

**EZ-Link<sup>®</sup> Sulfo-NHS-LC-Biotinylation Kit****21435**

1776.2

<b>Number</b>	<b>Description</b>
21435	<p><b>EZ-Link<sup>®</sup> Sulfo-NHS-LC-Biotinylation Kit</b>, sufficient biotin and other reagents for approximately 10 labeling reactions each containing 1-10 mg of antibody or other protein</p> <p><b>Kit Contents:</b></p> <p><b>EZ-Link<sup>®</sup> Sulfo-NHS-LC-Biotin</b>, 25 mg  Molecular Weight: 556.59  Spacer Arm: 22.4 Å</p> <p><b>BupH<sup>™</sup> Phosphate Buffered Saline Pack</b>, 1 pack, 0.1 M sodium phosphate, 0.15 M sodium chloride; pH 7.2 when reconstituted in 500 ml of ultrapure water</p> <p><b>Zeba<sup>™</sup> Desalt Spin Columns, 5 ml</b>, 10 columns, for 500-2,000 µl samples, 7,000 MWCO</p> <p><b>HABA</b>, 1 ml, 10 mM in 0.01 N NaOH</p> <p><b>Affinity Purified Avidin</b>, 10 mg</p> <p><b>Storage:</b> Upon receipt store vials of biotin reagent and avidin at -20°C. Store remaining kit components kit at 4°C. Kit is shipped at ambient temperature.</p>

**Table of Contents**

Introduction .....	2
Important Product Information .....	2
Procedure for Biotinylating Proteins .....	3
A. Calculations .....	3
B. Biotin Labeling Reaction .....	3
C. Buffer Exchange and Remove Excess Biotin Reagent Using a Desalting Column .....	4
HABA Assay for Measuring the Level of Biotin Incorporation .....	4
A. Reagent Preparation .....	4
B. Procedure for Estimating Biotin Incorporation .....	4
C. Calculations for Moles of Biotin Per Mole of Protein .....	5
Troubleshooting the HABA Assay .....	6
Website Resources .....	6
Related Pierce Products .....	6
Product References .....	6

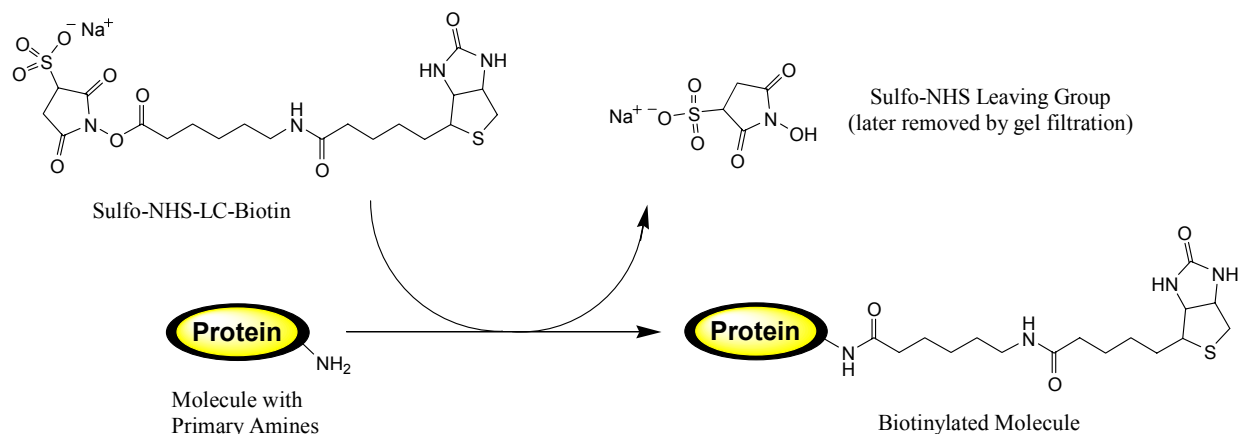
## Introduction

The EZ-Link<sup>®</sup> Sulfo-NHS-LC-Biotinylation Kit provides the reagents required for labeling antibodies, proteins and other macromolecules containing primary amino groups with biotin. Besides the biotin reagent and reaction buffer, the kit includes desalting columns for purifying the labeled molecule and HABA and avidin for measuring biotin incorporation. The kit is structured for labeling 1-10 mg of protein in a 1 ml reaction volume but is easily adapted to smaller or larger scales.

