

FluoReporter® Biotin-XX Protein Labeling Kit (F2610)

Quick Facts

Storage upon receipt:

- 4°C
- Do not freeze

Introduction

The FluoReporter® Biotin-XX Protein Labeling Kit provides a method for efficiently biotinylating antibodies and other proteins. The kit contains biotin-XX succinimidyl ester, which reacts with primary amines of proteins or other biomolecules to form stable biotin conjugates. The long spacer between the biotin and the reactive group in biotin-XX succinimidyl ester enhances the ability of the conjugated biotin to interact with the relatively deep biotin-binding sites of avidin and streptavidin.^{1,2} Also included in the kit are a gel filtration column to purify the biotinylated protein from excess biotin, avidin–HABA complex for quantitating the degree of biotinylation and biotinylated goat IgG for use as a control. This kit contains sufficient reagents for at least five biotinylation reactions of 5–20 mg each.

Materials

Kit Contents

- **Avidin with 4'-hydroxyazobenzene-2-carboxylic acid (avidin–HABA complex)** (Component A), 30 mL
- **Biotinylated goat IgG conjugate** (Component B), 1 mL of a 2 mg/mL solution in phosphate-buffered saline, pH 7.2, containing 2 mM sodium azide
- **Dimethylsulfoxide (DMSO)** (Component C), 1 mL
- **6-((6-((biotinoyl)amino)hexanoyl)amine)hexanoic acid, succinimidyl ester (biotin-XX, SE)** (MW = 568, Component D), 20 mg
- **Purification column** (Component E), gel filtration column with a 15 mL bed volume

Storage and Handling

Upon receipt, all kit reagents should be stored refrigerated at 4°C until required for use. When stored properly, they should be stable for approximately six months.

Materials Required but Not Provided

- **PBS**—Dissolve 0.36 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1.02 g of Na_2HPO_4 and 8.77 g of NaCl in 750 mL deionized water, adjust the pH to 7.2 with 1 M NaOH or 1 M HCl if necessary and bring the volume to 1000 mL with deionized water.
- **1 M sodium bicarbonate**—Dissolve 8.4 g of NaHCO_3 in 100 mL deionized water; the pH should be about 8.3–8.5.

Protocol

The degree of biotinylation will depend on the molar ratio of biotin-XX succinimidyl ester to protein in the reaction, the number of reactive sites available on the protein, the pH of the medium, the time and temperature of incubation and other factors. Since many of these parameters will vary with the protein, it may be necessary to alter the conditions from those recommended here. As a guideline, about 3–5 biotins bound per protein are usually adequate. Higher degrees of biotinylation may increase the background or inactivate the protein. For an initial reaction protocol, we suggest incubating the protein with about 10 moles of biotin-XX succinimidyl ester per mole of protein at pH ~8.5 for 1–1.5 hours at room temperature (see below).

Procedure for Biotinyating 5–20 mg of Protein

1.1 If your protein is essentially salt-free, dissolve it at 5–15 mg/mL in 0.1 M sodium bicarbonate (dilute the 1 M stock, prepared in *Materials Required but Not Provided*, tenfold). Proteins dissolved in an amine-containing buffer, e.g. Tris or glycine, must be dialyzed before conjugation. It is usually preferable to dialyze first against a dilute buffer such as 20 mM sodium phosphate. Because some proteins denature when exposed to basic pH for relatively long periods of time, add 1/10 volume of 1 M sodium bicarbonate immediately before conjugation. For optimal purification of biotinylated protein, the volume of protein solution should not exceed 1.5 mL, and the protein concentration should be above 2 mg/mL. For biotinyating small amounts of proteins (0.1–3 mg), we recommend our FluoReporter® Mini-biotin-XX Protein Labeling Kit (F6347).

1.2 Immediately before use, prepare a 20 mg/mL biotin-XX solution by weighing out about 2 mg biotin-XX succinimidyl ester (Component D) and dissolving in 0.1 mL DMSO (Component C) at room temperature. Vortex or sonicate until dissolved completely. This solution must be freshly prepared immediately before each use.

1.3 Calculate the volume of a 20 mg/mL biotin-XX solution needed to biotinylate your known quantity of protein as follows:

$$\text{mL of 20 mg/mL biotin-XX} = \frac{\text{mg protein}}{\text{MW of protein}} \times \text{MR} \frac{568}{20}$$

where MR = molar ratio of biotin-XX to protein in the reaction (for an initial reaction protocol, we recommend MR = 10) and 568 = molecular weight (MW) of biotin-XX succinimidyl ester.

For example, using 5 mg IgG and a 10:1 molar ratio of biotin-XX to protein gives:

$$\frac{5}{145,000} \times 10 \times \frac{568}{20} = 0.010 \text{ mL of 20 mg/mL biotin-XX}$$

1.4 While stirring, add the amount of 20 mg/mL biotin-XX solution calculated in step 1.3 to the protein solution prepared in step 1.1. Mix thoroughly.

