INSTRUCTIONS

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

21955

1790.4

Number	Description
21955	**EZ-Link Micro NHS-PEG$_4$-Biotinylation Kit**, sufficient reagents for approximately 8 labeling reaction each containing 50-200µg of antibody or other protein in 200-700µL reaction volumes

Kit Contents:

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

Molecular Weight: 588.67

Spacer Arm: 29 Å

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, 2mL, 10 columns, for 200-700µL samples, 7000 MWCO

Storage: Upon receipt store microtubes of biotin reagent at -20°C. Store the PBS saline pack at room temperature. Store the Zeba Desalting Column at 4°C. Kit is shipped at ambient temperature.

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Introduction

The Thermo Scientific™ EZ-Link™ Micro NHS-PEG$_4$-Biotinylation Kit provides the required reagents for labeling macromolecules containing primary amino groups and desalting columns for purifying the labeled molecule. The kit is structured for labeling 50-200µg of protein in 200-700µL. 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 provided in Thermo Scientific™ No-Weigh™ Format as convenient single-use microtubes, eliminating difficulties associated with weighing small quantities of reagent.

Biotin is a small, naturally occurring vitamin that binds with high affinity to avidin and avidin-like 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 biotin-avidin interaction is rapidly formed and 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™ Agarose (see Related Thermo Scientific Products) and detected in ELISA, dot blot and Western blot applications.

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N-Hydroxysuccinimide (NHS) esters are the most popular 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.

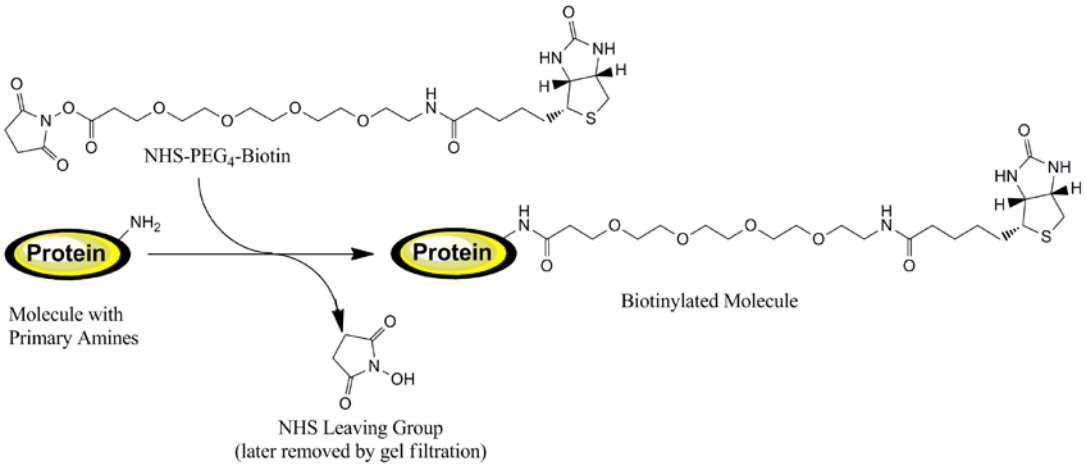

Figure 1. Reaction of NHS-PEG_4-Biotin with primary amine. If drawn to scale, the oval representing the protein would be many times larger than the structures and would likely contain several amino groups. Note that NHS is a leaving group (byproduct) in the reaction. The leaving group and any non-reacted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

Important Product Information

- Use reconstituted NHS-PEG_4-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-PEG_4-Biotin is moisture-sensitive. Immediately before use, puncture the microtube foil with a pipette tip, add solvent and mix by pipetting up and down. After use, cut off the used microtube and discard. Store the microtube strip at -20ºC 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 containing 50-200µg of protein in approximately 200-700µL. For smaller amounts of protein or reaction volumes, perform both the biotinylation reaction and buffer exchanges in a single Thermo Scientific™ Slide-A-Lyzer™ MINI Dialysis Device (see Additional Information and Related Thermo Scientific Products). For reaction volumes that are too large for processing with a desalting column, split the sample between two columns or use an appropriate Slide-A-Lyzer Dialysis Cassette for buffer exchanges.
- The biotin reagent is first prepared at ~12mg/mL before adding to the reaction mixture. To avoid having to pipette volumes less than 1µL when biotinylating low protein amounts, dilute the biotin reagent further (e.g., 1:10 dilution). To minimize hydrolysis in such a dilute solution, dissolve the biotin reagent in either anhydrous DMSO or DMF and make dilutions with the same solvent.

Procedure for Biotinylating Proteins

A. Calculations

The extent of biotin labeling depends on the size and distribution of amino groups on the protein, protein concentration and the reagent amount 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 50-fold molar excess of biotin reagent to label 50-200µg antibody (human IgG) for 30 minutes at room temperature resulted in 1-5 biotin groups per antibody molecule. Adjust the molar ratio of NHS-PEG_4-Biotin to protein to obtain the desired incorporation level.

