possible that some free biotin may be present in your sample after the purification step. The presence of free biotin, which can be determined by thin layer chromatography, will result in erroneously high calculated DOL values. Free biotin remaining after use of the spin filter can be removed by applying the conjugate to another spin filter or by extensive dialysis. Applying no more than 50 µL of conjugate to each spin filter is the best way to avoid contamination with free biotin.

• In rare cases, biotinylating a protein to any degree may destroy its biological activity.

Determination of Biotin Degree of Labeling (Optional)

As long as at least one accessible biotin is attached to a protein, it can be recognized by biotin binding proteins like streptavidin and avidin used for detection. However, optimizing biotinylation of your protein for optimal detection requires that you establish the relationship between the amount of reactive biotin added to the protein and the number of biotins that become attached to it. The FluoReporter® Biotin Quantitation Assay Kit requires as little as 1 µL of biotinylated protein and is ideal for use in conjunction with the Biotin-XX Microscale Protein Labeling Kit.

* PBS = phosphate-buffered saline (10 mM potassium phosphate, pH 7.2, 150 mM NaCl)
† MWCO = molecular weight cutoff

References

1. Methods Mol Biol 80, 173 (1998); **2.** Methods Mol Biol 45, 223 (1995); **3.** Biochem J 94, 23C (1965).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
B30010	Biotin-XX Microscale Protein Labeling Kit *for 20-100 µg protein* *3 labelings*	1 kit
B30756	Biotin-XX Microscale Protein Labeling Kit with FluoReporter® Biotin Quantitation Assay Kit *includes B30010 and F30751*	1 kit
F30751	FluoReporter® Biotin Quantitation Assay Kit *for biotinylated proteins* *5 determinations*	1 kit
F2610	FluoReporter® Biotin-XX Protein Labeling Kit *5 labelings of 5-20 mg protein each*	1 kit
F6347	FluoReporter® Mini-biotin-XX Protein Labeling Kit *5 labelings of 0.1-3 mg protein each*	1 kit

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