FluoReporter® Biotin Quantitation Assay Kit *for biotinylated proteins* (F30751)

Quick Facts

Storage upon receipt:

- ≤–20°C

Ex/Em: 495/519 nm

Introduction

The FluoReporter® Biotin Quantitation Assay Kit for biotinylated proteins provides a sensitive fluorometric assay for accurately determining the number of biotin labels on a protein. The assay is based on the displacement of a ligand tagged with a quencher dye from the biotin binding sites of Biotective™ Green reagent (Figure 1). The assay can detect from 4 to 80 pmol of biotin in a sample (Figure 2), providing a 50-fold higher sensitivity than the HABA biotin binding assay described by Green.[1]

To expose any biotin groups in a multiply labeled protein that are sterically restricted and inaccessible to the Biotective Green reagent, the kit includes protease and an optional protocol for digesting the protein. With this preliminary digestion, biotin assay values agree well with MALDI-TOF determinations. The signal window of this assay has a Z′ factor of 0.93 (Figure 3). With excitation/emission maxima of 495/519 nm, this assay is compatible with any fluorescence-based microplate reader capable of detecting fluorescein (FITC) or Alexa Fluor® 488 dye; it can also be scaled up for fluorometer-based experiments.

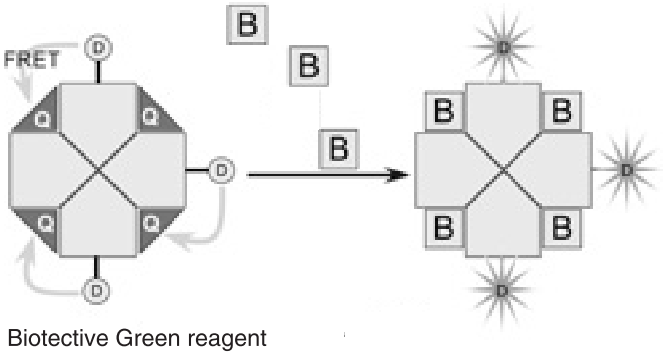

Figure 1. Schematic representation of the FluoReporter® biotin quantitation assay. The assay uses Biotective Green reagent, which consists of avidin labeled with a fluorescent dye (D) and with quencher dye ligands (Q) occupying the biotin binding sites. Through fluorescence resonance energy transfer (FRET), the ligand quenches the fluorescence. Biotin (B) attached to a protein displaces the quencher dye from Biotective Green reagent, yielding fluorescence proportional to the amount of added biotin.

Unlike the HABA biotin binding assay, which requires ~1 mg of protein sample, the FluoReporter® biotin quantitation assay requires only a minimum of 600 ng of a singly biotinylated IgG of MW 150,000. For proteins of lower molecular weight or multiple biotin labels, less protein can be used.

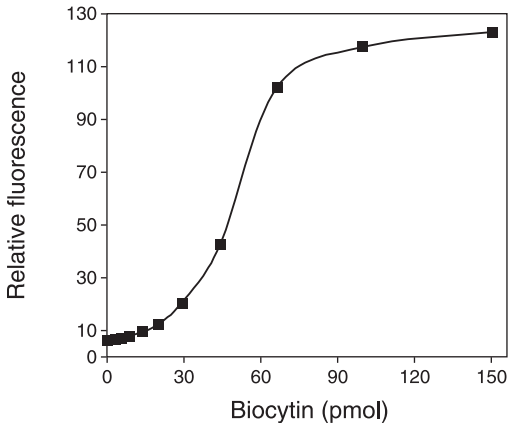

Figure 2. Standard curve showing dynamic range of FluoReporter® Biotin Quantitation Assay Kit. Each reaction consisted of 1X PBS, 1X Biotective Green reagent, and biocytin in a total volume of 100 µl. After a 5 minute incubation at room temperature in the dark, fluorescence was measured in a microplate reader using excitation at 485 ± 10 nm and fluorescence emission at 530 ± 12.5 nm.

