INSTRUCTIONS

EZ-Link™ Sulfo-NHS-Biotinylation Kit

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21425

Number	Description
21425	**EZ-Link Sulfo-NHS-Biotinylation Kit,** sufficient biotin and other reagents for approximately 10 labeling reactions each containing 1-10mg of antibody or other protein

Kit Contents:

EZ-Link Sulfo-NHS-Biotin (sulfosuccinimidyl-6-[biotin-amido]hexanoate), 25mg

Molecular Weight: 443.43

Spacer Arm: 13.5Å

BupH™ Phosphate Buffered Saline Pack, 1 pack, 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 when reconstituted in 500mL of ultrapure water

Zeba™ Spin Desalting Column, 5mL, 10 columns, for 500-2000µL samples, 7000 MWCO

HABA, 1mL, 10mM in 0.01 N NaOH

Affinity Purified Avidin, 10mg

Storage: Upon receipt, store Avidin and vial of biotin reagent (with desiccant) at -20ºC. Store remaining kit components at 4°C. Kit is shipped at ambient temperature.

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Introduction

The Thermo Scientific EZ-Link Sulfo-NHS-Biotinylation Kit provides the reagents required for labeling antibodies, proteins and other macromolecules containing primary amino groups. Besides the biotin reagent and reaction buffer, the kit includes desalting columns for purifying the labeled molecule and HABA and avidin to measure the level of biotin incorporation. The kit is structured for labeling 1-10mg of protein in a 1mL, but the procedure is easily adapted to smaller or larger scales.

Biotin is a naturally occurring vitamin that binds with high affinity to avidin and streptavidin proteins. Because of its small size (244Da), 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_2) 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. The sulfo-NHS-ester reagent in this kit is water-soluble, enabling reactions to be performed in the absence of organic solvents such as DMSO or DMF.

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

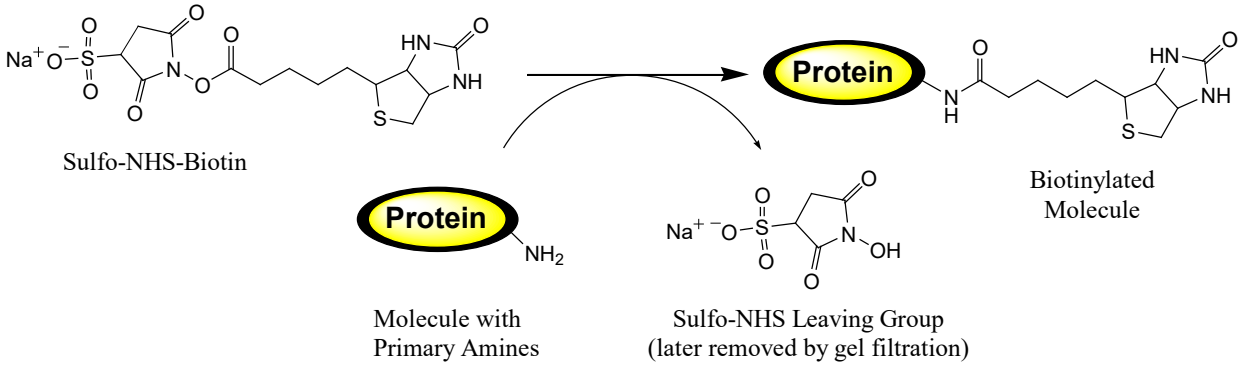

Figure 1. Reaction of Sulfo-NHS-Biotin with primary amine. If drawn to scale, the oval representing the protein would be many times larger than the structures and would likely contain several amino groups. Note that Sulfo-NHS is a leaving group (byproduct) in the reaction. The leaving group and any non-reacted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

Important Product Information

- Sulfo-NHS-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 compete with the intended 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 0.5-2mL. For smaller amounts of protein and/or smaller reaction volumes, perform both the biotinylation reaction and buffer exchanges in a single Thermo Scientific Slide-A-Lyzer MINI Dialysis Unit (See Additional Information and Related Thermo Scientific Products). For reaction volumes larger than can be processed with a desalting column, split the sample between two columns or use an appropriate Slide-A-Lyzer™ Dialysis Cassette for performing buffer exchanges. For processing small volumes (i.e., 10-150µL) of peptides and other low molecular weight molecules, the Thermo Scientific Pierce C18 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. 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 that used a 20-fold molar excess of biotin reagent to label 1-10mg/mL antibody (IgG) resulted in 4-6 biotin groups per antibody molecule. Adjust the molar ratio of Sulfo-NHS-Biotin to protein to obtain the level of incorporation desired.

1. Calculate millimoles of Sulfo-NHS-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 reagent per protein sample

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

$$\text{mmol Biotin} \times \frac{443\ \text{mg}}{\text{mmol Biotin}} \times \frac{500\ \mu\text{L}}{2.2\ \text{mg}} = \mu\text{L Biotin Solution}$$

   - 443 = Molecular weight of Sulfo-NHS-Biotin
   - 500 = Microliters of water in which 2.2mg of Sulfo-NHS-Biotin is dissolved to make 10mM

Example: For 1mL of a 2mg/mL IgG (150,000 MW) solution, ~27µL of 10mM Sulfo-NHS-Biotin will be added.

