Conjugation-Ready HRP-Maleimide (HRP-M) Cat# 6294, 5 mg

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INTRODUCTION

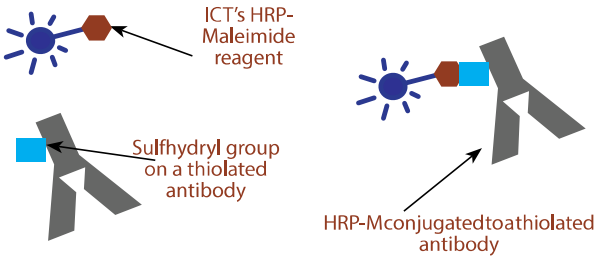
Horseradish peroxidase (HRP) enzymes are commonly used as signal reporters in ELISAs, Western blots, and other laboratory techniques. HRP reacts with a substrate (such as ICT's TMB 1-Component HRP Microwell Substrate, SUBT, catalog #6337) to generate the signal color in the assay that can be detected visually or quantified with a spectrophotometer.

To be useful in an assay, the HRP must first be conjugated to a protein or antibody. A stable HRP conjugation linkage is essential to generate reliable results in an assay. To facilitate the conjugation reaction with the antibody or protein, HRP can be linked in advance to maleimide groups, which can then subsequently react with free sulfhydryl groups on the target protein. ICT's Conjugation-Ready HRP-Maleimide (HRP-M) reagent makes it easy for scientists to create their own detection conjugates.

Maleimide conjugation linkers, like HRP-M, are quite stable at pH 6.5-7.5 and specifically react with free sulfhydryl groups (SH) on the target protein, forming a stable thioether linkage[1,2]. At this pH range of 6.5-7.5, maleimide is only minimally susceptibile to hydrolysis; this may be due to the fact that the maleimide ring structure is not directly linked to the aromatic cyclohexane ring[3]. (At more alkaline pH (>8.0), maleimide hydrolyzes at a faster rate into non-reactive maleamic acid. This reduces the efficiency of conjugation as the maleimide functional groups become inactivated prior to coupling to free SH on the antibody or protein.)

FIGURE 1: HRP-M CONJUGATED TO THIOLATED ANTIBODY

ICT's Conjugation-Ready HRP-Maleimide reagent (HRP-M) makes it easy for scientists to create their own detection conjugates. The maleimide is linked to the HRP, which facilitates a stable bond with sulfhydryl (SH) groups on the target antibody (shown) or protein.



Following ICT's protocol, the IgG or protein to be labeled must first be treated with Traut's Reagent (2-Iminothiolane) to introduce free thiols (SH groups) that enable maleimide mediated conjugation. Thiolated protein samples are subsequently incubated with HRP-M, leading to the covalent linkage of HRP to the antibody or protein target. This manual also includes a procedure to determine the ratio of HRP to protein to verify that the conjugation was successful (optional).

Older conjugation methods which utilized glutaraldehyde or periodate oxidation to form a Schiff-base have their drawbacks[4,5]. Glutaraldehyde methods are difficult to control as they often lead to excessive cross-linking and precipitation[2]. Periodate oxidation of the carbohydrate moiety of HRP can be a reasonably successful conjugation method[5], but this method also has its drawbacks. The oxidation step necessary to form aldehydes on HRP can also inactivate the enzyme, and the Schiff-base linkage must be reduced and stabilized to form a permanent covalent bond between the aldehyde on the HRP and the amino group on the protein.

Utilization of HRP-M to facilitate the conjugation reaction avoids HRP enzyme inactivation associated with periodate oxidation procedures, while still controlling the ratio of HRP to the target protein. The 2-step approach described here (thiolation then conjugation) eliminates the risk of uncontrolled cross-linking reactions. These lead to formation of large multimeric protein aggregates and subsequent precipitation events. This is an unavoidable occurrence when using glutaraldehyde techniques.

If you have any questions, please visit www.immunochemistry.com or call us at 1-800-824-8540.

This product is for research use only. Not for use in diagnostic procedures.

CONTENTS

- 1 vial of HRP-Maleimide (5 mg) #6294.
  Synonyms: HRP-M, Horseradish-Peroxidase-Maleimide.
  Molecular Wt: 40,000 plus 2-3 x 223 g/mole Maleimide
- Manual
- Desiccant

RECONSTITUTION

Just prior to addition to the thiolated antibody or protein, reconstitute HRP-M with 0.5 mL diH_2O. Do not reconstitute until immediately before it will be added to the thiolated protein. Once in an aqueous environment (pH 6.5-7.5), the reagent becomes reactive and the maleimide functional groups retain their ability to covalently react with free SH groups for about 2 hours at 30°C.

STORAGE

Store the lyophilized reagent at or below -20°C until the expiration date. It is often stable for 3 years or more when stored below -20°C.