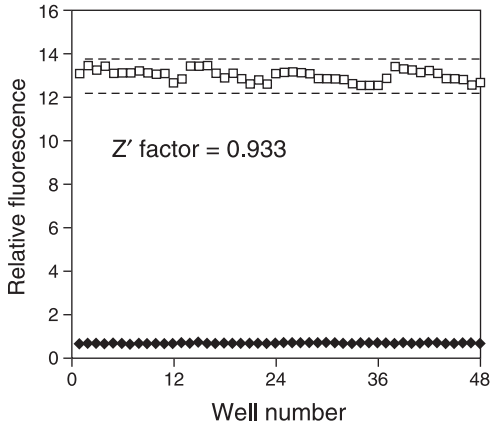

Figure 3. Z′ factor determination for FluoReporter® Biotin Quantitation Assay Kit. The Z′ factor, a dimensionless measure of an assay's signal window,[2] provides a simple method to evaluate a high-throughput assay. For this assay, 48 wells of 1X PBS, 1X Biotective Green reagent, 100 pmol biocytin in 100 µl (□) were measured alongside 48 wells of 1X PBS, 1X Biotective Green reagent without biocytin (◆). The reactions were incubated and measured as described in Figure 2. The dashed lines bracketing the upper data set represent ±3 standard deviations from the mean of 48 replicates.

Materials

Kit Contents

- **Biotective Green reagent** (Component A) lyophilized, 5 vials
- **Biocytin** (Component B) 200 µM, 100 µl
- **Protease** (Component C) lyophilized, 5 × 5 U (note **A**)
- **10X phosphate-buffered saline (10X PBS)** (Component D) 4 ml
- **Biotinylated goat anti–mouse IgG** (Component E) (note **B**) 2 mg/ml, 37.5 µl

This kit contains sufficient reagents to assay 5 samples independently using 8 wells in triplicate for the standard curve and 3 dilutions of the sample in triplicate (totaling 33 wells per assay). However, fewer wells may be used to conserve sample, and a single standard curve can be used for multiple samples in the same experimental session. Biocytin (biotinylated lysine) is provided as a standard for the assay because it more closely represents the form of biotin upon proteolytic cleavage. Biotinylated goat anti–mouse antibody is also provided as a positive control and biotinylated protein standard.

Storage and Handling

Upon receipt, components should be stored at ≤–20°C until required for use. When stored properly, the kit components should be stable for at least 6 months. Before opening a vial, allow it to warm to room temperature.

Protocol

The following procedure is designed for use with a fluorescence multiwell plate scanner, typically in 96-well format, using a final reaction volume of 100 µl per well. The volumes recommended here are sufficient for 33–35 wells; the kit provides sufficient material for ~175 microplate wells.

Stock Solution Preparation

Allow components to warm to room temperature before preparing the stock solutions.

1.1 Prepare 10 ml of 1X PBS by adding 1 ml of 10X PBS (Component D) to 9 ml of deionized water. This 1X PBS is sufficient to prepare all of the stock solutions, standards, and sample dilutions for two assays (requiring 33 wells per assay as described above).

1.2 Reconstitute one vial of Biotective Green reagent (Component A) with 1.75 ml of 1X PBS. This 2X reagent solution can be stored at 2–6°C for up to 5 days; DO NOT FREEZE. Each vial contains enough reagent for 33–35 reaction wells.

1.3 As needed, reconstitute one vial of protease (Component C) in 50 µl of 1X PBS. This makes a 100 U/ml stock solution and is sufficient for treating 50 samples. If the protease stock solution is not going to be used up the same day, aliquot and freeze the unused portion at ≤–20ºC.

Protease Digestion of Biotinylated Protein (Optional)

This optional protease digestion is recommended in order to expose the biotin groups on proteins. By making the biotin fully accessible to the Biotective Green reagent, the treatment maximizes the accuracy of the assay. If you are confident that the degree of labeling is less than 1, you may skip this step.

2.1 Dissolve or dilute the biotinylated protein sample in 1X PBS to a total volume of 50 µl.

2.2 Add 1 µl of the protease stock solution.

2.3 If desired, the biotinylated goat anti–mouse IgG (Component E) may be used as a positive control and digested as well. We recommend diluting 3 µl of Component E with 154 µl 1X PBS and then adding 3 µl of the protease stock solution.

