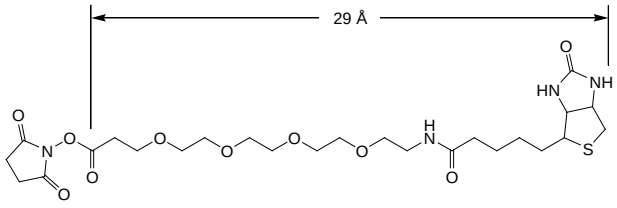
INSTRUCTIONS

EZ-Link NHS-PEG$_4$-Biotin

MAN0016360
Rev. A.0
Pub. Part No. 2161299.9

Number	Description
21329	**EZ-Link NHS-PEG$_4$-Biotin, No-Weigh Format,** 8 × 2mg microtubes
21330	**EZ-Link NHS-PEG$_4$-Biotin,** 25mg
21362	**EZ-Link NHS-PEG$_4$-Biotin,** 50mg
21363	**EZ-Link NHS-PEG$_4$-Biotin,** 1g



Molecular Weight: 588.67
Spacer Arm: 29Å
Net Mass Addition: 473.22
Solubility: 10mg/mL in water
Storage: Upon receipt store at -20°C protected from moisture. Product is shipped with an ice pack.

Introduction

The Thermo Scientific™ EZ-Link™ NHS-PEG$_4$-Biotin reagent enables simple and efficient biotin labeling of antibodies, proteins and any other primary amine-containing macromolecule. The hydrophilic polyethylene glycol (PEG) spacer arm imparts water solubility that is transferred to the biotinylated molecule. Consequently, antibodies that have been 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 Thermo Scientific™ No-Weigh™ Format of NHS-PEG$_4$-Biotin consists of 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 and detected using avidin, streptavidin or Thermo Scientific™ NeutrAvidin™ Products.

N-Hydroxysuccinimide (NHS) esters are the most commonly used biotinylation reagents. In pH 7-9 buffers, NHS-biotin reagents react efficiently with primary amino groups (-NH_2) by nucleophilic attack, forming an amide bond and releasing the NHS. 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.

Cell surface biotinylation has emerged as an important tool for studying the expression and regulation of receptors and transporters, differentiation of plasma membrane proteins from those localized to organelle membranes, and distribution of membrane proteins in polarized epithelial cells. Because NHS-PEG$_4$-Biotin dissolves readily in polar solutions, it does not permeate the cell membrane. As long as the cell remains intact, only primary amines exposed on the surface will be biotinylated with NHS-PEG$_4$-Biotin.

Pierce Biotechnology
3747 N. Meridian Road
PO Box 117
Rockford, IL 61105 USA
(815) 968-0747
(815) 968-7316 fax
Thermofisher.com

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. To avoid moisture condensation onto the product, vial must be equilibrated to room temperature before opening.
- No-Weigh Microtube Handling: 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 NHS-ester reaction.

Example Procedure for IgG Biotinylation using NHS-PEG_4-Biotin

The following protocol is an example application for this product. Specific applications require optimization. This protocol typically results in ~3-5 biotin molecules per molecule of IgG. Adjust the molar ratio of NHS-PEG_4-Biotin to protein to optimize for the level of labeling desired.

A. Additional Materials Required

- Reaction Buffer: Phosphate Buffered Saline (PBS; 0.1M phosphate, 0.15M sodium chloride; pH 7.2, Product No. 28372), or other non-amine containing buffer at pH 7.0-8.5
- Method for removing nonreacted biotin reagent (buffer exchange): Thermo Scientific™ Slide-A-Lyzer™ MINI Dialysis Units for 10-100µL sample volumes (Product No. 69576); Slide-A-Lyzer Dialysis Cassette Kit for 0.5-3.0mL sample volumes (Product No. 66382); or Thermo Scientific™ Zeba™ Spin Desalting Columns (Product No. 89889-89894)
- Optional: Thermo Scientific™ Pierce™ Biotin Quantitation Kit (Product No. 28005) or Fluorescence Biotin Quantitation Kit (Product No. 46610) for determining biotin incorporation

B. Calculations

The amount of biotin reagent to use for each reaction depends on the amount and concentration of the protein to be labeled. To control the extent of labeling, adjust the molar ratio of biotin to protein. For dilute protein solutions (e.g., 2mg/mL) use a greater molar-fold excess of biotin compared to a concentrated protein solution (e.g., 10mg/mL). For example, use ≥ 20-fold molar excess for IgG at 2mg/mL and ≥ 12-fold molar excess for IgG at 10mg/mL.

1. Calculate millimoles of biotin reagent 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 = Molar fold excess of biotin

2. Calculate microliters of 20mM biotin reagent solution (prepared in Step B.3) to add to the reaction:

$$\text{mmol Biotin} \times \frac{1{,}000{,}000\ \mu\text{l}}{\text{L}} \times \frac{\text{L}}{20\ \text{mmol}} = \mu\text{L Biotin}$$

Example: For 1mL of 2mg/mL IgG (150,000 MW), ~13 µL of 20mM biotin reagent will be added.

