INSTRUCTIONS

EZ-Link™ Desthiobiotinylation and Pull-Down Kit

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16138

Number	Description
16138	**EZ-Link Desthiobiotinylation and Pull-Down Kit**, sufficient desthiobiotin reagent and desalting columns for five labeling and clean-up reactions. Each reaction containing 0.2-5mg of protein in 500-1,500µL reaction volumes. Sufficient High Capacity Streptavidin Agarose Resin and buffers for 40 pull-down reactions (50µL each) **Kit Contents:** **EZ-Link Sulfo-NHS-LC-Desthiobiotin, No-Weigh Format,** 2 × 1mg vials Molecular Weight: 526.54 Spacer Arm: 17.3Å Solubility: Soluble in DMSO, DMF and aqueous buffers **Zeba Spin Desalting Column**, **7K MWCO, 5mL**: 5 columns, for 500-1,500µL samples **High Capacity Streptavidin Agarose Resin,** 2mL of settled resin. Supplied: 50% aqueous slurry containing 0.02% sodium azide as a preservative **BupH Phosphate Buffered Saline (PBS) Pack,** 1 pack, results in 0.1M sodium phosphate, 0.15M NaCl, pH 7.2 when reconstituted with 500mL of ultrapure water **Biotin Elution Buffer**, 8mL (4mM biotin, 20mM Tris, 50mM NaCl) **Storage:** Upon receipt, store vials of EZ-Link Sulfo-NHS-LC-Desthiobiotin at -20°C. Store PBS solution at room temperature or 4°C once reconstituted. Store the remaining kit components at 4°C. Kit is shipped at ambient temperature.

Introduction

The Thermo Scientific™ EZ-Link™ Desthiobiotinylation and Pull-Down Kit makes it possible to perform an efficient pull-down assay with high protein recovery and reproducibility under mild conditions. This kit uses EZ-Link Sulfo-NHS-LC-Desthiobiotin to label target proteins on primary amines, Thermo Scientific™ Zeba™ Spin Desalting Columns to clean up the labeling reaction, and immobilized High Capacity Streptavidin Agarose Resin and buffers to perform pull-down assays. Pull-down assays use the tagged or labeled bait protein coupled to a resin to capture a prey protein contained in a cell lysate or other unpurified protein mixtures. This technique can identify novel interactions between a known protein (bait) and previously undiscovered target (prey), or it can confirm the interaction between a known (bait) and a known target (prey) protein.

Desthiobiotin is a biotin analogue that binds to streptavidin with less affinity than biotin (K_d of 10^{-11}M versus a K_d of 10^{-16}M, respectively).[1-4] Unlike biotinylated proteins, desthiobiotinylated bait proteins and their interacting partners can be readily and specifically eluted under mild conditions when captured on streptavidin by using the provided Biotin Elution Buffer. The soft release characteristics of desthiobiotin minimize the isolation of naturally biotinylated molecules that can interfere with results and also eliminate the use of harsh elution conditions that can disassociate complexes and/or damage the target protein or cell. This technique is ideal when using native or recombinant proteins that are not expressed with a fusion tag and when isolating captured proteins under native conditions, such as targeting intact cells or cell surface proteins.

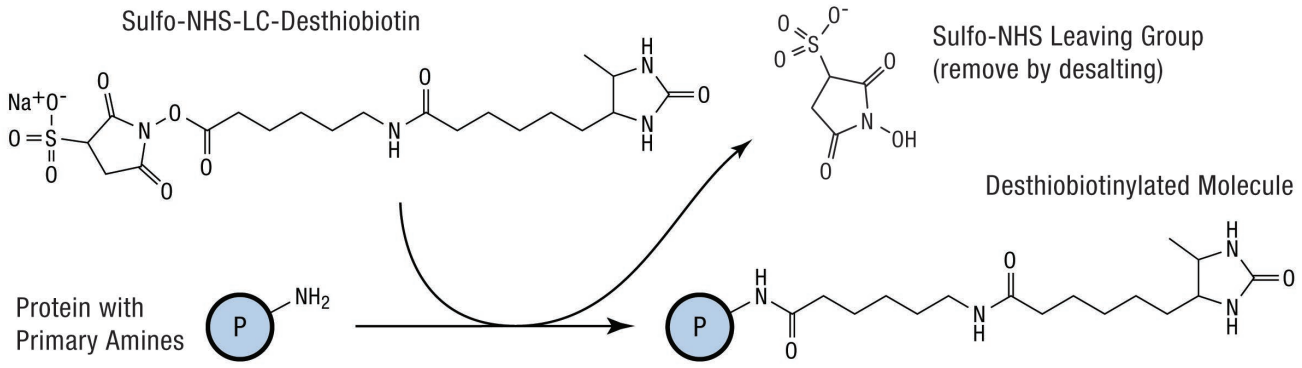

Figure 1. Reaction of Sulfo-NHS-LC-desthiobiotin with a primary amine. If drawn to scale, the circle representing the protein would be many times larger than the structures and would likely contain several amino groups. Note that Sulfo-NHS is a leaving group (byproduct) in the reaction. The leaving group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

