INSTRUCTIONS

Pierce® Biotin Quantitation Kit

1423.8

Number	Description
28005	**Pierce Biotin Quantitation Kit**
	Kit Contents:
	No-Weigh™ HABA/Avidin Premix, 24 microtubes
	ImmunoPure® Biotinylated Horseradish Peroxidase (40 kDa), 5 mg
	Storage: Upon receipt store at 4°C. Product is shipped at ambient temperature.

Introduction

Antibodies or other proteins are often biotinylated with reagents such as EZ-Link® NHS-PEO_4-Biotin (Product No. 21329). HABA (4´-hydroxyazobenzene-2-carboxylic acid) is a reagent that enables a quick estimation of the mole-to-mole ratio of biotin to protein. The Thermo Scientific Pierce Biotin Quantitation Kit contains a premix of HABA and avidin and a biotinylated horseradish peroxidase (HRP) positive control. The HABA/Avidin Premix is supplied in convenient No-Weigh Microtube packaging, which eliminates the difficulties associated with weighing small quantities of reagent.

Because biotin is a relatively small molecule, it can be conjugated to many proteins without altering the biological activity of the protein. A protein can be conjugated with several biotin molecules, each of which can bind one molecule of avidin, thereby greatly increasing the sensitivity of many assays. The bond formation between biotin and avidin is rapid and once formed is unaffected by most extremes of pH, organic solvents and other denaturing agents.[1-3]

To quantitate biotinylation, a solution containing the biotinylated protein is added to a mixture of HABA and avidin. Because of its higher affinity for avidin, biotin displaces the HABA and the absorbance at 500 nm decreases proportionately. By this method, an unknown amount of biotin present in a solution can be quantitated in a single cuvette by measuring the absorbance of the HABA-avidin solution before and after addition of the biotin-containing sample. The change in absorbance relates to the amount of biotin in the sample by the extinction coefficient of the HABA-avidin complex.

Important Product Information

- Following any biotin-labeling reaction, the biotinylated protein sample to be assayed must be dialyzed or desalted to remove nonreacted and hydrolyzed biotinylation reagent.
- Samples must be in one of the recommended buffers (PBS or TBS, see Reagent Preparation Section) for the assay. Avoid buffers containing potassium (such as Modified Dulbecco's PBS), which will cause precipitation in the assay. Other buffers may interfere and should not be used unless first validated by comparison to results using PBS or TBS.
- Slight color variation between the HABA/Avidin Premix microtubes does not affect product performance.

Reagent Preparation

Sample buffer	Phosphate-buffered saline (PBS) containing 100 mM sodium phosphate, 150 mM NaCl; pH 7.2 (Product No. 28372) or Tris-buffered saline (TBS) containing 25 mM Tris, 150 mM NaCl; pH 7.2 (Product No. 28376) **Note:** Avoid using buffers containing potassium salts (see Important Product Information).
Biotinylated HRP (Positive Control)	To make a 1 mg/ml solution, add 5 ml of ultrapure water to the vial of biotinylated HRP. Mix with a pipette tip and allow the biotinylated HRP to solubilize. Complete solubilization requires approximately 5 minutes at room temperature. Store reconstituted biotinylated HRP in single-use aliquots (i.e., 120 µl) at -20°C until ready to use.

Procedure for Quantitation of Moles of Biotin per Mole of Protein – Cuvette Format

Note: The Biotinylated HRP may be used as a positive control to verify assay performance. See product specification sheet for the biotinylation level.

1. Equilibrate the No-Weigh HABA/Avidin Premix to room temperature.
2. Add 100 µl of ultrapure water to one microtube of the No-Weigh HABA/Avidin Premix. Mix with pipette tip.
3. Pipette 800 µl of PBS or other sample buffer into a 1 ml cuvette. Use this cuvette with PBS to zero the spectrophotometer.
4. Add the 100 µl of the HABA/Avidin Premix solution from step 1 to the cuvette. Mix by inversion.
5. Measure the absorbance of the solution in the cuvette at 500 nm and record the value as A_{500} HABA/avidin.
6. Add 100 µl of biotinylated protein sample or biotinylated HRP (positive control) to the cuvette containing HABA/avidin and mix well.
7. Measure the absorbance of the solution in the cuvette at 500 nm and record the value as A_{500} HABA/avidin/biotin sample once the value remains constant for at least 15 seconds. If the A_{500} HABA/avidin/biotin sample is ≤ 0.3, dilute the sample and repeat the assay.

   Note: Dilutions must be accounted in the calculation step.
8. Proceed to the Calculation of Moles of Biotin Per Mole of Protein section.

