INSTRUCTIONS

EZ-Link™ NHS-PEG Solid Phase Biotinylation Kit: *spin columns*

21450

1474.3

Number	Description
21450	**EZ-Link NHS-PEG Solid Phase Biotinylation Kit: *spin columns*,** contains sufficient material for eight biotinylation reactions each consisting of 100-1000 µg IgG

Kit Contents:

HisPur™ Ni-NTA Spin Columns, 0.2mL resin bed, 8 columns

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

Molecular Weight: 588.67

Spacer Arm Length: 29Å

BupH™ Phosphate Buffered Saline Pack, 1 each

4 M Imidazole Stock Solution, 5mL

Pierce™ Microcentrifuge Tubes – 2mL, 30 each

Storage: Upon receipt store at 4°C. Product is shipped at ambient temperature.

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Introduction

The Thermo Scientific EZ-Link NHS-PEG Solid Phase Biotinylation Kit allows for efficient biotinylation of purified IgG-class antibodies. This solid-phase biotinylation method uses Ni-NTA agarose resin to first immobilize purified IgG. The antibody is then biotinylated by adding a solution of NHS-PEG$_4$-Biotin. Excess biotin is washed from the column, and the antibody is eluted in a buffered imidazole solution. The reaction results in approximately 3-5 biotin molecules per antibody molecule. Although this solid-phase format has been optimized using human IgG, it may be used with other mammalian antibodies. The nickel-chelated agarose binds IgG through a histidine-rich cluster on the Fc region at the junctures of the Cγ2 and Cγ3 domains that is highly conserved across all mammalian IgGs.[1-4] Purified IgG from sheep, mouse, goat, rat and rabbit will bind to nickel-chelated resin.

This kit includes Thermo Scientific No-Weigh NHS-PEG_4-Biotin packaged in convenient pre-measured microtubes, eliminating difficulties associated with weighing small quantities of reagent. NHS-PEG_4-Biotin (see Figure 1 in Appendix A for molecular structure) reacts with primary amines, primarily ε-amine groups on available lysine residues. The N-hydroxysuccinimide (NHS) ester reacts with amines by nucleophilic attack, forming an amide bond and releasing the NHS. The resulting biotinylated antibody retains biological activity because biotin is a relatively small molecule. An antibody conjugated with several biotin molecules can each bind one molecule of avidin, 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.[5] The hydrophilic polyethylene glycol (PEG) spacer arm of NHS-PEG_4-Biotin imparts water solubility that is transferred to the biotinylated antibody, thus NHS-PEG_4-Biotin reduces aggregation of labeled antibodies stored in solution.[6]

This solid-phase method is advantageous compared with solution-phase protocols as it facilitates reagent delivery and removal of spent product and there is more control over reaction conditions Although the time required for protocol completion is comparable to solution-phase protocols, antibody immobilization eliminates the need for desalting or dialysis to remove excess biotin, resulting in excellent antibody recovery.

Important Product Information

- Use this kit only with purified IgG. Antibodies in serum or ascites must be purified before using this kit. Do not use this kit for IgM or IgY, Fab, or antibody fragments that do not contain a Fc region, as they do not bind efficiently to the nickel-chelated agarose.
- This protocol has been optimized for 0.1-1mg of antibody. The antibody preparation must be free of chelating agents such as EDTA and EGTA.
- Bovine serum albumin (BSA) is often added to commercial antibody preparations as a stabilizer and is present in molar excess to the antibody. BSA will decrease specific biotinylation because it contains available histidine residues and binds to the nickel-chelated agarose and is then biotinylated and eluted along with the antibody. Remove BSA before using this kit. BSA removal is a fast and simple process; see Appendix B for suggested albumin removal products.