Important Product Information

- This is a general use kit compatible with a variety of samples and applications. The kit provides a starting point and a recommended protocol; however, it can be optimized for any desired application and sample. Some optimization may be required in order to make this kit appropriate for your application. To aid in any optimization or customization, a majority of kit components or alternatives are available separately. Please see the Related Thermo Scientific Products Section for information.
- This kit and the desalting columns provided are designed for processing desthiobiotinylation reactions involving 0.2-5mg of protein in approximately 500-1500µL. Desalting is required to eliminate any hydrolyzed and unreacted desthiobiotinylation reagent that will interfere with downstream binding to streptavidin. Smaller or larger reaction volumes can be processed using a wide range of our desalting columns suited for your desired sample size. See our full line of Zeba Spin Desalting Columns for samples of 20µL to 4mL (Product No. 89877-89894). To ensure sufficient removal of free desthiobiotin label, use 30% less sample volume than the maximum recommended for any appropriate volume of desalting resin.
- Sulfo-*N*-Hydroxysuccinimide (Sulfo-NHS) esters are among the most popular targeting chemistry for a wide variety of labeling reagents. Sulfo-NHS-activated desthiobiotin reacts efficiently with primary amine groups ($-NH_2$) to form stable amide bonds. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide that are available as targets for labeling. It is essential that the molecules to be labeled are contained in a buffer that is free of primary amines (e.g., Tris-HCl) with a pH between 7-9. If necessary, dialyze or otherwise desalt the sample into an amine-free buffer such as phosphate buffered saline.
- The Sulfo-NHS-containing reagents are very moisture sensitive and readily hydrolyze to become inactive. To maximize reliability and ease of handling, the EZ-Link Sulfo-NHS-LC-Desthiobiotin is provided in a convenient No-Weigh™ screw-cap vial that is designed to be solubilized and used in the container provided. This eliminates difficulties associated with weighing small quantities of reagent and the risk of hydrolysis, which can occur because of exposure to water or humidity as a result of opening a bottle multiple times. For best results, dissolve the EZ-Link Sulfo-NHS-LC-Desthiobiotin in organic solvents such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF) just before use. This will reduce or eliminate any hydrolysis before addition to a labeling reaction. In addition, any unused solution will remain stable at -20°C for 2 months when stored with a desiccant in the supplied foil pouch. Do not use old organic solvents, as they may have become contaminated with trace amounts of water after long-term storage.

  Note: If required, EZ-Link Sulfo-NHS-LC-Desthiobiotin is also soluble in aqueous buffers, which can be used as alternatives to organic solvents; however, the reagent will begin hydrolyzing immediately; must be used quickly; and any unused reagent must be discarded.

Additional Materials Required

- Water-miscible organic solvent such as DMSO (Product No. 85190) or DMF (Product No. 20673)
- 15mL conical collection tubes (Fisher Product No. 05-539-12)
- 1.5-1.7mL snap-cap microcentrifuge tubes

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 or room temperature. PBS will be used for buffer exchange, resin equilibration and as a wash buffer.

Procedure for Desthiobiotinylating Proteins

A. Calculations

The extent of desthiobiotin labeling depends on the size and distribution of amino groups on the protein and the amount of reagent used. This protocol provides instructions for protein labeling at 15X molar excess of label to protein. This is a recommended starting point as this typically produces labeling of 100% of the target protein molecules over a range of protein concentrations. Depending on your sample and application, a range of 5-25X can also be used. For example, compared to reactions involving concentrated protein solutions, labeling reactions with dilute protein solutions may require a greater-fold molar excess of desthiobiotin reagent to achieve the same incorporation level. For concentrated samples with an abundance of lysine residues, a lower molar excess may be desired to prevent over-labeling. In addition, proteins of differing molecular weights will also require different amounts of labeling reagents. Adjust calculations and labeling reagent's starting concentration as appropriate. **Perform all calculations before starting an experiment.**

