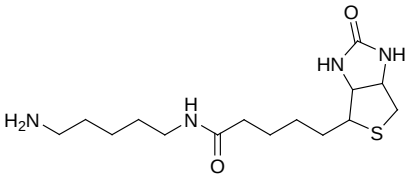
INSTRUCTIONS

EZ-Link® Pentylamine-Biotin

21345

0379.5

Number	Description
21345	**EZ-Link Pentylamine-Biotin,** 50mg Molecular Weight: 328.48 Spacer Arm: 18.9Å



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

Introduction

The Thermo Scientific EZ-Link Pentylamine-Biotin (5-biotinamidopentylamine) is a water-soluble biotinylation reagent containing a terminal primary amine. The amine group of this reagent can be reacted with protein carboxyl groups, which occur at carboxy termini, aspartate residues and glutamate residues. The reaction is mediated by EDC (Product No. 22980, 22981), the most common water-soluble carbodiimide crosslinker. EDC activates carboxyl groups to bind to the $–NH_2$ group of Pentylamine-Biotin, forming an amide linkage. A highly efficient EDC reaction can be achieved using Sulfo-NHS (Product No. 24510) in a two-step reaction, which increases conjugation yield dramatically compared with using EDC alone (see Appendix C: Efficient Coupling of Proteins Using EDC and Sulfo-NHS).

Example Procedure for Biotinylating BSA

The following method uses Pentylamine-Biotin in conjunction with EDC to selectively biotinylate carboxyl groups on bovine serum albumin (BSA). The specifics of this protocol can be modified for other applications (see Appendix B: Calculations). Polymerization of a peptide or protein may result if the molecule has both carboxyl groups and primary amines on its surface. Decreasing the amount of EDC in the reaction and/or increasing the amount of biotinylation reagent can minimize the extent of polymerization.

Note: EDC reactions are generally performed using MES buffer at pH 4.5-5. Avoid buffers containing primary amines (Tris, glycine, etc.) or carboxyls (acetate, citrate, etc.) as these buffers will interfere with the reaction. Phosphate buffers are also not recommended because they reduce conjugation efficiency, although this effect can be overcome by adding more EDC.

A. Materials Required

- MES Buffer: 0.1M MES [(2-N-morpholino) ethanesulfonic acid], pH 4.7-5.5 (Thermo Scientific BupH MES Buffered Saline Packs, Product No. 28390)
- BSA: Dissolve 2.5mg of BSA in 0.5mL of MES buffer.
- EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), Product No. 22980 or 22981
- Method for removal of non-reacted biotin (buffer exchange): Dialysis cassette (e.g., Thermo Scientific Slide-A-Lyzer Dialysis Cassette Kit, 10K MWCO, 0.5-3mL, Product No. 66382) or desalting column (e.g., Thermo Scientific Zeba Spin Desalting Columns, Product No. 89891)

B. Procedure

1. Dissolve Pentylamine-Biotin in water at 50mM (~16mg/mL).
2. Add an equal volume (i.e., 0.5mL) of the biotin solution to the BSA solution and mix.
3. Immediately before use, dissolve EDC in MES Buffer at 20mg/mL.
4. Add 50µL of the EDC solution to the solution from Step 2 and mix.
5. Incubate for 2 hours at room temperature with stirring.
6. Remove any precipitate that formed during the reaction by centrifugation.
7. Remove non-reacted Pentylamine-Biotin and EDC byproducts from the biotinylated protein by desalting or dialysis.

   Note: Biotinylated proteins may be stored using the same conditions as for the non-biotinylated protein. A typical storage condition is 4°C for two months.

Example Procedure for Assaying Factor XIII in Plasma

Lee *et al.* have developed an extensively optimized colorimetric Factor XIII assay with several advantages as compared to previous assays.[1] Their assay, based on Pentylamine-Biotin, is simple, quick, easily automated and ideal for routine use. Between-run coefficients of variation are ~5% and the method compares favorably with the more common radiometric filter-paper assay method.[2]

