INSTRUCTIONS

HABA

4′-hydroxyazobenzene-2-carboxylic acid

PIERCE
3747 N. Meridian Road
P.O. Box 117
Rockford, IL 61105

28010

0212.4

Number	Description
28010	**HABA,** 10 g

Storage: Upon receipt, store product at room temperature. Product is shipped at ambient temperature.

Molecular Weight: 242.23

Introduction

Antibodies or other proteins are often biotinylated with reagents such as EZ-Link® Sulfo-NHS-LC-Biotin (Product No. 21335). HABA (4′-hydroxyazobenzene-2-carboxylic acid) is a reagent that enables a quick estimation of the mole-to-mole ratio of biotin to protein. To quantify biotin label incorporation, 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 from its interaction with avidin and the absorption at 500 nm decreases proportionately. By this method, an unknown amount of biotin present in a solution can be evaluated 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.

Procedure for Measuring the Level of Biotin Incorporation

Note: The biotin-labeled protein sample must be desalted or dialyzed to remove all traces of nonreacted and hydrolyzed biotinylation reagent before performing the HABA assay. (This assay detects total biotin in the solution.)

A. Additional Materials Required

- Avidin (Product No. 21121)
- 1 N NaOH
- Phosphate Buffered Saline (PBS): 100 mM sodium phosphate, 150 mM sodium chloride; pH 7.2 (Product No. 28372)
- Cuvettes and spectrophotometer, or 96-well microplate and plate reader capable of measuring at 500 nm.

B. Reagent Preparation

HABA Solution	Add 24.2 mg HABA to 9.9 ml of ultrapure water and then add 0.1 ml of 1 N NaOH. If the HABA does not completely dissolve, add another 0.1 ml of 1 N NaOH and filter solution before use. Store HABA solution at 4°C.
HABA/Avidin Solution	Add 10 mg of avidin and 600 µl of the HABA Solution to 19.4 ml of PBS. Store solution at 4°C for up to 2 weeks.

C. Procedure (Cuvette Format)

1. Pipette 900 µl of HABA/Avidin Solution into a 1 ml cuvette and measure the absorbance at 500 nm. The A_{500} of this solution should be 0.9-1.3. Record the value as A_{500} HABA/Avidin.
2. Add 100 µl of biotinylated protein sample to the cuvette containing HABA/Avidin and mix well.
3. Measure the absorbance of the solution at 500 nm. Once the absorbance value remains constant for at least 15 seconds, record the value as A_{500} HABA/Avidin/Biotin Sample. If the A_{500} HABA/Avidin/Biotin Sample is ≤ 0.3, dilute sample and repeat assay. Proceed to Calculations (Section E).

D. Procedure (Microplate Format)

1. Add 180 µl of the HABA/Avidin Solution to a microplate well. Thoroughly mix plate on orbital shaker or equivalent.
2. Measure the absorbance of the solution in the well at 500 nm and record the value as A_{500} HABA/avidin.
3. Add 20 µl of biotinylated sample to the well containing the HABA/Avidin Solution. Mix as described above.
4. 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. If the A_{500} HABA/Avidin/Biotin Sample is ≤ 0.15, dilute sample and repeat assay.
5. Proceed to the Calculations (Section E).

E. Calculations for Moles of Biotin Per Mole of Protein

Note: The HABA Calculator, which is available from the Technical Resources menu from the Pierce website, will calculate the moles of biotin per 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:

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

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

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

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. $$\frac{\text{mmol biotin}}{\text{mmol protein}} = \frac{(1.25 \times 10^{-6}) \times 10}{4.6 \times 10^{-6}} = \frac{12.5 \times 10^{-6}}{4.6 \times 10^{-6}} = 2.72 \text{ biotin molecules per IgG molecule}$$

Troubleshooting

Problem	Cause	Solution
ΔA_{500} in HABA assay is ≤ 0	The protein sample has no or a 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 sample contributes to absorbance	Filter protein sample to remove particulate
High levels of biotinylation	Nonreacted biotin was not removed	Dialyze or desalt sample before performing the assay

Related Pierce Products

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

21335 **EZ-Link® Sulfo-NHS-LC-Biotin**, 100 mg, biotin reagent with 22.4 Å spacer arm

69576 **Slide-A-Lyzer® MINI Dialysis Unit Kit,** for 10-100 µl sample volumes, 10 units plus float

66382 **Slide-A-Lyzer® Dialysis Cassette Kit,** for 0.5-3 ml sample volumes, 10 cassettes, floats and syringes

20347 **Immobilized Streptavidin Gel**, 2 ml

21126 **ImmunoPure® Streptavidin, Horseradish Peroxidase Conjugated**, 1 mg

28005 **EZ™ Biotin Quantitation Kit**

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