SAFETY DATASHEETS (SDS)

SDS are available at www.immunochemistry.com.

REQUIRED MATERIALS

- Protein (5-10 mg/mL in PBS, pH 7.4) to be labeled with HRP
- PBS, pH 7.3
- G-25 desalting column
- PBS containing 5 mM EDTA, pH 7.3
- PBS containing 10 mM EDTA, pH 7.8
- Traut's Reagent (2-Iminothiolane)
- Spectrophotometer at A_{280} and A_{324}
- Dithiodipyridine (DTP), 5 mM
- DiH_2O
- Centrifuge concentrators
- L-Cysteine
- Iodoacetamide
- 1M boric acid, pH 9.7
- Dialysis supplies

PROTOCOL

A. Protein Thiolation

HRP-M specifically reacts with free sulfhydryl groups (SH) on the target protein, forming a stable thioether linkage at pH 6.5-7.5[1,2]. Most proteins possess very few free cysteine residues given their tendency to form disulfide linkages with other cysteine residues, leading to cystine structures. Therefore, it is often necessary to add free/reactive SH groups to the target protein. Several commercial thiolating reagents are available, including 2-Iminothiolane (Traut's Reagent) and N-Succinimidyl-S-acetylthioacetate (SATA). About 5-8 SH groups per IgG is recommended for good coupling to HRP-M. For this procedure, we use Traut's Reagent and IgG as the example protein.

1. Adjust the IgG/protein sample to a concentration of 5-10 mg/mL in PBS. If sodium azide is present, remove it by dialyzing the sample against PBS, pH 7.3 (4 hours at room temperature, or overnight at 2-8°C) as sodium azide will inhibit the thiolation reaction.
2. Equilibrate a G-25 desalting column in PBS with 5 mM EDTA, pH 7.3. This will be used to remove the free thiols from the sample. The size of the desalting column bed should be at least 20X the final volume of the thiolated protein solution.
3. Transfer the IgG/protein to a clean glass container with a stir bar.
4. Prepare a buffer of PBS with 10 mM EDTA at pH 7.8 to dissolve Traut's thiolation reagent.
5. Dissolve Traut's Reagent in the buffer described above to yield a stock concentration of 10 mg/mL.

FIGURE 2: THIOLATION

HRP-M links to proteins through free SH groups, which often must be added to the protein using Traut's reagent prior to conjugation.

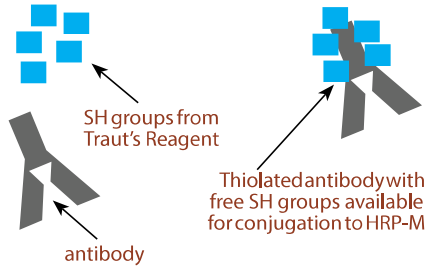
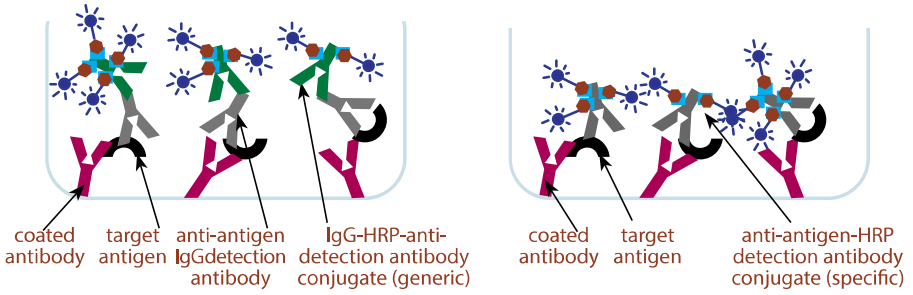


FIGURE 3: HRP CONJUGATE USED IN ANTIGEN DETECTION ELISA

HRP-conjugated antibodies are used in ELISAs to generate the signal in the assay. By directly conjugating the HRP to the specific top detection antibody (right), one layer of the sandwich can be eliminated, thereby greatly simplifying the development and optimization of the assay.