A. Materials Required

- 96-well microplates (e.g., Product No. 15040)
- Bentonite powder

B. Material Preparation

Citrated Human Plasma	Collect blood in vacutainer® tubes containing 38g/L sodium citrate (one part sodium citrate to nine parts blood). Centrifuge at 2500 × *g* for 10 minutes at 4°C. Analyze within 8 hours of collection.
Human Thrombin Solution	Reconstitute human thrombin to 250 NIH units/mL with 25% glycerol/75% water (v/v), pH 7.4 (1000 NIH units/mg protein); stable at -20°C for five months
N,N-Dimethylcasein Solution (bovine)	Prepare at 10g/L in Tris-buffered saline (TBS, 40mM Tris, pH 8.3, 150mM NaCl) containing 50mM DTT; stable at -20°C for five months
Pentylamine-Biotin Solution	Dissolve 5mM Pentylamine-Biotin in TBS
$CaCl_2$ Solution	Prepare 50mM $CaCl_2$ in TBS; stable at -20°C for five months
EDTA Solution	Prepare 200mM EDTA in TBS; stable at -20°C for five months
Wash Buffer	10mM sodium phosphate, 2.7mM potassium chloride, 120mM sodium chloride, pH 7.4, 2.0mM β-mercaptoethanol, 0.5g/L Tween®-20, 0.5g/L sodium azide
Streptavidin-β-galactosidase Solution	Immediately before use, prepare a 1µg/mL solution in wash buffer
PNPG Solution	Immediately before use prepare 1g/L p-nitrophenyl-β-galactopyranoside in 50mM sodium phosphate, pH 7.2, 1.5mM $MgCl_2$

C. Biotin Incorporation into Substrate

Note: Perform all steps at room temperature. For optimal results analyze samples in duplicate.

1. Add bentonite to tubes containing citrated plasma to a final concentration of 40mg/mL.
2. Incubate for 10 minutes and centrifuge at 9000 × *g* for 1 minute.
3. Transfer 50µL of supernatant from each sample to microplate wells and add 10µL of thrombin solution. Incubate for 20 minutes.
4. Add 20µL of $CaCl_2$ Solution, 20µL of *N*,*N*-Dimethylcasein Solution, and 100µL of Pentylamine-Biotin Solution to each well containing the sample or control. Substitute 20µL of EDTA solution for the $CaCl_2$ solution for controls. Mix well.
5. Incubate for 40 minutes with mixing.
6. Quench the reaction by adding 20µL of EDTA Solution.

D. Immobilization of Substrate and Detection

1. Add 200µL of solution from Step C6 into wells of a new microplate. Incubate for 60 minutes with shaking.

   Note: Use a new microplate to ensure linear results.
2. Decant the liquid and wash the wells 3 × 10 minutes with wash buffer.
3. Add 150µL of Streptavidin-β-galactosidase Solution to each well and incubate for 30 minutes with shaking.
4. Decant the liquid and wash the wells 3 × 10 minutes with wash buffer.
5. Add 200µL of the PNPG Solution and incubate for 20 minutes.
6. Measure the absorbance at 405nm. Blanks should give an absorbance of < 0.01.

Appendix

A. Determination of Biotin Incorporation

Biotin incorporation can be estimated using the HABA (4´-hydroxyazobenzene-2-carboxylic acid] method (e.g., Thermo Scientific 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 500nm. Biotin is then added to the solution and, because of its higher affinity for avidin, biotin 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.

B. Calculations

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

Note: Adding EDC at 10-fold molar excess to the protein results in sufficient conjugation. Alternatively, add EDC to a final reaction concentration of 0.05-0.1M. Polymerization of a peptide or protein may result if the molecule has both carboxyl groups and primary amines on its surface. Decreasing the amount of EDC in the reaction and/or increasing the amount of biotinylation reagent can minimize the extent of polymerization.

1. Calculate millimoles of Pentylamine-Biotin to add to the reaction for a 20-fold molar excess:

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{20\ \text{mmol biotin}}{\text{mmol protein}} = \text{mmol biotin reagent}$$

2. Calculate microliters of 50mM Pentylamine-Biotin to add to the reaction:

$$\text{mmol Biotin} \times \frac{328.48\ \text{mg}}{\text{mmol Biotin}} \times \frac{1000\ \mu\text{L}}{16.5\ \text{mg}} = \mu\text{L biotin reagent}$$

- 20 = Recommended molar fold excess of biotin for 2mg/mL IgG sample
- 328.48 = Molecular weight of Pentylamine-Biotin
- 1000 = Microliters of water in which 21mg of Pentylamine-Biotin is dissolved for a 50mM solution

Example: For 1mL of a 2mg/mL IgG (150,000 MW) solution, 5µL of 50mM Pentylamine-Biotin will be added.

