INSTRUCTIONS

Maleimide Activated NeutrAvidin® Protein

31007

0712.4

Number	Description
31007	**Maleimide Activated NeutrAvidin Protein,** 5mg

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

Introduction

Thermo Scientific Maleimide Activated NeutrAvidin Protein is for directly preparing NeutrAvidin Protein conjugates with proteins, peptides and other molecules that contain a free sulfhydryl (-SH) group. NeutrAvidin Protein has been maleimide-activated using Sulfo-SMCC, a heterobifunctional crosslinker that contains an *N*-hydroxysuccinimide ester and a maleimide group. The activated NeutrAvdin Protein presents an available maleimide group that reacts with sulfhydryl-containing molecules.

NeutrAvidin Protein (60KDa) is a modified avidin derivative with several key features that provide a biotin-binding protein with exceptionally low nonspecific binding properties. NeutrAvidin Protein does not contain carbohydrates, rendering lectin-binding activity to undetectable levels.[1] Additionally, the isoelectric point of NeutrAvdin Protein is 6.3 ± 0.3, which is much lower than native avidin and not as acidic as streptavidin. The relatively neutral isoelectric point is produced by careful modification of charged groups on the protein's surface. This controlled process ensures that sufficient lysines remain available such that enzymes and other conjugates can be successfully prepared through traditional amine-reactive chemistries.

Another advantage of NeutrAvdin Protein is that it does not contain the RYD domain that is analogous to the universal recognition sequence for a variety of cell adhesion receptors. The lack of this sequence eliminates the potential for binding to numerous cell types, through interaction with various membrane receptor molecules.

Important Product Information

- Reconstitute this reagent immediately before use. When in solution, the maleimide moiety may hydrolyze and become non-reactive; therefore, do not prepare stock solutions for storage. Discard any unused reconstituted reagent.
- Molecules for conjugation must have free -SH group(s) available. Disulfide bonds can be reduced to produce free sulfhydryls using the Thermo Scientific Immobilized TCEP Disulfide Reducing Gel (Product No. 77712), which enables reduction while recovering the sample in the absence of reducing agents. Additionally, 2-mercaptoethylamine•HCl (Product No. 20408) selectively cleaves the disulfide bonds between the heavy chains of IgG while preserving the disulfide linkages that connect the heavy and light chains.
- Sulfhydryls can be added to molecules using *N*-succinimidyl S-acetylthioacetate (SATA, Product No. 26102) or 2-iminothiolane•HCl (Traut's Reagent, Product No. 26101), which modifies primary amines.
- Avoid sulfhydryl-containing components during conjugation, as these will react with the maleimide group thereby inhibiting and reducing conjugation efficiency of the intended molecule.

Procedure for Conjugating Antibodies to Maleimide-activated NeutrAvidin Protein

Note: This protocol can be modified for molecules other than antibodies.

A. Choose either 2-MEA or SATA to Prepare IgG

Use one of two strategies to ensure that sulfhydryl groups are made available on an antibody for conjugation. One strategy is to reduce native disulfide bonds in the antibody using 2-mercaptoethylamine•HCl (Method 1), which selectively cleaves between the heavy chains of IgG. The result is monovalent antibodies with sulfhydryls available for conjugation. This

method preserves an intact and available antigen-binding site; however, antibody avidity is lowered as each half antibody has only one binding site.

A second strategy is to add sulfhydryl groups to antibodies with SATA (Method 2), a sulfhydryl-containing modification reagent that reacts with primary amines ($-NH_2$) present on the side-chain of lysine residues. The reaction results in antibodies that contain protected sulfhydryl groups that can be exposed when desired. With this sulfhydryl addition method there is no risk of completely reducing and fragmenting antibodies; however, disruption of antigen-binding capability is possible from modification of antigen-binding sites, especially if binding sites contain many lysine residues.

Note: Maleimides react with sulfhydryls at pH 6.5-7.5 to form stable thioether bonds. At pH values > 7.5, reactivity toward primary amines and hydrolysis of the maleimide group can occur.

B. Materials Required

Note: Reagents required for the SATA method are available individually or as a complete kit (i.e., Sulfhydryl Addition Kit, Product No. 23460).

- Phosphate-buffered Saline (PBS): 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 (Product No. 28372)
- Antibody: Dissolve 5mg of IgG in 1mL of PBS.
- 2-Mercaptoethylamine•HCl (2-MEA, Product No. 20408) or SATA (Product No. 26102)
- For the SATA reaction, Dimethylformamide (Product No. 20673) is needed for dissolving SATA, and Hydroxylamine•HCl (Product No. 26103) is needed for deacetylation
- Maleimide Conjugation Buffer: 100mM sodium phosphate, 5-10mM EDTA, pH 7.2 (Product No. 77164)
- Desalting column for buffer exchange (e.g., Thermo Scientific Zeba Spin Desalting Columns, Product No. 89893)

C. Prepare IgG to Present Sulfhydryl Groups Using Either Method 1 or 2

- **Method 1: Partially Reduce Antibodies to Produce Sulfhydryls using 2-MEA**

1. Add 100µL of Maleimide Conjugation Buffer to a 6mg vial of 2-MEA.
2. Add the prepared IgG to the vial containing the 2-MEA Solution and incubate for 90 minutes at 37°C.
3. Allow solution to cool to room temperature. While the solution is cooling pre-equilibrate a desalting column with Maleimide Conjugation Buffer.
4. Separate 2-MEA from reduced IgG using a desalting column and the Maleimide Conjugation Buffer. Collect 0.5mL fractions. Measure absorbance of each fraction at 280nm to locate the protein peak.