1. Calculate millimoles of NHS-PEG$_4$-Biotin to add to the reaction for a 50-fold molar excess:

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{50 mmol Biotin}}{\text{mmol protein}} = \text{mmol Biotin}$$

   - 50 = Recommended molar fold excess of biotin per 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 solvent in which 2.0mg of NHS-PEG$_4$-Biotin is dissolved to make 20mM

Example: For 0.7mL of a 0.29mg/mL IgG (150,000 MW) solution, 3µL of 20mM NHS-PEG$_4$-Biotin will be added.

$$0.7\ \text{mL IgG} \times \frac{0.29\ \text{mg IgG}}{1\ \text{mL IgG}} \times \frac{1\ \text{mmol IgG}}{150{,}000\ \text{mg IgG}} \times \frac{50\ \text{mmol Biotin}}{1\ \text{mmol IgG}} = 0.0000676\ \text{mmol Biotin}$$

$$0.0000676\ \text{mmol Biotin} \times \frac{589\ \text{mg}}{\text{mmol Biotin}} \times \frac{170\ \mu\text{L}}{2.0\ \text{mg}} = 3.4\ \mu\text{L Biotin Solution}$$

B. Biotin-labeling Reaction

1. Dissolve 50-200µg of protein in 200-700µL 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 200-700µL samples by dialysis or using a desalting column included in this kit. This kit contains 10 single-use desalting columns and eight microtubes of biotin reagent. Two columns may be used for buffer exchange, but the remaining eight columns are needed to remove excess biotin reagent after performing the biotinylation reaction for each of the eight reactions.

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 4ºC.

3. With a pipette tip, puncture the foil top on the biotin reagent microtube. To prepare 20mM, add 170µL of solvent (e.g., water, PBS, DMF or DMSO; see the Important Product Information Section) to the tube and mix by pipetting up and down.

4. Add the appropriate volume of NHS-PEG$_4$-Biotin solution (see calculations in Section A) to the protein solution.

5. Incubate the reaction on ice for two hours or at room temperature for 30-60 minutes. 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.

C. Buffer Exchange and Excess Biotin Removal

1. 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.

2. Equilibrate the column by adding 1mL 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.

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 < 400µL, add 100µL ultrapure water stacker on top of the absorbed sample to maximize recovery.

4. 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.

Troubleshooting

Problem	Possible Cause	Solution
Lack of biotinylation	No amines available on molecule of interest	Use a biotinylation reagent that targets a different functional group or convert sulfhydryl to amine using Aminoethyl-8 (Product No. 23010)
	Buffer contained primary amines	Use a non-amine-containing buffer
	Reagent was not reactive; caused by hydrolysis of the NHS ester	Use reagent immediately upon reconstitution
	Not enough biotin reagent was added to the reaction mixture	Increase the molar excess of biotin reagent to protein
Low level of biotinylation	Carrier protein was added to purified IgG to help stabilize it	Remove carrier protein before biotinylation to reduce competition for labeling
Protein is non-functional	Excessive biotinylation	Reduce the molar excess of biotinylation reagent, or reduce time or temperature for biotinylation
Low protein recovery after desalting	No stacker used	Apply a stacker above sample
	Unstable protein	Equilibrate column in a suitable buffer

Additional Information

Please visit the website for additional information including the following:

- Tech Tip #14: Perform labeling and other reactions in Slide-A-Lyzer Dialysis Cassettes
- Tech Tip #43: Protein stability and storage

Related Thermo Scientific Products

21925 **EZ-Link Micro Sulfo-NHS-Biotinylation Kit**

21326 **EZ-Link Sulfo-NHS-Biotin, No-Weigh Format,** 8 × 1mg microtubes

21935 **EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit**

21327 **EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format,** 8 × 1mg microtubes

21445 **EZ-Link Sulfo-NHS-SS-Biotinylation Kit**

21328 **EZ-Link Sulfo-NHS-SS-Biotin, No-Weigh Format,** 8 × 1mg microtubes

21945 **EZ-Link Micro Sulfo-NHS-SS-Biotinylation Kit**

21329 **EZ-Link NHS-PEG$_4$-Biotin, No-Weigh Format,** 8 × 2mg microtubes

20347 **Pierce™ Streptavidin Agarose Resin,** 2mL

General References

Ali, M.K. and Bergson, C. (2003). Elevated intracellular calcium triggers recruitment of the receptor cross-talk accessory protein calcyon to the plasma membrane. *J Biol Chem* **278(51):**51654-63.

Boroto, A., *et al.* (2003). Impaired trafficking and activation of tumor necrosis factor-alpha-converting enzyme in cell mutants defective in protein ectodomain shedding. *J Biol Chem* **278(28):**25933-9.

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.

Li, H. and Pajor, A.M. (2003). Mutagenesis of the *N*-glycosylation site of hNaSi-1 reduces transport activity. *Am J Physiol Cell Physiol* **285:**C1188-96.

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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