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

$$0.000266\ \text{mmol Biotin} \times \frac{328.48\ \text{mg}}{\text{mmol Biotin}} \times \frac{1000\ \mu\text{L}}{16.5\ \text{mg}} = 5\ \mu\text{L biotin reagent}$$

C. Efficient Coupling of Proteins Using EDC and Sulfo-NHS (Product No. 24510)

A highly efficient EDC reaction can be achieved using EDC/sulfo-NHS-coupled reactions, which increases conjugation yield dramatically compared with using EDC alone. EDC reacts with carboxyl groups first and forms an unstable amine-reactive intermediate. Failure to quickly react with an amine will result in hydrolysis of the intermediate, regeneration of the carboxyl, and release of an N-substituted urea. This result is especially a problem when targeting molecules in dilute solutions. Adding Sulfo-NHS modifies a carboxyl group to an amine-reactive NHS ester and extends the half-life of the activated carboxylate. Although a higher yield of conjugation results, the final product of this two-step reaction is identical to using EDC alone.

Related Thermo Scientific Products

46610	**Fluorescence Biotin Quantitation Kit**
20036	**Bioconjugate Techniques,** 1202 pages, softcover
28005	**Pierce Biotin Quantitation Kit**
22980	**EDC,** 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 5g
20227	**Immobilized Monomeric Avidin Kit**

Cited References

1. Lee, K.Y. *et al*. (1988). Colorimetric assay of blood coagulation factor XIII in plasma. *Clin Chem* **34**:906-10.
2. Lorand, L. *et al*. (1972). A filter paper assay for transamidating enzymes using radioactive amine substrates. *Anal Biochem* **50**:623-31.

General References

Lehninger, A.L. (1982). *Principles of Biochemistry*, p. 707, Worth Publishers, Inc., New York.

Chaiet, I. and Wolf, F.J. (1964). The properties of streptavidin, a biotin-binding protein produced by Streptomycetes. *Arch Biochem Biophys* **106**:1-5.

Gitlin, G. *et al*. (1987). Studies on the biotin-binding site of avidin. *Biochem J* **242**:923-6.

Green, N.M. (1975). Avidin. in Adv. In Protein Chemistry, Academic Press, New York, Anfinsen, C.B., Edsall, J.T. and Richards, F.M., eds. b85-133.

Bondi, A. *et al*. (1982). The use of β-galactosidase as a tracer in immunocytochemistry. *Histochemistry* **76**:153-8.

Lojda, Z. (1970). Indigogenic methods for glycosidases. *Histochemie* **23**:266-78.

Hosli, P. *et al*. (1965). Purification, composition, and molecular weight of the β-galactosidase of Escherichia coli K12. *J Biol Chem* **240**:2468-77.

Wallenfels, K. and Malholtra, O.P. β-Galactosidase. In: Boyer, P.D.; Lardy, B.; Myrback, K.; eds. The Enzymes. 2nd edition. Academic Press, N.Y., pp. 409-30.

Jeon, W.M. *et al*. (1989). Colorimetric assay for cellular transglutaminase. *Anal Biochem* **182**:170-5.

Tween is a trademark of Croda International Plc.

Vacutainer is a trademark of Becton, Dickinson and Company Corp.

This product ("Product") is warranted to operate or perform substantially in conformance with published Product specifications in effect at the time of sale, as set forth in the Product documentation, specifications and/or accompanying package inserts ("Documentation") and to be free from defects in material and workmanship. Unless otherwise expressly authorized in writing, Products are supplied for research use only. No claim of suitability for use in applications regulated by FDA is made. The warranty provided herein is valid only when used by properly trained individuals. Unless otherwise stated in the Documentation, this warranty is limited to one year from date of shipment when the Product is subjected to normal, proper and intended usage. This warranty does not extend to anyone other than the original purchaser of the Product ("Buyer").

No other warranties, express or implied, are granted, including without limitation, implied warranties of merchantability, fitness for any particular purpose, or non infringement. Buyer's exclusive remedy for non-conforming Products during the warranty period is limited to replacement of or refund for the non-conforming Product(s).

There is no obligation to replace Products as the result of (i) accident, disaster or event of force majeure, (ii) misuse, fault or negligence of or by Buyer, (iii) use of the Products in a manner for which they were not designed, or (iv) improper storage and handling of the Products.

Current product instructions are available at *www.thermoscientific.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.