   Note: Separation of 2-MEA from reduced IgG is critical, as residual 2-MEA will interfere with coupling. To determine if adequate separation has been achieved, perform a Thermo Scientific Pierce BCA Protein Assay (Product No. 23225) to identify the location of 2-MEA (see the Additional Information Section).
5. Pool fractions that contain reduced IgG. The protein concentration should be ~2.5mg/mL. Immediately proceed to Section D to minimize disulfide formation.

   Note: To precisely determine protein concentration, use the Thermo Scientific Coomassie Plus (Bradford) Assay Kit (Product No. 23236).

- **Method 2: Add Sulfhydryl Groups to Antibodies using SATA**

  Note: This protocol can be modified for primary amine-containing molecules other than antibodies.

1. Add 200µL of DMF to 2mg of SATA.
2. Add 20µL of SATA solution to the prepared IgG, which yields a 25-fold molar excess of SATA. Discard unused reconstituted SATA.
3. Incubate for 30 minutes at room temperature.

   Note: The SATA-modified protein contains a protected sulfhydryl and is, therefore, stable and may be stored. Once the sulfhydryl is deacetylated, conjugation must be performed rapidly to minimize disulfide formation.

4. For deacetylation, add 100µL of Maleimide Conjugation Buffer to 5mg of Hydroxylamine•HCl. Add the SATA-modified IgG to the solution.
5. Incubate for 2 hours at room temperature. During this incubation, pre-equilibrate the desalting column with Maleimide Conjugation Buffer.
6. Separate nonreacted SATA from the modified IgG using a desalting column and the Maleimide Conjugation Buffer. Collect 0.5mL fractions. Measure absorbance of each fraction at 280nm to locate the protein peak

   Note: Separation of nonreacted SATA from modified IgG is critical as residual SATA will interfere with conjugation.
7. Pool fractions that contain the modified IgG. The protein concentration should be ~2.5mg/mL. Immediately proceed to Section D to minimize disulfide formation.

   Note: To precisely determine protein concentration, use the Coomassie Plus (Bradford) Assay Kit (Product No. 23236).

D. Conjugation of IgG to Maleimide Activated NeutrAvdin Protein

This method uses approximately three-fold molar excess of activated NeutrAvdin Protein to IgG. For SATA-modified IgG the result will be 1-3 moles of NeutrAvdin Protein incorporated per mole of IgG. For 2-MEA-reduced IgG the result will be 1 mole of NeutrAvdin Protein incorporated per half antibody. Other molar ratios may be used.

1. Add 2mL of the pooled protein to the vial of activated NeutrAvdin Protein.
2. Incubate reaction for 1 hour at room temperature. To increase NeutrAvdin Protein incorporation, extend reaction time up to 12 hours.
3. For long-term storage, remove EDTA from conjugate by dialysis or using a desalting column. Use Thermo Scientific Pierce Peroxidase Conjugate Stabilizer (Product No. 31503) or add glycerol to 50% and store at -20°C.

Additional Information

A. Determine Location of Protein and Reducing Reagents by BCA Protein Assay

Separation of 2-MEA from reduced IgG is critical, as residual 2-MEA will interfere with coupling. To determine if adequate separation has been achieved, perform a Thermo Scientific BCA Protein Assay (Product No. 23225) to identify locations of 2-MEA and the NeutrAvidin Protein conjugate.

1. Using a gravity-flow-desalting column, place microcentrifuge tubes in a rack and collect fractions as the solution flows through the column.
2. Prepare BCA Working Reagent according to the instructions supplied with the kit. Pipette 200µL of Working Reagent into one microplate well for each fraction collected.
3. Add 5µL from each fraction to the wells. The 2-MEA reacts immediately producing an intense blue to purple product.

   Note: Do not use greater than 5µL of sample, as the EDTA content of the buffer will interfere with the assay.
4. After 15-30 minutes, wells containing protein will turn blue to purple. A blank (or green) well between protein-containing samples and 2-MEA indicates excellent separation.

B. Information Available from the Internet

Visit our web site for additional information including the following items:

- Tech Tip #43: Protein stability and storage
- Tech Tip #6: Extinction coefficient guide

General Reference

Hiller, Y, *et al.* (1987). Biotin binding to avidin. Oligosaccharide side chain not required for ligand association. *Biochem J* **248:**167-71.

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.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce