INSTRUCTIONS

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

21930 1476.2

Number	Description
21930	**EZ-Link Maleimide-PEG Solid Phase Biotinylation Kit: *spin columns*,** contains sufficient material for eight biotinylation reactions each consisting of 0.1-1mg of IgG
	Kit Contents:
	HisPur Ni-NTA Spin Columns, 0.2mL, 8 each
	No-Weigh™ Maleimide-PEG$_2$-Biotin, 8 × 2mg microtubes
	Molecular Weight: 525.63
	Spacer Arm Length: 29.1Å
	BupH™ Tris Buffered Saline Pack, 1 pack
	Bond-Breaker™ TCEP Solution, Neutral pH (0.5M)**,** 5mL
	4M Imidazole Stock Solution, 5mL
	Pierce™ Microcentrifuge Tubes – 2mL, 30 each
	Storage: Upon receipt store at 4°C. Product shipped at ambient temperature.

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Introduction

The Thermo Scientific EZ-Link Maleimide-PEG Solid Phase Biotinylation Kit allows for efficient biotinylation of IgG-class antibodies. This method uses nickel-chelated agarose to first immobilize purified IgG. The antibody-disulfide bonds are then reduced by adding a solution of trialkylphosphine Tris(2-carboxyethyl) phosphine (TCEP). Excess reducing reagent is washed from the column, and the reduced sulfhydryl groups are biotinylated with Maleimide-PEG$_2$-Biotin. After removal of excess biotin, the antibody is eluted in a buffered imidazole solution. The reaction results in approximately two to four 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 solid-phase biotinylation method uses high-quality, easy-to-use reagents. Thermo Scientific Bond-Breaker TCEP is an odorless, neutral pH solution that retains room temperature stability for 12 months and is more effective for reduction of

antibody disulfide bonds than DTT.[5] TCEP is also compatible with immobilized metal affinity chromatography (IMAC), making it ideal for use with this method. The Thermo Scientific No-Weigh Maleimide-PEG_2-Biotin (Figure 1), which reacts with free sulfhydryls, is packaged in convenient pre-measured microtubes, eliminating difficulties associated with weighing small quantities of reagent. Each biotin molecule conjugated to the antibody can 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.[6] The polyethylene glycol (PEG_2) spacer arm has a hydrophilic property that is transferred to the final biotin conjugate, which reduces aggregation of labeled antibodies stored in solution.[7]

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. Less time is required for protocol completion, and antibody immobilization eliminates the need for desalting or dialysis to remove excess biotin, resulting in excellent antibody recovery.

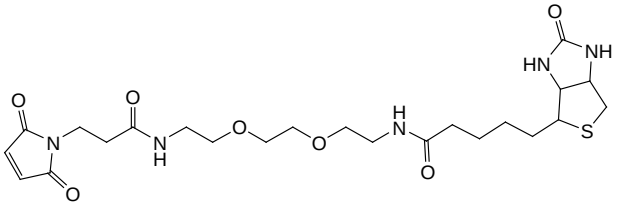

Figure 1. **Molecular structure of Maleimide-PEG_2-Biotin.**

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 A 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.
- Prepare No-Weigh Maleimide-PEG_2-Biotin immediately before use. When in solution, the maleimide moiety may hydrolyze and become non-reactive; therefore, stock 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 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
- Rotating platform or microcentrifuge tube nutator

Material Preparation

Tris Buffered Saline (TBS)	Reconstitute contents of the Thermo Scientific BupH Tris Buffered Saline (TBS) 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 using up to 10mg IgG for each reaction.
Elution Buffer	Prepare 6mL of Elution Buffer by diluting 300µL of the 4M Imidazole Stock Solution with 5.7mL of TBS.
Antibody Binding Solution	Dilute purified IgG (0.1-1mg) with TBS to 500µL to 1mL. The volume of the Antibody Binding Solution to use 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 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.