Procedure for Quantitation of Moles of Biotin per Mole of Protein – Microplate Format

Note: The Biotinylated HRP may be used as a positive control to verify assay performance. See product specification sheet for the biotinylation level.

1. Equilibrate the No-Weigh HABA/Avidin Premix to room temperature.
2. Add 100 µl of ultrapure water to one microtube of the No-Weigh HABA/Avidin Premix. Mix with pipette tip.
3. Pipette 160 µl of PBS into a microplate well.
4. Add 20 µl of the HABA/Avidin Premix solution from step 1 to the PBS in the well. Place microplate on an orbital shaker or equivalent to mix.
5. Measure the absorbance of the solution in the well at 500 nm and record the value as A_{500} HABA/avidin.
6. Add 20 µl of biotinylated sample or Biotinylated HRP (positive control) to the well containing the HABA/avidin reaction mixture. Mix as described above.
7. Measure the absorbance of the solution in the well at 500 nm and record the value as A_{500} HABA/avidin/biotin sample once the value remains constant for at least 15 seconds.
8. Proceed to the Calculation of Moles of Biotin Per Mole of Protein section.

Calculation of Moles of Biotin Per Mole of Protein

Note: The HABA Calculator, which is available from the Technical Resources menu from our web site, will calculate the moles of biotin/mole of protein upon entering the required values.

These calculations are based on the Beer Lambert Law (Beer's Law): $\mathbf{A_\lambda = \varepsilon_\lambda bC}$

Where:

A is the absorbance of the sample at a particular wavelength (λ). The wavelength for the HABA assay is 500 nm. There are no units for absorbance.

ε is the absorptivity or extinction coefficient at the wavelength (λ). For HABA/avidin samples at 500 nm, pH 7.0 extinction coefficient is equal to 34,000 $M^{-1}cm^{-1}$.

b is the cell path length expressed in centimeters (cm). A 10 mm square cuvette has a path length of 1.0 cm. Using the recommended microplate format volumes, the path length is typically 0.5 cm.

C is the concentration of the sample expressed in molarity (= mol/L = mmol/ml).

The values needed for calculating the number of moles of biotin per mole of protein or sample are as follows:

- Concentration of the protein or sample used, expressed as mg/ml
- Molecular weight (MW) of the protein, expressed as grams per mole (e.g., HRP = 40,000; IgG =150,000)
- Absorbance at 500 nm for HABA/avidin reaction mixture (**A_{500} H\A**)
- Absorbance at 500 nm for HABA/avidin/biotin reaction mixture (**A_{500} H\A\B**)
- Dilution factor, if the sample is diluted before adding to the HABA/avidin reaction mixture

1. Calculation #1 is for the concentration of biotinylated protein in mmol/ml (before any dilution for the assay procedure):

$$\text{mmol protein per ml} = \frac{\text{protein concentration (mg/ml)}}{\text{MW of protein (mg/mmol)}} = Calc\#1$$

2. Calculation #2 is for the change in absorbance at 500 nm:

   - Cuvette:

     $\Delta A_{500} = (0.9 \times A_{500}\ H\backslash A) - (A_{500}\ H\backslash A\backslash B) = Calc\#2$

   - Microplate:

     $\Delta A_{500} = (A_{500}\ H\backslash A) - (A_{500}\ H\backslash A\backslash B) = Calc\#2$

Note: The cuvette format requires the 0.9 correction factor to adjust for dilution of the H\A mixture by the biotinylated protein sample. The microplate format does not require this correction factor because the dilution effect is exactly offset by the increased height and light path length of solution in the well.

3. Calculation #3 is for the concentration of biotin in mmol per ml of reaction mixture:

$$\frac{\text{mmol biotin}}{\text{ml reaction mixture}} = \frac{\Delta A_{500}}{(34{,}000 \times b)} = \frac{Calc\#2}{(34{,}000 \times b)} = Calc\#3$$

Note: ***b*** is the light path length (cm) of the sample. Use ***b*** = 1 with the cuvette format. Use ***b*** = 0.5 with the microplate format when using a standard 96-well plate and the volumes specified in the procedure. The exact path length is the height of the solution through which the plate reader measures the absorbance.