Biotin is a small naturally occurring vitamin that binds with high affinity to avidin and streptavidin proteins. Because of its size (244 Da), biotin can be conjugated to many proteins without altering their biological activities. The labeled molecule then can be detected in ELISA, dot blot or Western blot methods using streptavidin or avidin probes.

*N*-Hydroxysuccinimide (NHS) ester-activated biotins are the most popular type of biotinylation reagent. NHS esters react efficiently with primary amino groups (-NH<sub>2</sub>) in pH 7-9 buffers to form stable amide bonds (Figure 1). Because antibodies and other proteins generally contain multiple lysine (K) residues in addition to the N-terminus of each polypeptide, they have multiple primary amines available as targets for labeling with NHS-activated reagents. EZ-Link<sup>®</sup> Sulfo-NHS-LC-Biotin is water soluble, enabling reactions to be performed in the absence of organic solvents such as DMSO or DMF.

The EZ-Link<sup>®</sup> Sulfo-NHS-LC-Biotinylation Kit combines the basic reagents, tools and easy-to-follow instructions for biotin-labeling amine-containing macromolecules. With this kit, even researchers who have never before labeled antibodies or other proteins can expect to obtain results comparable to those obtained in commercial laboratories.



**Figure 1.** Reaction of Sulfo-NHS-LC-Biotin with primary amine. If drawn to scale, the oval representing the protein would be many times larger than the structures and would likely contain several amino groups. Note that NHS is a leaving group (byproduct) in the reaction. The leaving group and any non-reacted biotin reagent are removed during the desalting step.

## Important Product Information

- Sulfo-NHS-LC-Biotin is moisture-sensitive. Store the vial of biotin reagent at -20°C with desiccant. To avoid moisture condensation onto the product, equilibrate vial to room temperature before opening.
- As directed in the procedure, dissolve the biotin reagent immediately before use. The NHS-ester moiety readily hydrolyzes and becomes non-reactive; therefore, weigh and dissolve only a small amount of the reagent at a time, and do not prepare stock solutions for storage. Discard any unused reconstituted reagent.
- Avoid buffers containing primary amines (e.g., Tris or glycine) as these will compete with the reaction (see Figure 1). If necessary, dialyze or otherwise desalt to exchange the protein sample into an amine-free buffer such as phosphate-buffered saline (one packet is included in this kit).
- The desalting columns provided in this kit are best suited for processing biotinylation reactions involving 1-10 mg of protein in approximately 0.5-2 ml. For smaller amounts of protein and/or smaller reaction volumes, perform both the biotinylation reaction and buffer exchanges in a single Slide-A-Lyzer<sup>®</sup> MINI Dialysis Unit. For reaction volumes that are larger than can be processed with a desalting column, split the sample between two columns or use an appropriate Slide-A-Lyzer<sup>®</sup> Dialysis Cassette for buffer exchanges. For processing small volumes (i.e. 10-150 µl) of peptides and other low molecular weight molecules, the PepClean<sup>™</sup> C-18 Spin Columns (Product No. 89870 or 89873) may be used.

## Procedure for Biotinylating Proteins

### A. Calculations

The extent of biotin labeling depends on the size and distribution of amino groups on the protein and the amount of reagent used per protein concentration. Compared to reactions involving concentrated protein solutions, labeling reactions with dilute protein solutions require a greater fold molar excess of biotin reagent to achieve the same incorporation level. Experiments performed at Pierce that used a 20-fold molar excess of biotin reagent to label 1-10 mg/ml antibody (IgG) resulted in 4-6 biotin groups per antibody molecule. Adjust the molar ratio of Sulfo-NHS-LC-Biotin to protein to obtain the level of incorporation desired. Instructions for using Sulfo-NHS-LC-Biotin (Product No. 21335) for labeling cells is available for download from the Pierce web site.

