INSTRUCTIONS

Fluorescence Biotin Quantitation Kit

46610 2127.0

Number	Description
46610	**Fluorescence Biotin Quantitation Kit**
	Kit Contents:
	DyLight™ Reporter, 1.3 ml
	Biocytin Control, 1 mM, 100 µl
	20X Phosphate Buffered Saline, 3 ml
	Storage: Upon receipt store at 4°C. Product shipped with an ice pack.

Introduction

The Thermo Scientific Fluorescence Biotin Quantitation Kit requires only 10 µl of sample to accurately measure the biotinylation level of biomolecules. This microplate-based assay is easy to perform by adding the Thermo Scientific DyLight Reporter (i.e., fluorescent avidin and HABA premix) to the biotinylated samples and diluted biocytin standards. The avidin fluoresces when the weakly interacting HABA (4´-hydroxyazobenzene-2-carboxylic acid) is displaced by the biotin. The amount of biotin is determined by comparing the sample's fluorescence to the biocytin standard curve. This assay requires half the sample volume and is much more sensitive than the microplate colorimetric HABA assay.

Important Product Information

- Biotinylated sample must be dialyzed or desalted to remove non-reacted or hydrolyzed biotinylation reagent before performing the assay. Excess biotin in the sample will result in overestimation of biotinylation levels.
- Samples containing albumin (i.e., BSA) cannot be assayed using this kit because albumin interferes with HABA:avidin binding.
- Chromophoric proteins that absorb in the same range as the DyLight Reporter (494 nm) (e.g., horseradish peroxidase) cannot be assayed using this kit.
- For best results, assay samples in a neutral pH buffer (e.g., PBS or TBS).
- Biocytin standards must be prepared immediately before use.

Additional Materials Required

- Biotinylated protein or macromolecule, free of non-conjugated biotinylation reagent and of known protein/macromolecule concentration
- 1.5 ml microcentrifuge tubes
- Pipettors and disposable pipette tips
- Black opaque 96-well microplate
- Fluorescence plate reader capable of exciting at 494 nm and detecting fluorescence at 520 nm
- Ultrapure water

Protocol for Fluorescence Biotin Quantitation

A. Preparation of Diluted Biocytin Standards

1. Dilute 0.5 ml of 20X PBS with 9.5 ml ultrapure water to prepare 10 ml of 1X PBS. For long-term storage, add sodium azide to a final concentration of 0.02%.
2. Immediately before use prepare the standards according to Table 1 using the 1 mM Biocytin Control and 1X PBS as the diluent. There is sufficient volume of Biocytin Control Stock to prepare for 10 replications of each standard; if less total volume is desired, proportionally scale the biocytin and diluent volumes.

Table 1. Preparation of the biocytin standards (Working Range = 10-60 pmol/10 µl).

Vial	Diluent Volume (µl)	Volume and Biocytin Source	Final Biocytin Concentration (pmol/10 µl)
A	990	10 µl of Biocytin Control	100
B	40	160 µl of vial A	80
C	80	120 µl of vial A	60
D	120	80 µl of vial A	40
E	160	40 µl of vial A	20
F	180	20 µl of vial A	10
G	190	10 µl of vial A	5
I	200	0	0 = Blank

B. Preparation of DyLight Reporter Working Reagent (DWR)

1. Use the following formula to determine the total volume of DWR required:

   (# standards + # unknowns) × (# replicates) × (90 µl of DWR per sample) = total volume DWR required

   Example: (8 standards + 2 unknowns) × (3 replicates) × (90 µl) = 2,700 µl DWR required
2. Immediately before use, prepare the DWR by mixing 14 parts of 1X PBS with 1 part of DyLight Reporter. For the above example, combine 2.8 ml of 1X PBS with 200 µl of DyLight Reporter. Prepare sufficient volume of DWR based on the number of samples to be assayed.

C. Biotin Quantitation Assay

1. Dilute sample 1:1, 1:10 and 1:20 with 1X PBS to ensure that the concentration is within the assay's working range.

   Note: If an estimate of the biotinylation level is known, dilute sample with 1X PBS to contain 10-60 pmol of biotin/10 µl.
2. Pipette 10 µl replicates of each standard or unknown sample into a microplate well. Add 90 µl of the DWR to each well.
3. Incubate plate for 5 minutes at room temperature. Measure the fluorescence at excitation/emission 494/520 nm using a fluorescent microplate reader. Optimize the gain setting using a well containing the 100 pmol biocytin/10 µl standard.

   Note: For best results, measure fluorescence within 25 minutes of adding the DWR. Fluorescence intensity decays with time, reducing the working range of the standard curve.
4. Prepare a standard curve by plotting the average fluorescence intensity measurement for each biocytin standard vs. picomoles of biocytin per 10 µl volume (e.g., values for this protocol range from 0 to 100 pmol of biocytin). Use the standard curve to determine the picomoles of biotin present in each unknown sample.

   Note: To simplify determination of picomoles of biotin per sample, prepare a reverse plot of the linear range (10-60 pmol biocytin/10 µl) of the standard curve. Use a spreadsheet or statistical software to generate a linear regression equation (i.e., $y = mx + b$). Use the resulting equation to determine picomoles of biotin (y) in an unknown sample by inserting the sample's average fluorescence intensity (x).
5. Divide the calculated picomoles of biotin by the picomoles of protein contained in the 10 µl sample to obtain the moles of biotin per mole of protein ratio.

Troubleshooting

Problem	Possible Cause	Solution
Standards and samples produced less fluorescence than expected	Fluorescence measured at incorrect excitation/emission	Measure excitation/emission at 494/520 nm
	Suboptimal gain setting	Optimize the gain setting using one of the top standard replicates (i.e., 100 pmol/10 µl)
Samples produced less fluorescence than expected, but standard curve is okay	Sample diluted below the assay's working range	Prepare a new dilution series of the sample
	Sample is not biotinylated	Confirm biotinylation using an alternative method, such as a plate assay or dot blot
	Sample molecule absorbs in the same range as the DyLight Reporter	Use the traditional colorimetric HABA assay (Product No. 28005) for biotin quantitation
Samples produced more fluorescence than expected, but standard curve is okay	Sample not diluted within the assay's working range	Prepare a new dilution series of the sample
	Sample contains an interfering substance (e.g., albumin)	Remove interfering substance from sample before quantitation
Standard curve was flat	Standards were prepared and stored in advance	Prepare standards immediately before use

Related Thermo Scientific Products

28022 **Biocytin**, 100 mg

28348 **20X Phosphate Buffered Saline**, 500 ml

21925 **Micro Sulfo-NHS-Biotinylation Kit**

21935 **Micro Sulfo-NHS-LC-Biotinylation Kit**

21945 **Micro Sulfo-NHS-SS-Biotinylation Kit**

21955 **Micro Sulfo-NHS-PEG$_4$-Biotinylation Kit**

Visit our website for a complete list of biotinylation reagents and kits.

Patents pending on DyLight 488, 549, 649 and 750 Dyes.

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