EZ-Link™ Maleimide Activated Horseradish Peroxidase Kit

Catalog Number 31494

Doc. Part No. 2160234 Pub. No. MAN0011204 Rev. B

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

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.

Contents and storage

Table 1 Kit contents for EZ-Link™ Maleimide Activated Horseradish Peroxidase Kit.

Component	Amount	Storage
EZ-Link™ Maleimide Activated Horseradish Peroxidase[1]	5 mg	Store at 4°C
Conjugation Buffer (10X)[2]	20 mL	
BupH™ Phosphate Buffered Saline Pack[3]	1 each	
2-Mercaptoethylamine-HCl	6 mg	
SATA	2 mg	
Dimethylformamide	1 mL	
Hydroxylamine-HCl	5 mg	
Polyacrylamide desalting column[4]	1 x 10 mL	

[1] Total weight is approximately 25 mg as a result of buffer components and salts lyophilized along with the activated protein.
[2] Contains 100 mM sodium phosphate and 100 mM EDTA; pH 7.0.
[3] Results in 0.1 M sodium phosphate, 0.15 M NaCl; pH 7.2 when reconstituted with 500 mL of ultrapure water.
[4] Contains 2 porous discs and a porous disc insertion tool.

Conjugate 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 the required sulfhydryl group can be chemically added.

Material preparation

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

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 100 µL of Maleimide Conjugation Buffer (1X) to the 6 mg 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 30 mL 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 0.5 mL fractions. Measure absorbance of each fraction at 280 nm 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" on page 2 Section).
6. Pool fractions that contain reduced IgG. The protein concentration should be approximately 2.5 mg/mL. Immediately proceed to "Conjugation of IgG to EZ-Link™ maleimide activated HRP" on page 2 to minimize disulfide formation.

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

Method 2: Add sulfhydryl groups to antibodies using SATA

1. Add 200 µL of Dimethylformamide to the vial containing the 2 mg of SATA.
2. Add 20 µL 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 100 µL 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 5–10 mm 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 1 mm 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 30 mL 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 0.5 mL fractions. Measure the absorbance of each fraction at 280 nm 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 ~2.5 mg/mL. Immediately proceed to "Conjugation of IgG to EZ-Link™ maleimide activated HRP" on page 2 to minimize disulfide formation.

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

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 2 mL 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

Determine the 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 (Cat. No. 23225) to identify which fractions contain 2-MEA and the HRP conjugate.

1. Prepare BCA™ Working Reagent according to the instructions supplied with the kit. Pipette 200 µL of Working Reagent into one microplate well for each fraction collected.

2. Add 5 µL from each fraction into the wells. The 2-MEA will react immediately producing an intense color.

   Note: Do not use greater than 5 µL 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.

Related products

Product	Cat. No.
EZ-Link™ Maleimide Activated Horseradish Peroxidase, 5 mg	31485
SuperSignal™ West Pico Chemiluminescent Substrate, 500 mL	34080
SuperSignal™ West Dura Extended Duration Substrate, 100 mL	34075
SuperSignal™ West Femto Maximum Sensitivity Substrate, 100 mL	34095
CL-XPosure™ Film, 5" × 7" sheets, 100 sheets/pkg	34090
Restore™ Western Blot Stripping Buffer, 500 mL	21059

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.

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.

Revision history: Pub. No. MAN0011204 B

Revision	Date	Description
B	13 May 2024	Removing bottom caps to correspond with product change.
A.0	17 October 2015	New document for EZ-Link™ Maleimide Activated Horseradish Peroxidase Kit.