1. Calculate millimoles of Sulfo-NHS-LC-Biotin to add to the reaction for a 20-fold molar excess:

$$\text{ml protein} \times \frac{\text{mg protein}}{\text{ml protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{20 \text{ mmol Biotin}}{\text{mmol protein}} = \text{mmol Biotin}$$

- 20 = Recommended molar fold excess of biotin per 1-10 mg/ml protein sample

2. Calculate microliters of 10 mM Sulfo-NHS-LC-Biotin (prepared in Step B.3) to add to the reaction:

$$\text{mmol Biotin} \times \frac{557 \text{ mg}}{\text{mmol Biotin}} \times \frac{400 \mu\text{l}}{2.2 \text{ mg}} = \mu\text{l Biotin Solution}$$

- 557 = Molecular weight of Sulfo-NHS-LC-Biotin
- 400 = Microliters of water in which 2.2 mg of Sulfo-NHS-LC-Biotin is dissolved to make a 10 mM solution

**Example:** For 1 ml of a 2 mg/ml IgG (150,000 MW) solution, ~27  $\mu\text{l}$  of 10 mM Sulfo-NHS-LC-Biotin will be added.

$$1 \text{ ml IgG} \times \frac{2 \text{ mg IgG}}{1 \text{ ml IgG}} \times \frac{1 \text{ mmol IgG}}{150,000 \text{ mg IgG}} \times \frac{20 \text{ mmol Biotin}}{1 \text{ mmol IgG}} = 0.000266 \text{ mmol Biotin}$$

$$0.000266 \text{ mmol Biotin} \times \frac{557 \text{ mg}}{\text{mmol Biotin}} \times \frac{400 \mu\text{l}}{2.2 \text{ mg}} = 26.9 \mu\text{l Biotin Solution}$$

### B. Biotin Labeling Reaction

1. Remove the vial of Sulfo-NHS-LC-Biotin from freezer and equilibrate it to room temperature before opening in step 3.
2. Dissolve 1-10 mg protein in 0.5-2 ml of phosphate-buffered saline (PBS) according to the calculation made in section A. Prepare the BupH™ PBS as directed on the package label.

**Note:** Protein already dissolved in amine-free buffer at pH 7.2-8.0 may be used without buffer exchange. Proteins in Tris or other amine-containing buffers must be exchanged into PBS. Perform buffer exchange of 0.5-2.0 ml samples by dialysis or using one of the desalting columns included in this kit, as described in Section C. Be aware that this kit contains only 10 single-use desalting columns, sufficient for 10 biotinylation procedures when used only for Section C.

3. Immediately before use, prepare 10 mM Sulfo-NHS-LC-Biotin by dissolving 2.2 mg in 400  $\mu\text{l}$  ultrapure water.
4. Add the appropriate volume of Sulfo-NHS-LC-Biotin solution (see calculations in Section A) to the protein solution.
5. Incubate reaction on ice for two hours or at room temperature for 30-60 minutes.

**Note:** There is no harm in reacting longer than the specified time other than the possibility of ordinary protein degradation or microbial growth.

**Note:** Although excess non-reacted and hydrolyzed biotin reagent remains in the solution, it is often possible to perform preliminary tests of the labeled protein by ELISA or Western blot. Once function has been confirmed, buffer exchange the labeled protein for optimal performance and stability using the procedure in Section C: Buffer Exchange using a Desalting Column. If the HABA Assay will be performed to determine biotin incorporation, the protein must be purified by buffer exchange first.