1. Calculate amount of EZ-Link Sulfo-NHS-LC-Desthiobiotin required for a labeling reaction:

Step 1: Determine mg of target protein in sample:	Equation: (sample volume in mL) × (sample concentration in mg/mL) = mg of protein
Step 2: Convert mg protein in sample to mmol:	Equation: (mg of protein) / (molecular weight of the protein) = mmol of protein
Step 3: Determine number of mmol label needed for desired molar excess:	Equation: (mmol protein) × (desired molar excess) = mmol of EZ-Link Sulfo-NHS-LC-Desthiobiotin needed
Step 4: Determine amount of label solution required and convert to µL:	Equation: (mmol label required) / (concentration of label stock in mM) × 10^6µL/L = µL of stock solution to add to sample

Example calculations for a typical antibody labeling are provided below as a combined equation. Starting sample: Volume: 1mL, Concentration: 1mg/mL IgG, Approximate IgG molecular weight: 150,000, Concentration of EZ-Link Sulfo-NHS-LC-Desthiobiotin stock solution: 10mM, Desired molar excess: 15X

Example: (1mL sample) × (1mg/mL IgG) / (150,000mg IgG/mmol) × (15-fold excess) / (10mmol/L EZ-Link Sulfo-NHS-LC-Desthiobiotin) × 10^6µL/L = **10µL of 10mM EZ-Link Sulfo-NHS-LC-Desthiobiotin required**

B. Prepare Desthiobiotin Solution

Note: The Sulfo-NHS-LC-containing reagents are very moisture sensitive and readily hydrolyze to become inactive. See the Important Product Information Section above for instructions on proper handling and storage.

1. Remove one 1mg vial of EZ-Link Sulfo-NHS-LC-Desthiobiotin. Return the unused vials of reagent to the provided pouch and store desiccated at 4°C.

2. Prepare a 10mM solution of EZ-Link Sulfo-NHS-LC-Desthiobiotin. Unscrew the cap to the EZ-Link Sulfo-NHS-LC-Desthiobiotin reagent vial and solubilize the entire contents with the addition of 190µL of DMSO or DMF and mix by pipetting up and down.

   Note: If an alternative to a 10mM stock concentration is desired, use the following calculations to determine the volume needed to reconstitute the 1mg vial.

   Final volume (XµL) = [(1mg / 526.54mg/mmol) / (desired stock concentration mM)] × 10^6µL/L

C. Desthiobiotin Labeling Reaction

1. Ensure sample to be labeled has a starting concentration of between 0.2mg/mL and 2mg/mL and is in an amine-free buffer, such as PBS at pH 7.2-8. Ensure sample volume is between 500µL and 1.5mL for efficient removal of unreacted label in Section D, Step 3.

   Note: If starting sample contains Tris or other amine-containing buffers, buffer exchange into PBS. Buffer exchange can be performed by desalting or dialysis [e.g., Zeba Spin Desalting Columns 7K, 5mL (Product No. 89891) or Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 2mL (Product No. 88404)].

   Note: This kit contains five Zeba Spin Desalting Columns, 7K MWCO, 5mL. These are included to remove any unreacted label in Section D, Step 3. One or more of the provided columns can be used for buffer exchange of the starting sample (if needed); however, this will reduce the number of labeling reactions that can be processed. Do not re-use desalting columns.

2. Add the appropriate volume of EZ-Link Sulfo-NHS-LC-Desthiobiotin solution to the protein solution to achieve the desired molar excess of labeling reagent (see calculations in Section A).
3. Dispose of any unused labeling reagent. Alternatively, any unused EZ-Link Sulfo-NHS-LC-Desthiobiotin solution can be stored at -20ºC for up to 2 months only if the reagent has been prepared in a high-quality anhydrous DMSO or DMF.
4. Incubate the reaction on ice for two hours or at room temperature for 30-60 minutes.

   Note: There is no harm in reacting longer than the specified time other than the possibility of ordinary protein degradation or microbial growth.

D. Buffer Exchange and Removal of Excess Desthiobiotin Reagent Using a Desalting Column

Note: This kit contains desalting columns formatted to remove unreacted labeling reagent from samples between 500-1500µL in volume. If sample volumes are outside of this range, see our full product line of Zeba Spin Desalting Columns for a format suited to your desired sample size. Because of the larger size of desthiobiotinylation reagents and the high molar excess used for labeling, use 30% less sample volume than the maximum recommended for any appropriate volume of desalting column to ensure removal of unreacted tag.