  Note: Although gelatin, which often is also added to antibody preparations, will bind to the nickel-chelated agarose, it is present in low amounts (usually ~0.2%) and will not significantly affect yields.
- Use reconstituted No-Weigh NHS-PEG_4-Biotin immediately. The NHS-ester moiety readily hydrolyzes and becomes nonreactive; therefore, solutions cannot be prepared for storage. Discard any unused reconstituted reagent.
- The degree of biotinylation can be determined by performing the HABA assay (Product No. 28005); however, 0.2M imidazole (Elution Buffer) interferes with the HABA assay. Dilute on-column biotinylated IgG 1:1 with phosphate-buffered saline (PBS) before use in the HABA assay to reduce imidazole concentration to 0.1M.
- Protein assays can be used to determine concentration of eluted IgG. When determining concentration of IgG in Elution Buffer, use Thermo Scientific Coomassie Plus (Bradford) Protein Assay Reagent (Product No. 23236). The Thermo Scientific BCA Protein Assay cannot be used because imidazole interferes with the assay chemistry.

Additional Materials Required

- 0.2µm, 500mL filter sterilization unit
- Test tubes and test tube rack
- Rotating platform or microcentrifuge tube nutator

Material Preparation

PBS	Reconstitute contents of the Thermo Scientific BupH Phosphate Buffered Saline (PBS) pack with 500mL of ultrapure water. Filter-sterilize solution using a 0.2µm filter apparatus and store at 4°C. When stored properly, there is sufficient buffer for eight antibody biotinylation reactions of up to 1mg IgG each.
Elution Buffer	Prepare 1mL of Elution Buffer by diluting 50µL of the 4M Imidazole Stock Solution with 950µL of PBS.
Antibody Binding Solution	Adjust volume of purified IgG (0.1-1mg) with PBS to 500µL to 1mL. The volume of the Antibody Binding Solution will depend on the antibody concentration. To ensure proper mixing of the resin during binding, the volume must be at least 500µL. Use the lowest possible volume (500µL) to maximize antibody binding. Volumes greater than 1mL can be used, but decreased binding efficiency will result.

Procedure for Solid-Phase Biotinylation

A. Equilibration of Thermo Scientific HisPur Ni-NTA Resin

1. Remove the bottom tab from the HisPur Ni-NTA Spin Column by gently twisting. Place column into a centrifuge tube.
2. Note: Use 2mL centrifuge tubes for the 0.2µL spin column.
3. Centrifuge column at 700 × g for 2 minutes to remove storage buffer. Discard the flow-through.
4. Equilibrate column with two resin-bed volumes of PBS. Allow buffer to enter the resin bed.
5. Centrifuge column at 700 × g for 2 minutes to remove buffer. Discard the flow-through.
6. Place the bottom plug in the column and proceed immediately to Step B1.

B. Antibody Binding

The antibody must be purified. If BSA is present in the antibody preparation, remove it before using this kit. See Appendix B for a list of suggested purification products.

1. Add the prepared Antibody Binding Solution to the HisPur Ni-NTA Spin Column. Insure the bottom plug and cap are securely fastened.
2. Invert tube several times to suspend the resin. Incubate 10 minutes at room temperature with gentle rocking motion on a rotating platform. DO NOT VORTEX.

   Note: The resin must remain suspended during binding. If necessary, invert the tube manually every 2-3 minutes to keep the resin in suspension.
3. Remove the bottom plug. Centrifuge the column in a centrifuge tube at 700 × *g* for 2 minutes and discard the flow-through.
4. Add 0.5 mL of PBS to the tube. Invert tube several times to wash the resin.
5. Centrifuge at 700 × *g* for 2 minutes and discard the flow-through.
6. Repeat Steps 4-5 three additional times to complete washing and proceed immediately to Step C1.

C. Antibody Biotinylation

1. Apply bottom plug to column.
2. Puncture seal of one No-Weigh NHS-PEG_4-Biotin Microtube with a pipette tip and dissolve tube contents by adding 200µL of PBS. Gently pipette up and down.
3. Add 190µL of PBS to the column.
4. Add 10µL of NHS-PEG_4-Biotin to the column.
5. Cap top of column with a screw cap. Mix by gentle flicking.