4. Calculation #4 is for the mmol of biotin per mmol of protein:

$$= \frac{\text{mmol biotin in original sample}}{\text{mmol protein in original sample}}$$

$$= \frac{\text{(mmol per ml biotin in reaction mixture)(10)(dilution factor)}}{\text{mmol per ml protein in original sample}}$$

$$= \frac{(Calc\#3) \times 10 \times \text{dilution factor}}{Calc\#1}$$

Note: The original biotinylated protein sample was diluted 10-fold in the reaction mixture. Therefore, a multiplier of 10 is used in this step to convert the biotin concentration in the reaction mixture to the biotin concentration in the original sample. If the original sample was diluted before performing the assay, then the dilution factor must be used as well. Calculation #4 yields the biotin:protein molar ratio (average # of biotin molecules per protein molecule).

EXAMPLE: In this example, the labeled protein is IgG (MW 150,000) at 0.69 mg/ml. The absorbance measurements were A_{500} H\A = 0.904 and A_{500} H\A\B =0.771.

1. $$\text{mmol biotinylated protein per ml} = \frac{0.69\ \text{mg/ml}}{150{,}000\ \text{mg/mmol}} = 4.6 \times 10^{-6}$$

2. $$\Delta A_{500} = (0.9 \times 0.904) - 0.771 = 0.0426$$

3. $$\frac{\text{mmol biotin}}{\text{ml reaction mixture}} = \frac{0.0426}{34{,}000} = 1.25 \times 10^{-6}$$

4. $\frac{\text{mmol biotin}}{\text{mmol protein}} = \frac{(1.25\times10^{-6})\times10}{4.6\times10^{-6}} = \frac{12.5\times10^{-6}}{4.6\times10^{-6}} = 2.72$ biotin molecules per IgG molecule

Troubleshooting

Problem	Possible Cause	Solution
The change in the 500 nm absorbance (ΔA_{500}) is ≤ 0	The protein sample has no or a very low level of biotinylation because of limited accessible functional groups on the protein	Repeat biotinylation with alternative chemistry (e.g., amine reactive rather than sulfhydryl reactive, or use a higher molar ratio of biotinylation reagent)
	Incomplete mixing of reagent	Completely solubilize and mix HABA/Avidin before diluting
	Particulate in protein sample contributing to absorbance	Filter protein sample to remove particulate
	Buffer contains potassium ions	Use PBS buffer containing no potassium ions
Calculated biotinylation level of the Biotinylated HRP control is lower than specified	Incomplete solubilization of the Biotinylated HRP	Do not allow pipette tip to touch the lyophilized HRP
		Allow the Biotinylated HRP to dissolve completely (≥ 10 minutes)
Extremely high levels of biotinylation	Nonreacted biotin was not removed	Dialyze or desalt sample before performing the assay

Additional Information

A. Optional Pronase Digestion

Note: Pronase can be used to digest the protein to expose biotin groups that may be buried within the molecule and sterically hindered from binding to avidin. This Pronase method is optional and is not normally necessary because for most applications it is sufficient to quantify the number of biotin groups available on the surface of the protein molecule.

1. Prepare 1% Pronase (w/v) in ultrapure water.
2. Heat 100 µl of biotinylated protein sample at 56°C for 10 minutes.
3. Add 10 µl of 1% pronase to the sample and digest overnight at room temperature.
4. Quantitate biotinylation as previously described.

B. Please visit our web site for additional information on this product including the following:

- Use the HABA Calculator to determine the moles of biotin/mole of protein in your sample. Enter the absorbance values, protein molecular weight and protein concentration and the biotin/protein molar ratio will be calculated for you. The HABA Calculator can be accessed from the Technical Resources menu.

Related Products

21329 **No-Weigh™ Premeasured NHS-PEO_4-Biotin Microtubes,** 8 × 2 mg

21329 **EZ-Link NHS-PEO_4-Biotin,** 25 mg

21331 **EZ-Link Sulfo-NHS-SS-Biotin,** 100 mg

21334 **EZ-Link PEO-Iodoacetyl Biotin,** 50 mg

21901 **EZ-Link PEO-Maleimide Activated Biotin,** 50 mg

29139 **Biotinylated Horseradish Peroxidase,** 5 mg

References

1. Green, N.M. (1975). Avidin. In Adv. in Protein Chemistry. Academic Press, New York. **29,** 85-133.
2. Green, N.M., *et al.* (1971). The use of bifunctional biotinyl compounds to determine the arrangement of subunits in avidin. *Biochem. J.* **125,** 781-791.
3. Green, N.M. (1965). A spectrophotometric assay for avidin and biotin based on binding of dyes by avidin. *Biochem. J.* **94,** 23c-24c.

Pronase is a trademark of Calbiochem.

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There is no obligation to replace Products as the result of (i) accident, disaster or event of force majeure, (ii) misuse, fault or negligence of or by Buyer, (iii) use of the Products in a manner for which they were not designed, or (iv) improper storage and handling of the Products.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.