   Note: Use 2mL centrifuge tubes for the 0.2mL spin columns.
2. Centrifuge column at 700 × *g* for 2 minutes to remove storage buffer. Discard the flow-through.
3. Equilibrate column with two resin-bed volumes of PBS. Allow buffer to enter the resin bed.
4. Centrifuge column at 700 × *g* for 2 minutes to remove buffer. Discard the flow-through.
5. 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 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 top 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, manually invert the tube 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 0.5mL of TBS to the tube. Invert tube several times to wash the resin.
5. Centrifuge at 700 × *g* for 2 minutes and collect fraction in a centrifuge tube.
6. Repeat Steps 4-5 three additional times to complete washing and proceed immediately to Step C1.

C. Antibody Reduction

Note: Biotinylation protocols vary in amount of diluted TCEP added to the column.

1. Apply bottom plug to column.
2. Dilute TCEP by adding 2µL of 0.5M TCEP to 200µL of TBS.
3. Add the appropriate amount of TBS and diluted TCEP for the amount of antibody being biotinylated (as indicated in Table 1) directly to the column. Add the TBS first and then add the diluted TCEP.

Table 1. Amount of diluted TCEP to add to the bound antibody.

Antibody Amount (mg)	TBS Volume (µL)	Diluted TCEP Volume (µL)	TCEP Final Molarity (mM)
0.1	192	8	0.2
0.11-0.3	190	10	0.4
0.31-0.44	175	25	0.6
0.45-0.7	160	40	1
0.71-0.84	120	80	2
0.85-1	80	120	3

4. Cap the column top with a screw cap and mix by gently flicking the column.
5. Incubate for 30 minutes at room temperature.

   Note: Flick the column occasionally during incubation to keep the resin from settling. DO NOT VORTEX.
6. Remove bottom cap from column and centrifuge at 500 × *g* for 30 seconds. Discard flow-through and place column back into the same tube.
7. Add 400µL of TBS to the column. Centrifuge at 500 × *g* for 30 seconds. Discard flow-through and place column back into the same tube.
8. Repeat Step 7 four additional times to wash the column.

D. Antibody Biotinylation

1. Apply bottom plug to column.
2. Add 190µL of TBS to the column.
3. Puncture the seal of one No-Weigh Maleimide-PEG_2-Biotin Microtube with a pipette tip and dissolve tube contents by adding 200µL of TBS. Gently pipette up and down.
4. Add 10µL of biotinylation reagent 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 TBS to the column. Centrifuge the column at 700 × *g* for 2 minutes and discard the flow-through.
9. Repeat Step 8 four additional times to wash the column.

E. Antibody Elution

1. Apply bottom plug to the column. Place column in a new 2mL 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 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 TBS) 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	Biotinylation reagent hydrolyzed before use	Reconstitute Maleimide-PEG_2-Biotin immediately before use and always use a new tube of biotinylation reagent for each reaction

Appendix

A. 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.

B. Determination of Biotin Incorporation

Biotin incorporation can be estimated using the HABA (4´-hydroxyazobenzene-2-carboxylic acid) method. In solution, the HABA dye binds avidin, forming a complex with maximal absorption at 500nm. When biotin is added to the solution, its higher affinity for avidin displaces the HABA and the absorption at 500nm 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. The Thermo Scientific Pierce Biotin Quantitation Kit (Product No. 28005) contains a premix of HABA and avidin and a biotinylated protein control supplied in convenient No-Weigh Microtube packaging, which eliminates the difficulties associated with weighing small quantities of reagent.

Related Thermo Scientific Products

28005	**Pierce Biotin Quantitation Kit**
23236	**Coomassie Plus (Bradford) Protein Assay Kit**
69715	**Pierce Microcentrifuge Columns, 2mL**
21126	**Streptavidin, Horseradish Peroxidase Conjugated**
21324	**Streptavidin, Alkaline Phosphatase Conjugated**
15120	**Pierce Streptavidin Coated Plates**

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