INSTRUCTIONS

EZ-Link® Maleimide-PEG Solid Phase Biotinylation Kit: *pre-packed column*

21920

1475.1

Number	Description
21920	**EZ-Link® Maleimide-PEG Solid Phase Biotinylation Kit: *pre-packed column*,** contains sufficient material for eight biotinylation reactions each consisting of 1-10 mg of IgG

Kit Contents:

Immobilized Nickel Chelated Column, 1 ml

Binding capacity: >10 mg human IgG

Maleimide-PEG$_2$-Biotin No-Weigh™ Microtubes, 8 x 2 mg

Molecular Weight: 525.63

Spacer Arm Length: ~29.1Å

BupH™ Tris Buffered Saline Pack, 1 pack

Bond-Breaker® TCEP Solution, Neutral pH (0.5 M)**,** 5 ml

4 M Imidazole Stock Solution, 5 ml

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

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Introduction

The 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. 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. 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 two-unit 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.

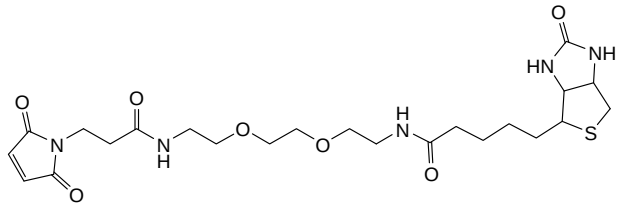

Figure 1. Molecular structure of Maleimide PEG_2-Biotin.

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 1-10 mg of antibody.
- 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 (dissolve) one microtube of 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, 0.2 M 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 0.1 M.
- Protein assays can be used to determine concentration of eluted IgG. When determining concentration of IgG in Elution Buffer, use Coomassie Plus Protein Assay Reagent (Product No. 23236). The BCA Protein Assay cannot be used because imidazole interferes with the assay chemistry.
- When properly washed and stored, the nickel-chelated column can be re-used up to 10 times without significant loss of binding capacity.

Additional Materials Required

- 0.2 µm, 500 ml filter sterilization unit
- Test tubes and test tube rack

Material Preparation

Tris Buffered Saline (TBS)	Reconstitute contents of the BupH Tris Buffered Saline (TBS) pack with 500 ml ultrapure water. Filter-sterilize solution using a 0.2 µm filter apparatus and store at 4°C. When stored properly, there is sufficient for eight antibody biotinylation reactions using up to 10 mg IgG for each reaction.
Elution Buffer	Prepare 6 ml of Elution Buffer by diluting 300 µl of the 4 M Imidazole Stock Solution with 5.7 ml TBS.
Antibody Binding Solution	Dilute purified IgG to be biotinylated (1-10 mg) with TBS to a volume of 6 ml. If the antibody concentration is too dilute to make a 6 ml binding solution, dilute the antibody 1:1 with TBS and load the entire volume of Antibody Binding Solution on the column. Any volume may be applied provided the total amount of IgG is less than or equal to 10 mg.

Procedure for Solid-phase Biotinylation

A. Antibody Binding

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

1. Equilibrate Nickel-Chelated Column and TBS buffer to room temperature before use.
2. To prevent air bubbles from being drawn into the gel, open a Nickel-Chelated Column first by removing the top cap and pouring off the storage solution (contains 0.01% sodium azide). Next, remove the bottom cap from the column. Place the column in a test tube.
3. Equilibrate the column by adding 15 ml TBS and allowing the solution to drain through the gel bed.
4. Apply the Antibody Binding Solution to the column and allow it to flow completely into the gel. The column will stop flowing when the liquid level reaches the top disc.
5. Wash the column with 12 ml TBS.

B. Antibody Reduction

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

1. Add 6 ml of TBS to a new test tube and add TCEP according to the amount of antibody being biotinylated as indicated in Table 1. Add TBS first, then TCEP.

Table 1. TCEP solution preparation.

Antibody Amount (mg)	TCEP Volume (µl)	TCEP Final Molarity (mM)
1	3	0.25
2	5.5	0.45
3	8.4	0.7
4	11	0.9
5	13	1.1
6	16	1.3
7	19	1.6
8	22	1.8
9	24	2
10	27	2.2

2. Apply the TCEP solution to the column.
3. After the solution has flowed completely through the gel bed, cap the bottom and top of the column.
4. Incubate 30 minutes at room temperature.
5. After incubation, remove the top and bottom caps from the column.
6. Wash the column with 15 ml TBS.

C. Antibody Biotinylation

1. Add 2.5 ml TBS to a new test tube.
2. Puncture the seal of one Maleimide-PEG_2-Biotin microtube with a pipette tip and dissolve tube contents in 200 µl of TBS. Solubilize contents by gently pipetting up and down.
3. Add the 200 µl biotinylation reagent to the test tube containing 2.5 ml TBS. Mix well and apply the biotin solution to the column.
4. After the solution has flowed completely into the gel bed, cap the bottom and top of the column.
5. Incubate 30 minutes at room temperature.
6. After incubation, remove the top and bottom caps from the column.
7. Wash the column with 15 ml TBS.

D. Antibody Elution

1. Place column in a new test tube.
2. Add 3 ml Elution Buffer to the column. Collect biotinylated antibody and store at 4°C for up to one month.

   Note: Biotinylated antibodies are generally stable when stored in Elution Buffer (0.2 M 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 aliquots at -20°C.
3. Regenerate the column by adding 3 ml Elution Buffer.
4. For storage, wash the column with 15 ml water containing 0.02% sodium azide. When approximately 3 ml of solution remains above the top disc, replace the bottom cap followed by the top cap on the column and store upright at 4°C. When properly washed and stored, the column can be re-used up to 10 times without significant loss of binding capacity.

Troubleshooting

Problem	Cause	Solution
Antibody does not bind to column	Fab fragments, IgM or IgY were used	Do not use antibodies without an Fc region, or IgM or IgY with this kit
	BSA is present in antibody preparation	Remove BSA before using kit
Antibody is not biotinylated	Maleimide-PEG_2-Biotin 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

The products listed below can be used for BSA removal from antibody preparations. The Pierce Antibody Clean-Up Kit uses Melon Gel Resin to bind the BSA, allowing the purified antibody to be collected in the flow-through. Choice of immobilized Proteins A, G or L is dependent upon antibody species being treated (see Appendix B for Tech Tip). 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 nickel-chelated agarose.

44600 **Pierce® Antibody Clean-Up Kit**

89948, 89878 **NAb™ Protein A Plus Spin Kits** (0.2mL or 1mL columns, respectively)

89949, 89979 **NAb Protein G Spin Kit,** (0.2mL or 1mL columns, respectively)

89951, 89981 **NAb Protein L Spin Kit,** (0.2mL or 1mL columns, respectively)

B. Additional Information

Please visit the web site for additional information on this product including the following items:

- Tech Tip #34: Binding characteristics for Protein A, Protein G, Protein A/G and Protein L
- Tech Tip #43: Protein stability and storage

C. Determination of Biotin Incorporation

Biotin incorporation can be estimated using the HABA [2-(4´-hydroxyazobenzene)-benzoic acid] method. In solution, the HABA dye binds avidin, forming a complex with maximal absorption at 500 nm. When biotin is added to the solution, its higher affinity for avidin 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. The Biotin Quantitation Kit (Product No. 28005) contains a premix of HABA and avidin and a biotinylated protein control.

Related Thermo Scientific Products

28010	**HABA**
21121	**Avidin**
23236	**Pierce Coomassie Plus Protein Assay Kit**

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