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

$$0.000266\ \text{mmol Biotin} \times \frac{1{,}000{,}000\ \mu\text{l}}{\text{L}} \times \frac{\text{L}}{20\text{mmol}} = 13.3\ \mu\text{L Biotin Reagent}$$

C. Reaction

For reaction volumes from 10 to 100µL, the buffer exchange and biotinylation can be conveniently performed in a single Slide-A-Lyzer MINI Dialysis Unit. For reaction volumes from 0.1 to 12mL, Slide-A-Lyzer Dialysis Cassettes can be used.

1. Dissolve or buffer exchange the IgG into Reaction Buffer.
2. Immediately before use, add 170µL of ultrapure water to 2mg of NHS-PEG_4-Biotin to prepare a 20 mM stock solution.

   Note: If using the No-Weigh NHS-PEG_4-Biotin, puncture foil with a pipette tip, add 170µL of water and mix by pipetting up and down. After use, cut the used microtubes from the unused microtubes and discard.

   Note: It is possible to make a stable concentrated (e.g., 100-200mM) stock solution of NHS-PEG_4-Biotin by dissolving the reagent in pure, moisture-free ("dry") DMSO (Product No. 20688) or DMF (Product No. 20673). With proper handling (i.e., complete exclusion of moisture), the stock may be stable for several months at -20°C.
3. Add the appropriate volume of the NHS-PEG_4-Biotin solution (see Calculations section) to the IgG solution.
4. Incubate reaction on ice for two hours or at room temperature for 30 minutes.
5. Remove nonreacted NHS-PEG_4-Biotin by dialysis or gel filtration. See instructions provided with the buffer exchange product.
6. Store the biotinylated protein using the same condition that is optimal for the non-biotinylated protein.

D. Determination of Biotin Incorporation (optional)

Biotin incorporation can be estimated using the HABA (4´-hydroxyazobenzene-2-carboxylic acid] method (e.g., Pierce Biotin Quantitation Kit, Product No. 28005). This method is based on the ability of the HABA dye to bind avidin forming a complex with maximal absorption at 500 nm. Biotin is then added to the solution and because of its higher affinity for avidin, biotin displaces the HABA and the absorption at 500 nm decreases proportionately. The absorbance of the HABA-avidin solution is measured before and after adding the biotin-containing sample. The change in absorbance relates to the amount of biotin in the sample.

Procedure for Biotinylating Cell Surface Proteins

Many variations of this procedure exist in the literature (see Product References). Labeling may be performed on cells in suspension or on adherent cells in culture plates. In the latter situation, diffusion of the NHS-PEG_4-Biotin to all surfaces of the cells will be limited, and labeling will occur predominately on the exposed surface. Culture media must be washed from cells; otherwise, amine-containing components will compete and quench the reaction to cell surface proteins. Using a more concentrated cell suspension is most effective because less biotin reagent is required in the reaction. Generally, a final concentration of 2-5mM biotin reagent is effective. NHS reactions occur more rapidly at high pH; therefore, pH 8.0 is used in the following example so that labeling can be completed as quickly as possible.

1. Wash cells three times with ice-cold PBS (pH 8.0) to remove amine-containing media and proteins from the cells.
2. Suspend cells at a concentration of ~25×10^6 cells/mL in PBS (pH 8.0).
3. Immediately before use, add 170µL of ultrapure water to 2mg of NHS-PEG_4-Biotin to make a 20mM stock solution.
4. Add ~100µL of the NHS-PEG_4-Biotin solution per milliliter of reaction volume (results in 2mM biotin reagent).
5. Incubate reaction mixture at room temperature for 30 minutes.

   Note: Performing this incubation at 4°C may reduce active internalization of the biotin reagent.
6. Wash cells three times with PBS + 100mM glycine to quench and remove excess biotin reagent and byproducts.

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 contains primary amines	Use a non-amine containing buffer
		Extensively dialyze or desalt sample into a buffer free of primary amines
	Reagent is non-reactive caused by hydrolysis of the NHS ester	Use reagent immediately upon reconstitution
	Moisture condensation on the product vial has caused hydrolysis of the NHS ester	Purchase new reagent and always allow it to equilibrate to room temperature before opening
Biotinylated protein does not function in downstream application	Excessive biotinylation	Reduce molar excess of biotinylation reagent, or reduce time or temperature for biotinylation
		Choose biotinylation reagent that targets different groups

General References

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Gretch, D.R., Suter, M. and Stinski, M.F. (1987). The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate herpes virus hydrophobic proteins or glycoproteins. *Anal. Biochem*. **163:**270-7.

Manning, J., *et al.* (1977). A method for gene enrichment based on the avidin-biotin interaction. Application to the Drosophila ribosomal RNA genes. *Biochemistry* **16:**1364-70.

Updyke, T.V. and Nicolson, G.L. (1984). Immunoaffinity isolation of membrane antigens with biotinylated monoclonal antibodies and immobilized streptavidin matrices. *J. Immunol. Meth.* **73:**83-95.

Product References

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Nielsen, P.A., *et al.* (2002). Molecular cloning, functional expression, and tissue distribution of a novel human gap junction-forming protein, connexin-31.9. *J. Biol. Chem.* **277(41):**38272-83.