1.5 Incubate the above mixture at room temperature for 1–1.5 hours with gentle stirring or shaking.

1.6 Proceed to protein purification steps.

Procedure for Purifying Biotinylated Protein

Please note that the provided column is designed to accommodate proteins of molecular weight ≥ 30 kDa. Purification of conjugates prepared from smaller proteins should be accomplished by extensive dialysis or other methods.

2.1 Remove the column's top cap first, then remove the bottom closure. This order of opening is important—otherwise air will enter the column tip, interfering with free liquid flow.

2.2 Wash the gel filtration column with about 50 mL PBS. The column has been packed in PBS with 0.1% sodium azide to inhibit bacterial growth.

2.3 Gently load the reaction mixture on top of the column. Rinse the reaction vessel with a few drops of PBS and load these washes on top of the column after the sample has been absorbed. Note that if a precipitate is present in the reaction mixture, the sample should be centrifuged before column loading; only the supernatant should be loaded onto the column.

2.4 Once the mixture has run into the column, elute the protein with PBS. Collect 1 mL fractions immediately after adding the PBS. To ensure good separation of the biotinylated protein from unreacted biotin-XX, the volume of each of the eluted fractions should not be greater than 1.2 mL.

2.5 Read the absorbance of each eluted fraction at 280 nm to identify protein-containing fractions.

2.6 The first protein-containing fractions to elute off the column contain the most concentrated biotinylated protein and may be combined. Fractions that elute later may be contaminated with free biotin.

2.7 Wash the column with at least 75 mL PBS containing 0.1% sodium azide and seal tightly.

2.8 Store the column at 4°C. If washed and stored properly, the column can be reused six to seven times.

2.9 Proceed with the assay to determine the degree of biotinylation.

Determination of Degree of Biotinylation

Principle of the Assay

When the dye HABA (4'-hydroxyazobenzene-2-carboxylic acid) is bound to avidin, an absorption band at 500 nm appears. This absorption decreases in proportion to the addition of biotin due to the competitive displacement of HABA. This FluoReporter® Biotin-XX Protein Labeling Kit includes biotinylated goat IgG as a control to ensure that this assay system is functioning properly. *The degree of substitution (moles of biotin per mole of goat IgG protein) of this standard is printed on the container's label.* For reliable results, duplicate assays should be run for each sample.

Assay Procedure

3.1 Adjust the concentration of a sample of biotinylated protein to 1 mg/mL or higher in PBS. The protein concentration can be determined using the molar or 1% extinction coefficient of the protein; for most IgGs the absorbance at 280 nm of a 1 mg/mL solution in a cuvette with a 1 cm pathlength is about 1.4.

3.2 Add 1 mL of avidin–HABA complex (Component A) to a series of glass test tubes or microfuge tubes. While vortexing, add 100 μ L of the biotinylated protein sample from step 3.1 to one of the tubes. For a positive control, add 50 μ L of the 2 mg/mL biotinylated IgG standard (Component B) and 50 μ L of PBS to one tube. For a negative control, add 100 μ L of PBS to another tube. Mix well and incubate all samples at room temperature for 5–10 minutes. Then, spin samples in a microcentrifuge for 5 minutes to remove any precipitate that may have formed (note that there may only be a trace of precipitate; samples should be centrifuged even when no precipitate is visible).

3.3 Blank a spectrophotometer at 500 nm with PBS.

3.4 Read and record the absorbance at 500 nm of the sample comprising 100 μ L of PBS and 1 mL of avidin–HABA complex.

3.5 Read and record the absorbance at 500 nm for each mixture of avidin–HABA with biotinylated protein prepared in step 3.2. Since a wet cuvette will dilute the HABA solution, making the absorbance reading inaccurate, it is important to dry the cuvette before putting samples in. We recommend rinsing the cuvette in methanol and thoroughly drying it between samples.

3.6 Subtract the absorbance value determined for each biotinylated protein sample (step 3.5) from the value for free avidin–HABA complex (step 3.4). The difference is ΔA_{500} for the biotinylated protein sample.

3.7 Calculate the degree of biotinylation (moles of biotin per mole of protein) as follows

$$\frac{\Delta A_{500} \times \text{BSF} \times \text{MW} \times 10^3}{\text{mg/mL protein} \times 0.1 \text{ mL}} = \frac{\text{moles of biotin}}{\text{mole of protein}}$$

where BSF = biotin sensitivity factor. This is a lot-specific constant for the avidin–HABA complex determined from a standard curve of ΔA_{500} versus known amounts of added biotin. *The BSF value is printed on the avidin–HABA complex container label.* The other factors are the concentration (mg/mL protein, from step 3.1), assay volume (0.1 mL, from step 3.2) and MW of the protein.

References

1. Methods Mol Biol 80, 173 (1998);
2. Methods Mol Biol 45, 223 (1995).

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

Cat #	Product Name	Unit Size
F2610	FluoReporter® Biotin-XX Protein Labeling Kit *5 labelings of 5-20 mg protein each*	1 kit

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

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