INSTRUCTIONS

EZ-Link® Maleimide Activated Horseradish Peroxidase

31485

0645.4

Number	Description
31485	**EZ-Link Maleimide Activated Horseradish Peroxidase,** 5mg

Note: Total weight is ~25mg as a result of buffer components and salts lyophilized along with the activated protein

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

Introduction

The Thermo Scientific EZ-Link Maleimide Activated Horseradish Peroxidase is for preparing horseradish peroxidase (HRP) conjugates with proteins, peptides and other ligands that contain a free sulfhydryl (–SH) group. HRP has been maleimide-activated using Sulfo-SMCC, a heterobifunctional crosslinker that contains an *N*-hydroxysuccinimide ester and a maleimide group. The activated HRP presents an available maleimide group that can react with sulfhydryl-containing molecules.

Procedure for Conjugating Antibodies to Maleimide-activated HRP

Note: This protocol can be modified for molecules other than antibodies that have either a disulfide bond that can be reduced to generate free –SH groups or an available primary amine so that the required –SH group can be added chemically.

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

One of two strategies may be used 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 to the activated HRP. 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, which 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 contains 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; however the maleimide group of Sulfo-SMCC is unusually stable up to pH 7.5.

B. Materials Required

Note: Reagents required for the SATA method are available individually or as a complete kit (i.e., Thermo Scientific 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: 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 Column, 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 BCA assay to identify 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 other primary amine-containing molecules.

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 HRP coupling.
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 HRP

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

1. Add 2mL of the pooled protein to the vial of activated HRP.
2. Incubate reaction for 1 hour at room temperature. To increase HRP 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 Pierce 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 protein assay (e.g., Thermo Scientific Pierce BCA Protein Assay, Product No. 23225) to identify location of 2-MEA and the HRP conjugate.

1. Place 1mL tubes in a rack and collect fractions as the solution flows through the desalting column.
2. Prepare Pierce 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 will react immediately producing an intense color.

   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 Our Website

Visit our website for additional information including the following items:

- Tech Tip # 43: Protein stability and storage
- Tech Tip # 6: Extinction coefficients guide
- Tech Tip # 7: Remove air bubbles from columns to restore flow rate
- Tech Tip #29: Degas solutions for use in affinity and gel filtration columns

Related Thermo Scientific Products

89893	**Zeba™ Spin Desalting Columns, 7K MWCO, 10mL,** 5/pkg
89894	**Zeba Spin Desalting Columns, 7K MWCO, 10mL,** 25/pkg
31495	**EZ-Link Activated Peroxidase,** 5mg
34080	**SuperSignal® West Pico Chemiluminescent Substrate,** 500mL
34075	**SuperSignal West Dura Extended Duration Substrate,** 100mL
34095	**SuperSignal West Femto Maximum Sensitivity Substrate,** 100mL
34090	**CL-XPosure™ Film,** 5” × 7” sheets, 100 sheets/pkg
21059	**Restore™ Western Blot Stripping Buffer,** 500mL

General References

Duncan, R.J.S., *et al*. (1983). A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal Biochem* **132:**68-73.

Hashida, S., *et al.* (1984). More useful maleimide compounds for the conjugation of Fab' to horseradish peroxidase through thiol groups in the hinge. *J Appl Biochem* **6:**56-63.

Imagawa, M., *et al*. (1982). Characteristics and evaluation of antibody- horseradish peroxidase conjugates prepared by using a maleimide compound, glutaraldehyde, and periodate. *J Appl Biochem* **4:**41-57.

Yoshitake, S., *et al.* (1979). Conjugation of glucose oxidase from *Aspergillus niger* and rabbit antibodies using *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexyl-methyl) maleimide. *Eur J Biochem* **101:**395-9.

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