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

$$0.000266\ \text{mmol Biotin} \times \frac{443\ \text{mg}}{\text{mmol Biotin}} \times \frac{500\ \mu\text{L}}{2.2\ \text{mg}} = 26.8\ \mu\text{L Biotin Solution}$$

B. Biotin Labeling Reaction

1. Remove the vial of Sulfo-NHS-Biotin from freezer and equilibrate it to room temperature before opening in Step 3.
2. Dissolve 1-10mg protein in 0.5-2mL of phosphate-buffered saline (PBS) according to the calculation made in Section A. Prepare the Thermo Scientific 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 0.5-2.0mL 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 a 10mM Sulfo-NHS-Biotin solution by dissolving 2.2mg in 500µL of ultrapure water.
4. Add the appropriate volume of Sulfo-NHS-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. If the HABA Assay will be performed to determine biotin incorporation, the protein must be purified by buffer exchange first (Section C).

Buffer Exchange and Remove Excess Biotin Reagent Using a Desalting Column

1. Prepare a Thermo Scientific Zeba Spin Desalting Column by breaking off the bottom reusable closure 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. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing outward in all subsequent centrifugation steps.
2. Equilibrate the column by adding 2.5mL 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 2-3 times.

3. Place column into a new 15mL collection tube and apply protein sample directly onto the center of the resin bed. Allow sample to absorb into the resin.

   Note: For samples < 1,000µL, add 100µL 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 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 absorbance at 500nm 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)	100mM sodium phosphate, 150mM sodium chloride; pH 7.2; Product No. 28372 **Note:** Avoid buffers containing potassium (e.g., Modified Dulbecco's PBS), which will cause precipitation in the assay. Empirically determine if other buffers are compatible by comparing to results obtained using TBS or PBS.
HABA/Avidin Solution	Add 10mg of avidin and 600µL of 10mM HABA to 19.4mL of PBS. If prepared correctly, the A_{500} of this solution will be 0.9-1.3 in a 1cm 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 900µL of HABA/Avidin Solution into a 1mL cuvette.
2. Measure the absorbance of the solution in the cuvette at 500nm and record the value as A_{500} HABA/Avidin.
3. Add 100µL of biotinylated protein sample to the cuvette containing HABA/Avidin and mix well.
4. Measure the absorbance of the solution in the cuvette at 500nm. Once the value remains fairly constant for at least 15 seconds, record the value as A_{500} HABA/Avidin/Biotin Sample. If the A_{500} HABA/Avidin/Biotin is < 0.3, dilute the biotinylated protein sample and repeat the assay, but remember to account for the dilution in subsequent calculations.
5. Proceed to Section C: Calculation of Moles of Biotin per Mole of Protein.

- **Procedure Option 2 – Microplate Format**

1. Pipette 180µL of HABA/Avidin Solution into a microplate well.
2. Measure the absorbance at 500nm of the solution in the well and record the value as A_{500} HABA/Avidin.
3. Add 20µL 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 500nm of the solution in the well. Once the value remains fairly constant for at least 15 seconds, record the value as A_{500} 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 website for performing these calculations.

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 500nm. There are no units for absorbance.

ε is the absorptivity or extinction coefficient at the wavelength (λ). For HABA/avidin samples at 500nm, 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 10mm square cuvette has a path length of 1.0cm. Using the recommended microplate format volumes, the path length is typically 0.5cm.

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 500nm for HABA/Avidin Solution (**A_{500} H\A**)
- Absorbance at 500nm for HABA/Avidin/Biotin reaction mixture (**A_{500} H\A\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):

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

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

- 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 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:

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

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

1. $$\text{mmol biotinylated protein per mL} = \frac{0.69\ \text{mg/mL}}{150{,}000\ \text{mg/mmol}} = 4.6\times10^{-6}$$

2. $$\Delta A_{500} = (0.9\times0.904) - 0.771 = 0.0426$$

3. $$\frac{\text{mmol biotin}}{\text{mL reaction mixture}} = \frac{0.0426}{34{,}000} = 1.25\times10^{-6}$$

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

Troubleshooting the HABA Assay

Problem	Possible Cause	Solution
ΔA_{500} in HABA assay is ≤ 0	The protein sample had 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 contributed 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.

Related Thermo Scientific Products

21217 **EZ-Link Sulfo-NHS-Biotin,** 50 mg

69576 **Slide-A-Lyzer MINI Dialysis Unit Kit**

66382 **Slide-A-Lyzer Dialysis Cassette Kit**

89870 **Pierce™ C18 Spin Columns,** 25 columns

89873 **Pierce C18 Spin Columns,** 50 columns

General References

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Chyung, J.H. and Selkoe, D.J. (2003). Inhibition of receptor-mediated endocytosis demonstrates generation of amyloid beta-protein at the cell surface. *J Biol Chem* **278(51)**:51035-43.

Frickel, E-M., *et al.* (2002). TROSY-NMR reveals interaction between Erp57 and the tip of the calreticulin P-domain. *PNAS* **99(4)**:1954-9.

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Ohnishi, T., *et al.* (2003). MD-2 is necessary for the toll-like receptor 4 protein to undergo glycosylation essential for its translocation to the cell surface. *Clin Diagn Lab Immunol* **10(3)**:405-10.

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition. The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0011559 B

Revision	Date	Description
B	31 July 2024	Correcting spin column usage.
A	17 October 2015	New document for EZ-Link™ Sulfo-NHS-Biotinylation Kit.