1. Prepare a Zeba Spin Desalting Column, 5mL by breaking off the bottom closure 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 upwards. Place the column in centrifuge with the mark facing outward in all subsequent centrifugation steps.
2. Equilibrate the column by adding 2.5mL of PBS to the top of the resin bed and centrifuge 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 500-1500µL of protein sample directly onto the center of the resin bed. Allow the sample to absorb into the resin.

   Note: For samples < 1000µL, add 100µL of ultrapure water on top of the absorbed sample to increase protein recovery.

4. Centrifuge the column at 1000 × *g* for 2 minutes. Collected flow-through containing the purified and labeled protein sample is now ready for coupling and pull-down experiments. Store the protein solution at appropriate conditions. Dispose of desalting column after use.

Procedure for Pull-down Interaction Assays

A. Procedure for Coupling Desthiobiotinylated Bait Protein to Streptavidin Resin

Note: The following protocol is based on a batch-binding and elution methodology for incubations and sample collection. Alternatively, Thermo Scientific™ Pierce™ Centrifuge Columns (Product No. 89868, 69705 or 69725) can be used with the same centrifugation conditions described below. This example protocol uses a 50µL resin bed. Alternative resin amounts may be used if desired. Directly scale all buffer usage accordingly.

1. Gently vortex the provided High Capacity Streptavidin Agarose Resin to suspend and transfer 100µL of resin slurry to a 1.7mL microcentrifuge tube using a 200µL pipette tip with the end cut off to ensure that no resin is caught in the tip.

   Note: Resin is provided as a 50% slurry.

2. Centrifuge at 500 × *g* for 1 minute to form resin pellet.
3. Being careful to not disturb the resin pellet, pipette out the storage buffer and discard.
4. Wash and equilibrate resin by adding 100µL of PBS, pH 7.4. Vortex gently to resuspend. Centrifuge at 500 × *g* for 1 minute. Remove and discard wash buffer with a pipette, being careful to not disturb the resin pellet. Repeat this step two additional times.
5. Once the resin is equilibrated, add 50-200µL of desalted desthiobiotinylation reaction from Step D.4. Base the amount added on the desired amount of desthiobiotinylated target bound to the resin (typical range is 10-100µg). For a 50µL resin bed, do not exceed 100µg of labeled protein to avoid increased amounts of unbound target protein. Vortex and incubate at room temperature for 10-30 minutes with gentle mixing.
6. Once binding reaction is complete, remove tubes from mixing and centrifuge at 500 × *g* for 1 minute. Recover flow-through and analyze by protein assay or SDS-PAGE to verify bound labeled protein.
7. Wash and equilibrate resin by adding 100µL of PBS, pH 7.4. Vortex gently to resuspend. Centrifuge at 500 × *g* for 1 minute. Remove and discard wash buffer with a pipette, being careful to not disturb the resin pellet. Repeat this step one additional time. Resin is now ready for performing a pull-down experiment.

B. Procedure for Pull-down and Protein Elution

Note: To reduce nonspecific protein binding, pre-clear the sample by incubating sample with uncoupled streptavidin resin before performing a pull-down experiment.

1. Add 50-400µL of lysate (or other sample containing suspected prey protein) to resin containing labeled bait protein from Step E.7. Pipette up and down to gently resuspend the resin pellet.

   Note: The methods and buffers used to prepare a sample can have a significant effect on maintaining or capturing protein interactions. For production of a cell lysate, use a gentle reagent-based lysis buffer such as Thermo Scientific™ Pierce™ IP Lysis Buffer (Product No. 87787) or a gentle mechanical lysis method such as liquid nitrogen grinding or pressure-based lysis. Optimization may be required for any specific experiment.

2. Incubate at 4°C for at least 60 minutes with gentle rocking motion on a rotating platform. DO NOT VORTEX.

   Note: Maximal binding may require a longer incubation time, which needs to be determined for each protein. Incubation may proceed at room temperature if lysate is deemed stable.

3. After incubation, centrifuge at 500 × *g* for 1 minute to form a resin pellet. Being careful to not disturb the resin pellet, pipette out the unbound fraction and save for analysis.
4. Wash resin by adding 100µL of PBS, pH 7.4 or other suitable wash buffer. Pipette up and down gently to resuspend. Centrifuge at 500 × *g* for 1 minute. Remove wash buffer with a pipette being careful to not disturb the resin pellet. Save for analysis if desired. Repeat this step two additional times.
5. To elute desthiobiotinylated bait protein and any captured protein interactions, add 50µL of Biotin Elution Buffer and resuspend resin pellet. Incubate at 37°C for 10 minutes with gentle mixing.