2.4 Incubate overnight at 37°C. This will ensure complete digestion of the biotinylated protein.

Biotin Assay

3.1 Prepare a 1.6 µM biocytin standard by adding 6.8 µl of 200 µM biocytin (Component B) to 843 µl of 1X PBS. Serially dilute the 1.6 µM biocytin in 1X PBS in triplicate columns of 50 µl per well of a 96-well microplate. Use 1X PBS without biocytin as a negative control in the final row. This dilution series will generate a standard curve of 0–80 pmol of biocytin.

3.2 Dilute the protease-digested samples in 1X PBS until each sample is believed to be within the assay's dynamic range of 4–80 pmol biotin. If a protein's biotinylation level is unknown, make a wide range of dilutions to ensure that a few will be within the desired range. We recommend a twofold dilution series that assumes that the degree of labeling is between 1 and 20.

Example: A scientist has biotinylated an IgG of MW 150,000. She makes a dilution series of 40, 20, 10, 5, and 2.5 pmol IgG per well, corresponding to 6 µg–375 ng per well.

3.3 Pipette 50 µl of the diluted samples into separate empty wells of the microplate. For the highest accuracy, we recommend that each sample be assayed in triplicate at each concentration. If you are using the positive control, pipet 50 µl of the digested positive control into each of three wells.

3.4 Begin the reactions by adding 50 µl of the reconstituted 2X Biotective Green reagent (from step 1.2) to each microplate well containing a sample or standard. Incubate for 5 minutes at room temperature, protecting the reactions from light.

3.5 Measure the resulting fluorescence in a microplate reader using typical fluorescein wavelengths (excitation/emission maxima ~485/530 nm). The reactions should be read <15 minutes after adding the Biotective Green reagent. After 15 minutes, the protease (if used) will begin to affect the assay.

Data Analysis

4.1 Plot the fluorescence of the biocytin standards on the x-axis against the amount of biocytin on the y-axis. Do not subtract background.

4.2 Use the quadratic fit in Microsoft Excel™ software to find the quadratic equation of the standard curve from 0 to 80 pmol biocytin. Insert the measured relative fluorescence of the samples as x values and solve for y (biotin amount).

4.3 An alternative to step 4.2 is to plot the standards and draw a straight line through the two points that have x values that bracket each biotinylated sample. Use the equation of this line to find the amount of biotin in each sample by inserting the measured fluorescence as the x value and solving for y.

Example: An experimental sample gave a signal of 4,000 relative fluorescence units (RFU). The standards that bracket it are 10 pmol and 20 pmol biocytin, which gave 2,000 and 9,000 RFU, respectively. The equation of the line between these two standard points is y = 0.00143x + 7.143, where x is fluorescence and y is biotin amount. Solving for y, the experimental sample contains 12.9 pmol. This amount can be used to determine the amount or concentration of biotin in the original sample.

4.4 Once you have calculated the amount (or concentration) of biotin in each original sample, divide that number by the amount (or concentration) of protein in the same sample. This will give the degree of labeling (DOL).

Example: An experimental sample was determined to contain 12.9 pmol biotin. Using a protein assay, the same sample was also determined to contain 4.3 pmol of protein. The DOL is (12.9 pmol biotin)/(4.3 pmol protein) = 3. Note that the calculated DOL is an average; the total population of labeled proteins will be distributed around that average.

The DOL of the positive control varies from lot to lot. This assay should arrive at a DOL within ±0.5 of the lot-specific DOL. We recommend using the Quant-iT™ protein quantitation assay (Q33210) to determine the amount of protein. If your sample contains detergents, we recommend the CBQCA protein quantitation assay (C6667).

Note

[**A**] Protease type XIV from *Streptomyces griseus*. One unit (U) is the amount that hydrolyzes casein to produce color equivalent to 1 μmol of tyrosine per min at pH 7.5 at 37°C, using Folin-Ciocalteu reagent.
[**B**] The degree of biotin labeling of this IgG varies from lot to lot. Please see the product label.

References

1. Biochem J 94, 23C (1965); **2.** J Biomolecular Screening 4, 67 (1999).

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 Kit *for biotinylated proteins* *5 determinations*	1 kit

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