INSTRUCTIONS

EZ-Link NHS-PEG$_4$-Biotinylation Kit

21455

1778.8

Number	Description
21455	**EZ-Link NHS-PEG$_4$-Biotinylation Kit,** sufficient biotin and other reagents for approximately 8 labeling reactions each containing 1-10mg of antibody or other protein

Kit Contents:

NHS-PEG$_4$-Biotin, No-Weigh Format, 8 × 2mg microtubes

Molecular Weight: 588.67

Spacer Arm: 29Å

Solubility: 10mg/mL in aqueous solutions

BupH Phosphate Buffered Saline (PBS) 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 0.01N NaOH

Affinity Purified Avidin, 10mg

Storage: Upon receipt store microtubes of biotin reagent and vial of avidin at -20°C. Store the PBS saline pack at room temperature. Store the remaining kit components at 4°C. Kit is shipped at ambient temperature.

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Introduction

The Thermo Scientific™ EZ-Link™ NHS-PEG$_4$-Biotinylation Kit enables simple and efficient biotin labeling of antibodies, proteins and any other primary amine-containing macromolecule. The hydrophilic polyethylene oxide (PEO), also called polyethylene glycol (PEG), spacer arm imparts water solubility that is transferred to the biotinylated molecule. Consequently, antibodies labeled with NHS-PEG$_4$-Biotin exhibit less aggregation when stored in solution compared to antibodies labeled with reagents having only hydrocarbon spacers. Specific labeling of cell surface proteins is another useful application for this water-soluble and membrane-impermeable reagent. The NHS-PEG$_4$-Biotin is packaged in Thermo Scientific™ No-Weigh™ format as convenient two-milligram aliquots, eliminating difficulties associated with weighing small quantities of reagent.

Biotin is a small naturally occurring vitamin that binds with high affinity to avidin and streptavidin proteins. Biotinylated proteins typically retain biological activity because the biotin group is relatively small. An antibody conjugated with several biotin molecules can amplify signal, thereby 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. Labeled proteins can be purified using immobilized streptavidin, avidin or Thermo Scientific™ NeutrAvidin™ Gel (see Related Thermo Scientific Products) and detected in ELISA, dot blot or Western blot applications.

N-Hydroxysuccinimide (NHS) esters are the most commonly used biotinylation reagents. In pH 7-9 buffers, NHS esters react efficiently with primary amino groups (-NH_2) by nucleophilic attack, forming an amide bond and releasing the NHS (Figure 1). Proteins typically have many sites for labeling, including the primary amine in the side chain of lysine (K) residues and the N-terminus of each polypeptide.

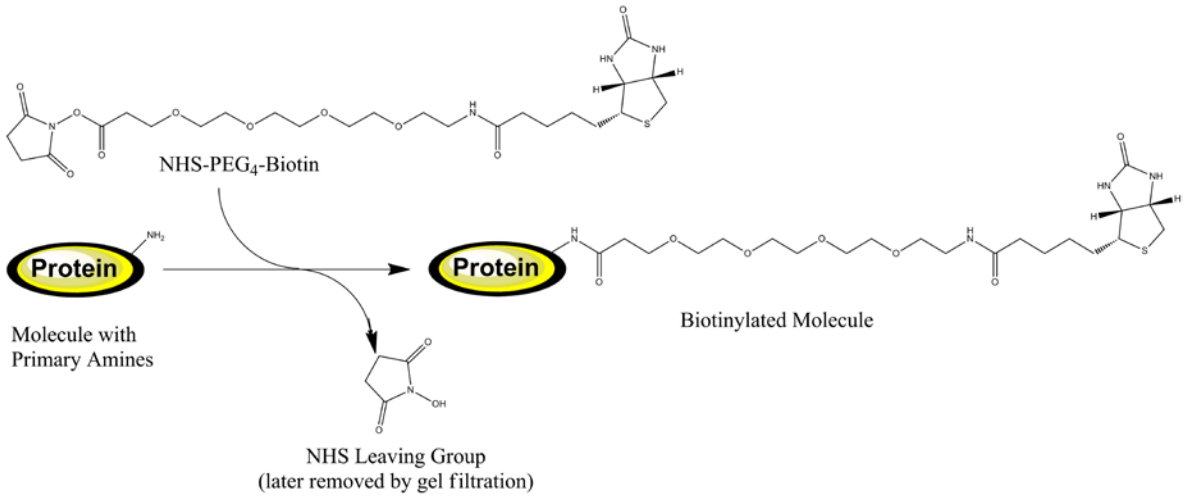

Figure 1. Reaction of NHS-PEG_4-Biotin with primary amine. If drawn to scale, the oval representing the protein would be many times larger than the structures and would likely contain several amino groups. Note that NHS is a leaving group (byproduct) in the reaction. The leaving group and any non-reacted biotin reagent are removed during the desalting step.