   Note: Incubation at 37°C is critical for full sample recovery.

6. Centrifuge tubes at 500 × *g* for 1 minute. Remove Biotin Elution Buffer with a pipette, being careful to not disturb the resin pellet. Save for analysis. Repeat Steps B.5-B.6 two additional times.

Troubleshooting

Problem	Possible Cause	Solution
Protein is not desthiobiotinylated	Reagent hydrolyzed and became non-reactive.	Do not store reagent in aqueous solutions or solvent that has been absorbed in water. Bring up reagents in anhydrous organic solvents such as DMSO or DMF.
	Protein sample contained secondary source of amines (e.g., Tris, glycine, Natzide or a Nucleophite).	Buffer exchange sample into an amine-free buffer by dialysis or desalting.
	Suboptimal reaction conditions for target protein concentration.	Optimize molar excess of desthiobiotin reagent.
	There were limited free or accessible amines in target molecule.	Modify protein or choose alternative reactive chemistry.
Binding capacity of streptavidin resin compromised	Non-reacted desthiobiotin was not removed effectively, resulting in resin-binding competition.	Desalt sample before performing assay, ensuring column capacities are not exceeded.
Interacting protein was not isolated	Weak or transient interaction.	Wash conditions too stringent; lower the number of washes and ionic strength of wash buffer.
	Poor expression level of prey protein.	Apply more protein sample.
		Increase incubation time.
		Increase amount of bait protein.
	Excessive desthiobiotinylation hindered target protein binding site.	Reduce molar excess of desthiobiotin reagent or use a reagent that targets a different functional group.
	Binding or sample preparation conditions were insufficient to maintain or allow protein interactions.	Try alternative buffers for sample preparation, binding or washing procedures.
High background or contaminating proteins	Isolation of naturally biotinylated proteins.	Reduce elution time and temperature.
		Dilute elution buffer 20-40%.
		Pre-clear sample by incubating with unlabeled streptavidin resin.
	Insufficient washing.	Increase number of washes or add trace amounts (0.2%) of non-ionic detergent (e.g., NP-40 detergent) to wash buffers.

Related Thermo Scientific Products

16129 **EZ-Link NHS-Desthiobiotin,** 50mg
16130 **EZ-Link Hydrazide-PEG$_4$-Desthiobiotin, No-Weigh Format,** 5 × 1mg vials
16131 **EZ-Link Amine-PEG$_4$-Desthiobiotin, No-Weigh Format,** 5 × 1mg vials
16133 **EZ-Link Phosphine-PEG$_4$-Desthiobiotin, No-Weigh Format,** 5 × 1mg vials
16136 **EZ-Link Sulfo-NHS-LC-Desthiobiotin, No-Weigh Format,** 5 × 1mg vials
28372 **BupH Phosphate Buffered Saline Packs,** 40 pack
89891 **Zeba Spin Desalting Columns, 7K MWCO, 5mL,** 5/pkg
89893 **Zeba Spin Desalting Columns, 7K MWCO, 10mL,** 5/pkg
20357 **High Capacity Streptavidin Agarose Resin,** 2mL
20673 **Dimethylformamide (DMF), Sequencing Grade,** 50mL
85190 **Dimethylsulfoxide (DMSO), Sequencing Grade,** 50mL

References

1. Green, N.M. (1970) *Meth Enzymol* **18A**:418.
2. Hirsch, J., *et al*. (2002) Easily reversible desthiobiotin binding to streptavidin, avidin, and other biotin-binding proteins: uses for protein labeling, detection and isolation. *Anal Biochem* **308**:343-57.
3. Hofmann, K., *et al.* (1982) Avidin binding of carboxyl-substituted biotin and analogues. *Biochem* **21**:978-84.
4. Hofmann, K., *et al.* (1984) Syntheses of biotinylated and dethiobiotinylated insulins. *Biochem* **23:**2547-53.

Thermo Fisher Scientific | 3747 N. Meridian Road | Rockford, Illinois 61101 USA

For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition. The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0011872 B

Revision	Date	Description
B	31 July 2024	Correcting spin column usage.
A	17 October 2015	New document for EZ-Link™ Desthiobiotinylation and Pull-Down Kit.