**C. Buffer Exchange and Remove Excess Biotin Reagent Using a Desalting Column**

1. Prepare a Zeba™ Desalt Spin Column by breaking off the bottom plug and placing the column into a 15 ml collection tube. Centrifuge the column at 1,000 × g for 2 minutes, discard the storage buffer and return column to the same collection tube.
2. Equilibrate the column by adding 2.5 ml of PBS to the top of the resin bed and centrifuging at 1,000 × g for 2 minutes. Discard the flow-through and repeat this step a total of 2-3 times.
3. Place column into a new 15 ml collection tube and apply the protein sample directly onto the center of the resin bed. Allow sample to absorb into the resin.  
**Note:** For samples < 1,000 µl, add 100 µl ultrapure water on top of the absorbed sample to increase protein recovery.
4. Centrifuge the column at 1,000 × g for 2 minutes. The collected flow-through solution is the purified protein sample. Store the protein solution in appropriate conditions.

**HABA Assay for Measuring the Level of Biotin Incorporation**

To estimate biotin 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 absorbance at 500 nm decreases proportionately. An unknown amount of biotin present in a solution is estimated 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.

**Note:** The biotin-labeled protein sample must be desalted or dialyzed to remove all traces of non-reacted and hydrolyzed biotinylation reagent before the HABA assay is performed.

**A. Reagent Preparation**

Phosphate-buffered Saline (PBS)      100 mM sodium phosphate, 150 mM sodium chloride; pH 7.2; Product No. 28372

HABA/Avidin Solution      Add 10 mg of avidin and 600 µl of 10 mM HABA to 19.4 ml of PBS. If prepared correctly, the A<sub>500</sub> of this solution will be 0.9-1.3 in a 1 cm cuvette. The solution is stable for two weeks at 4°C. If a precipitate forms in the HABA solution, it can be filtered and then used.

**B. Procedure for Estimating Biotin Incorporation**

• **Procedure Option 1 – Cuvette Format**

1. Pipette 900 µl of HABA/Avidin Solution into a 1 ml cuvette.
2. Measure the absorbance of the solution in the cuvette at 500 nm and record the value as A<sub>500</sub> HABA/Avidin.
3. Add 100 µl of biotinylated protein sample to the cuvette containing HABA/Avidin and mix well.
4. Measure the absorbance of the solution in the cuvette at 500 nm. Once the value remains fairly constant for at least 15 seconds, record the value as A<sub>500</sub> HABA/Avidin/Biotin Sample. If the A<sub>500</sub> HABA/Avidin/Biotin is < 0.3, dilute the biotinylated protein sample and repeat the assay, but remember to account for the dilution in during calculations.
5. Proceed to Section C: Calculation of Moles of Biotin Per Mole of Protein.

• **Procedure Option 2 – Microplate Format**

1. Pipette 180 µl of HABA/Avidin Solution into a microplate well.
2. Measure the absorbance at 500 nm of the solution in the well and record the value as A<sub>500</sub> HABA/Avidin.
3. Add 20 µl of biotinylated sample to the well containing the HABA/Avidin Solution. Mix the plate using an orbital shaker or plate mixer.
4. Measure the absorbance at 500 nm of the solution in the well. Once the value remains fairly constant for at least 15 seconds, record the value as A<sub>500</sub> HABA/Avidin/Biotin Sample.
5. Proceed to Section C: Calculation of Moles of Biotin Per Mole of Protein.

## C. Calculations for Moles of Biotin Per Mole of Protein

**Note:** An automatic HABA Calculator is available at the Pierce website for performing these calculations.

These calculations are based on the Beer Lambert Law (Beer's Law):  $A_{\lambda} = \epsilon_{\lambda} b C$

Where:

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

**$\epsilon$**  is the absorptivity or extinction coefficient at the wavelength ( $\lambda$ ). For HABA/avidin samples at 500 nm, pH 7.0 extinction coefficient is equal to 34,000 M<sup>-1</sup>cm<sup>-1</sup>.

