INSTRUCTIONS

EZ-Link® Maleimide Activated Horseradish Peroxidase Kit

31494

0234.5

Number	Description
31494	**EZ-Link Maleimide Activated Horseradish Peroxidase Kit,** contains sufficient reagents to label 5mg of IgG

Kit Contents:

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

Conjugation Buffer (10X), 20mL, contains 100mM sodium phosphate, 100mM EDTA; pH 7.0

BupH™ Phosphate Buffered Saline Pack, 1 each, results in 0.1M sodium phosphate, 0.15M NaCl; pH 7.2 when reconstituted with 500mL of ultrapure water

2-Mercaptoethylamine•HCl, 6mg

SATA, 2mg

Dimethylformamide, 1mL

Hydroxylamine•HCl, 5mg

Polyacrylamide Desalting Column, 1 × 10mL, contains bottom plugs (2), porous discs (2) and a porous disc insertion tool

Storage: Upon receipt store kit at 4°C. Kit is shipped at ambient temperature.

Introduction

The Thermo Scientific EZ-Link Maleimide Activated Horseradish Peroxidase Kit is for preparing horseradish peroxidase (HRP) conjugates directly from proteins, peptides, and other ligands that contain a free sulfhydryl 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. Two modification reagents are also included in this kit allowing flexibility in the method of producing free sulfhydryls on macromolecules. 2-Mercaptoethylamine•HCl is a mild reagent for reduction of IgG and $F(ab')_2$ fragments. SATA is a sulfhydryl-containing modification reagent that reacts with primary amines to present protected sulfhydryl groups, which can be exposed upon treatment with hydroxylamine.

Procedure for Conjugating Antibodies to Maleimide-activated HRP

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

A. Material Preparation

- Phosphate Buffered Saline (PBS): Dissolve the dry-blend buffer with 500mL of ultrapure water. For long-term storage of excess buffer, sterile-filter the solution and store at 4°C.
- Antibody: Dissolve 5mg of IgG in 1mL of PBS.
- Maleimide Conjugation Buffer (1X): Add 10mL of the Conjugation Buffer (10X) to 90mL of PBS.

B. Prepare IgG using either 2-MEA or SATA

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-MEA (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 only has 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 sulfhydryl groups 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 stable up to pH 7.5.

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

1. Add 100µL of Maleimide Conjugation Buffer (1X) to the 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 the solution to cool to room temperature. While the solution is cooling, pre-equilibrate the desalting column by adding 30mL of Maleimide Conjugation Buffer (1X) and allowing it to flow through.
4. To separate 2-MEA from reduced IgG, apply the IgG/2-MEA to the equilibrated desalting column.
5. Add Maleimide Conjugation Buffer (1X) to the column and collect 0.5mL fractions. Measure absorbance of each fraction at 280nm to locate the protein peak. Generally, fractions 6-10 will contain most of the protein.

   Note: Separation of 2-MEA from reduced IgG is critical. Residual 2-MEA will interfere with coupling. To determine if adequate separation has been achieved, perform a Thermo Scientific Pierce BCA Protein Assay to identify the location of 2-MEA (see the Additional Information Section).
6. Pool fractions that contain reduced IgG. The protein concentration should be ~2.5mg/mL. Immediately proceed to Section C 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

1. Add 200µL of Dimethylformamide to the vial containing the 2mg of SATA.
2. Add 20µL of SATA solution to the prepared IgG, which yields a 25-fold molar excess of SATA. Discard unused 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 Conjugation Buffer (10X) to the vial of hydroxylamine•HCl. Add the SATA-modified IgG to the solution.
5. Incubate for 2 hours at room temperature. During this incubation, prepare the desalting column. Invert column several times to resuspend the resin, then position the column upright in a test tube or clamp and allow the resin to settle for several minutes.
6. Remove the top cap from the column and carefully pipette the storage solution (contains 0.02% sodium azide) until 5-10mm of solution remains above the resin bed.
7. (Optional) Using the open end of the porous disc insertion tool, insert and slide a porous disc to within 1mm of the resin bed. This disc provides a stop-flow function that prevents disturbance and drying of the resin bed during use.
8. Twist off the column bottom end tab. Equilibrate the desalting column by adding 30mL of Maleimide Conjugation Buffer (1X) to the column and allowing it to flow through.
9. Apply the SATA-modified IgG to the equilibrated desalting column.

10. Add Maleimide Conjugation Buffer (1X) to the column and collect 0.5mL fractions. Measure the absorbance of each fraction at 280nm to locate the protein peak. Generally, fractions 6-10 will contain most of the protein.

    Note: Separation of nonreacted SATA from modified IgG is critical as residual SATA will interfere with HRP coupling.

11. Pool fractions that contain the modified IgG. The protein concentration should be ~2.5mg/mL. Immediately proceed to Section C to minimize disulfide formation.

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

C. Conjugation of IgG to EZ-Link 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 of, extend reaction time up to 12 hours.
3. For long-term storage, remove EDTA from conjugate by dialysis or gel filtration. Use 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

Separation of 2-MEA from reduced IgG is critical, as residual 2-MEA will interfere with coupling. Use the Pierce BCA Protein Assay (Product No. 23225) to identify which specific fractions contain 2-MEA and the HRP conjugate.

1. 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.
2. Add 5µL from each fraction into 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.
3. 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 Web

Please visit our website for additional information relating to this product including the following items:

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

Related Thermo Scientific Products

31485	**EZ-Link Maleimide Activated Horseradish 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.

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