Important Product Information

- Use reconstituted NHS-PEG4-Biotin immediately. The NHS-ester moiety readily hydrolyzes and becomes non-reactive; therefore, do not prepare solutions for storage. Discard any unused reconstituted reagent.
- NHS-PEG4-Biotin is moisture-sensitive. Immediately before use, puncture the microtube foil with a pipette tip, add water and mix by pipetting up and down. After use, cut the used microtube from the unused microtubes and discard. Store the microtube strip at -20°C in the foil pouch provided.
- Avoid buffers containing primary amines (e.g., Tris or glycine), as these will 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 1-10mg of protein in approximately 0.5-2mL. For smaller amounts of protein 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 that are 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 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 NHS-PEG_4-Biotin to protein to obtain the desired level of incorporation.

1. Calculate millimoles of NHS-PEG_4-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 1-10mg/mL protein sample

2. Calculate microliters of 20mM NHS-PEG_4-Biotin (prepared in Step B.3) to add to the reaction:

$$\text{mmol Biotin} \times \frac{589\ \text{mg}}{\text{mmol Biotin}} \times \frac{170\ \mu\text{L}}{2.0\ \text{mg}} = \mu\text{L Biotin Solution}$$

   - 589 = Molecular weight of NHS-PEG_4-Biotin
   - 170 = Microliters of water in which 2.0mg of NHS-PEG_4-Biotin is dissolved to make 20mM

Example: For 1mL of a 2mg/mL IgG (150,000 MW) solution, 13.3µL of 20mM NHS-PEG_4-Biotin will be added.

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

$$0.000266\ \text{mmol Biotin} \times \frac{589\ \text{mg}}{\text{mmol Biotin}} \times \frac{170\ \mu\text{L}}{2.0\ \text{mg}} = 13.3\ \mu\text{L Biotin Solution}$$

A. Biotin Labeling Reaction

1. 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 0.5-2.0 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.

2. Cut off one microtube of NHS-PEG_4-Biotin from the No-Weigh Microtube Strip. Return the unused strip of microtubes to its pouch and store desiccated at 4°C.
3. With a pipette tip, puncture the foil top on the biotin reagent microtube; add 170µL of water and mix by pipetting up and down to prepare a 20mM solution of NHS-PEG_4-Biotin.
4. Add the appropriate volume of NHS-PEG_4-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.

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

Prepare a Thermo Scientific™ Zeba™ Spin Desalting Column by breaking off the bottom plug and placing the column into a 15mL collection tube. Centrifuge the column at 1000 × *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.

1. Equilibrate the column by adding 2.5mL of PBS to the top of the resin bed and centrifuging at 1000 × *g* for 2 minutes. Discard the flow-through and repeat this step 2-3 times.
2. 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 < 1000µL, add 100µL ultrapure water on top of the absorbed sample to increase protein recovery.
3. Centrifuge the column at 1000 × *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 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 during calculations.
5. Proceed to Section C: Calculation of Moles of Biotin per Mole of Protein.

- **Procedure Option 2 – Microplate Format**

Pipette 180µL of HABA/Avidin Solution into a microplate well.

1. Measure the absorbance at 500nm of the solution in the well and record the value as A_{500} HABA/Avidin.
2. Add 20µL of biotinylated sample to the well containing the HABA/Avidin Solution. Mix the plate using an orbital shaker or plate mixer.
3. 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.
4. 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. $\frac{\text{mmol biotin}}{\text{mmol protein}} = \frac{(1.25\times10^{-6})\times10}{4.6\times10^{-6}} = \frac{12.5\times10^{-6}}{4.6\times10^{-6}} = 2.72$ biotin molecules per IgG molecule

Troubleshooting the HABA Assay

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., 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

Additional Information Available on Our Website

- 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 Thermo Scientific Products

21329 **No-Weigh NHS-PEG_4-Biotin,** 8 × 2mg microtubes, biotin reagent used in this kit

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

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

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

15120 **Streptavidin Coated Plates,** 5 plates (see catalog for a complete listing of plates)

20347 **Streptavidin Agarose Resin,** 2mL

20228 **Immobilized Monomeric Avidin Kit**

General References

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