**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 Solution ( $A_{500} H\backslash A$ )
- Absorbance at 500 nm for HABA/Avidin/Biotin reaction mixture ( $A_{500} H\backslash A\backslash B$ )
- Dilution factor, if the sample is diluted before adding it to the HABA/Avidin Solution

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

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

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

- Cuvette:

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

- Microplate:

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

**Note:** The cuvette format requires the 0.9 correction factor to adjust for dilution of the H\A Solution 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:

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

**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:

$$\begin{aligned} &= \frac{\text{mmol biotin in original sample}}{\text{mmol protein in original sample}} \\ &= \frac{(\text{mmol per ml biotin in reaction mixture})(10)(\text{dilution factor})}{\text{mmol per ml protein in original sample}} \\ &= \frac{(\text{Calc\#3}) \times 10 \times \text{dilution factor}}{\text{Calc\#1}} \end{aligned}$$

**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 HABA Assay calculation:** In this example, the labeled protein is IgG (MW 150,000) at 0.69 mg/ml. The absorbance measurements were  $A_{500\text{ HABA}} = 0.904$  and  $A_{500\text{ HABA}} = 0.771$ .

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 the HABA Assay

Problem	Cause	Solution
$\Delta A_{500}$ in HABA assay is $\leq 0$	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., sulfhydryl-reactive) or use a higher molar ratio of biotinylation reagent
	Incomplete mixing of reagent	Completely solubilize and mix HABA/Avidin before diluting
	Particles in sample contribute to absorbance	Filter protein sample to remove particles
High levels of biotinylation	Nonreacted biotin was not removed	Dialyze or desalt sample before performing the assay

### Website Resources

- HABA Calculator for computing the results associated with the HABA Assay measurements
- Tech Tip #14: Perform labeling and other reactions in Slide-A-Lyzer® Dialysis Cassettes

### Related Pierce Products

- 21335**      **EZ-Link® Sulfo-NHS-LC-Biotin**, 100 mg, biotin reagent used in this kit
- 69576**      **Slide-A-Lyzer® MINI Dialysis Unit Kit**, for 10-100  $\mu\text{l}$  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**      **ImmunoPure® Immobilized Streptavidin Gel**, 2 ml
- 21126**      **ImmunoPure® Streptavidin, Horseradish Peroxidase Conjugated**, 1 mg
- 89870**      **PepClean™ C-18 Spin Columns**

### Product References

- Coffman, K.T. *et al.* (2003). Differential EphA2 epitope display on normal versus malignant cells. *Cancer Res.* **63**:7907-12.
- Liu, H-B., *et al.* (2003). Estrogen receptor  $\alpha$  mediates estrogen's immune protection in autoimmune disease. *J. Immunol.* **171**:6936-40.
- Longhurst, C.M., *et al.* (1999). A CD9,  $\alpha_{\text{IIb}}\beta_3$ , integrin-associated protein, and GPIb/V/IX complex on the surface of human platelets is influenced by  $\alpha_{\text{IIb}}\beta_3$  conformational states. *Eur. J. Biochem.* **263**:104-11.
- Wu, P.C., *et al.* (1999). *In vivo* sensitivity of human melanoma to tumor necrosis factor (TNF)- $\alpha$  is determined by tumor production of the novel cytokine endothelial-monocyte activating polypeptide II (EMAPII). *Cancer Res.* **59**:205-12.
- Zhang, L., *et al.* (1999). Calpain inhibitor I increases  $\beta$ -amyloid peptide production by inhibiting the degradation of the substrate  $\gamma$ -secretase. *J. Biol. Chem.* **274**(13):8966-72.
- Zuk, P.A. and Elferink, L.A. (2000). Rab15 differentially regulates early endocytic trafficking. *J. Biol. Chem.* **275**(35):26754-64.

Sulfo-NHS Technology is protected by U.S. Patent #s 6,407,263, 5,872,261, 5,892,057 and 5,942,628.

Slide-A-Lyzer® Dialysis Cassette Technology is protected by U.S. Patent # 5,503,741 and other patent pending.

Slide-A-Lyzer® MINI Dialysis Unit Technology is protected by U.S. Patent # 6,039,871.

Current versions of product instructions are available at [www.piercenet.com](http://www.piercenet.com). For a faxed copy, call 800-874-3723 or contact your local distributor.

©Pierce Biotechnology, Inc., 2/2007. Printed in the USA.