6. Incubate 30 minutes at room temperature.

   Note: Flick the column occasionally during incubation to keep the resin from settling. DO NOT VORTEX.

7. Remove the bottom plug. Centrifuge the column at 700 × *g* for 2 minutes and discard the flow-through.
8. Add 400µL of PBS to the column. Centrifuge the column at 700 × *g* for 2 minutes and discard the flow-through.
9. Repeat Step 7 three additional times to wash the column.

D. Antibody Elution

1. Cap bottom of column. Place column in a new 2mL microcentrifuge tube.
2. Add 200µL of Elution Buffer to the column and incubate for 10 minutes at room temperature.
3. Elute antibody from the resin by centrifugation at 700 × *g* for 2 minutes.

   Note: After elution, some antibody will remain bound to the column. To increase the yield of biotinylated antibody, repeat Steps 2-3, collecting each fraction in a separate tube. To increase concentration of smaller amounts of antibody (i.e., 0.1-0.25mg), re-apply eluted antibody solution to the column and repeat Step 3. Discard resin after use.

4. Store biotinylated antibody at 4°C for up to one month.

   Note: Biotinylated antibodies are generally stable when stored in Elution Buffer (0.2M Imidazole in PBS) at 4°C; however, stability will depend on the specific antibody being used. If biotinylated antibodies are not to be used within one month, store them in single-use volumes at -20°C.

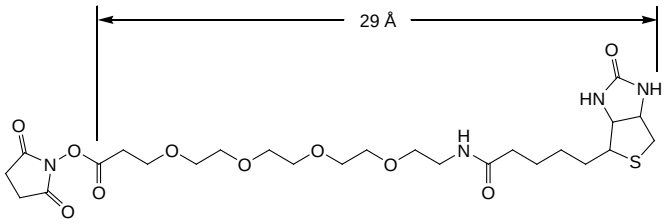
Troubleshooting

Problem	Cause	Solution
Antibody does not bind to column	BSA was present in antibody preparation	Remove BSA before using this kit
	Fab fragments, IgM or IgY were used	Do not use antibodies without an Fc region, or IgM or IgY with this kit
Antibody is not biotinylated	NHS-PEG_4-Biotin hydrolyzed before use	Reconstitute NHS-PEG_4-Biotin immediately before use and always use a new tube of biotinylation reagent for each reaction

Appendix

A. Structure of NHS-PEG_4-Biotin

The NHS ester of NHS-PEG_4-Biotin (Figure) reacts with amines by nucleophilic attack forming an amide bond. The hydrophilic polyethylene oxide (PEG) spacer arm (29Å) imparts water solubility that is transferred to the biotinylated antibody, thus NHS-PEG_4-Biotin reduces aggregation of labeled antibodies stored in solution.



B. Bovine Serum Albumin (BSA) Removal

Two methods exist for removing BSA and/or gelatin from antibody preparations. The first is to affinity purify the antibody using immobilized Proteins A, G or L. Antibody will bind to the immobilized protein, allowing BSA to be removed by washing. The antibody is eluted and the solution is adjusted to a neutral pH (according to the protocol). Dilute the eluted antibody 1:1 with PBS before adding to the HisPur Ni-NTA Spin Column. For more information about Protein A, G, and L binding characteristics, see our catalog or Tech Tip #34 from the website.

The second method is to use Thermo Scientific Melon Gel Resin (e.g., Product No. 45206), which will bind to the BSA and gelatin and allow the purified antibody to be recovered in the flow-through. For more information about Melon™ Gel Products and this method of removal, see Tech Tip #55 from the website.

Related Thermo Scientific Products

28005	**Pierce Biotin Quantitation Kit**
23236	**Coomassie Plus™ (Bradford) Protein Assay Kit**
69715	**Pierce Microcentrifuge Columns**
21126	**Streptavidin, Horseradish Peroxidase Conjugated**
21324	**Streptavidin, Alkaline Phosphatase Conjugated**
15120	**Streptavidin Coated Plates**

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