6. While stirring, add the 10 mg/mL Traut's stock at a 1:10 v/v ratio to the IgG. For example, add 100 µL Traut's to 900 µL IgG. This yields a final concentration of Traut's at 1 mg/mL in the reaction mixture.
7. While stirring, incubate under low light for 60 minutes at RT to allow the thiolation reaction to occur.
8. After the incubation period, ALL unreacted excess Traut's Reagent must then be completely removed from the IgG/protein sample for the HRP-M conjugation to succeed. To remove it, run the sample over the G-25 column (equilibrated in step 2) and collect the eluted protein, which comes off the column first. Monitor the fractions at A_{280} and put the fractions with higher protein concentrations on ice to preserve the free thiol integrity. Continue to monitor the fractions at A_{280} to verify that there is a clear separation between the IgG/protein fractions and the free Traut's Reagent.
9. Pool the fractions containing the highest protein concentrations into a polypropylene tube and put on ice. Record the total volume and concentration of the pool to determine the total mg of thiolated IgG/protein that was recovered. As an example, IgG has a $E_{0.1\%}$ (absorbance at 280 nm) value of 1.4 in most buffers, including PBS. To obtain the IgG concentration (mg/mL) in the pooled fractions of IgG-SH, simply divide the A_{280} absorbance value of the IgG-SH pool by 1.4 Absorbance Units/mg/mL for IgG.
   a. Total volume of pool: _____ mL.
   b. Concentration of pool: _____ A_{280} / 1.4 Absorbance units/mg/mL for IgG= _____mg/mL.
   c. Total mg recovered: _____ mL volume x _____ mg/mL = _____ mg.
10. To determine the number of thiol groups that were introduced to the IgG using Traut's, perform an optional thiolation quantitation assay. A simple, inexpensive method using dithiodipyridine (DTP) is outlined in section B.

B. OPTIONAL: Quantitation of Thiols using DTP

1. 5×10^{-6} mmole of protein is needed to perform the DTP assay. For IgG, this is equal to 0.75 mg. This is calculated by using 150,000 (1.5×10^5) as the typical molar weight reference point for IgG: 5×10^{-6} mmole x 1.5×10^5 mg/mmole IgG = 7.5×10^{-1} mg IgG = 0.75 mg IgG. Using the protein concentration that was determined above, calculate the volume of sample needed to obtain the required amount. For example, if the pool concentration of IgG-SH is 4 mg/mL, add 0.188 mL = 188 µL (0.75 mg ÷ 4 mg/mL = 0.188 mL).
   a. For IgG, calculate: 0.75 mg / _____ mg/mL concentration of pool = _____ mL.
2. Set up two 12x75 mm glass test tubes and label as "sample". Add the calculated volume of IgG-SH that will yield 0.75 mg IgG-SH. In most assays, this amount is usually less than 200 µL. Add PBS to bring the volume up to 200 µL. For example, if the calculated volume of IgG-SH is 188 µL, add 12 µL PBS to make the total volume 200 µL (200 µL - 188 µL= 12 µL).
3. Set up two 12x75 mm glass test tubes and label as "blank". Add 200 µL PBS to each tube.
4. Add 40 µL of 5 mM DTP to all tubes, mix, and incubate in the dark for 15 minutes at RT.
5. Add 760 µL of diH_2O to all tubes and mix. This brings the total volume in each tube to 1 mL.
6. Blank the spectrophotometer cuvette against each of the blank tubes at A_{324} OD; read the samples.
7. Calculate the molarity of thiol groups present in the IgG-SH prep. A good thiolation level for IgG coupling to HRP-Maleimide is about 5-8 SH per IgG. Determine the amount of SH-reacted DTP by dividing the A_{324} value of each sample tube by 19,800 (which is the molar extinction coefficient for SH-reacted DTP). The resulting molar value can be expressed as mmoles/mL, and is equivalent to the molarity of thiol groups in the IgG-SH solution. To determine the number of added thiols per IgG, simply divide this by 5×10^{-6} mmoles/mL (the molar amount of IgG used in the DTP assay).
   a. Calculate: _____ A_{324} / 19,800 = _____mmoles/mL of reacted DTP (molarity of thiol groups in the IgG-SH solution)
   b. Calculate: _____mmoles/mL / (5×10^{-6} mmoles/mL) = _____ thiols per IgG.

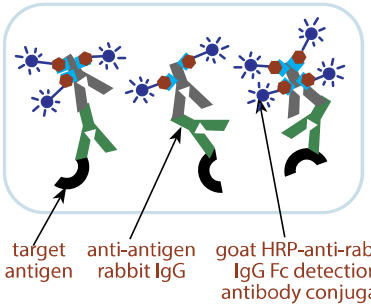
C. Conjugation of HRP-M to Thiolated IgG

1. Determine the number of HRP molecules to be conjugated to the thiolated IgG/protein. For many ELISA applications, a 4-fold molar excess of HRP over IgG is preferred. In the case of smaller proteins, it is often desirable to use an equal number of moles HRP per mole of protein.

2. Calculate the number of 5 mg lyophilized HRP-M vials that are required to give the desired molar excess ratio. Using IgG as an example, the molar weight of HRP is approximately 40,000 and the molar weight of IgG is approximately 150,000. Therefore, IgG-SH is approximately 4 times as large as HRP-M. This makes adding a 4-fold molar excess very easy; simply add 1 mg HRP-M for every 1 mg IgG-SH present in the pool. For example, if there is 10 mg IgG-SH in the pool, add 10 mg HRP-M for the coupling reaction.
3. Just prior to use, reconstitute the HRP-M. Each 5 mg vial should be reconstituted in 0.5 mL diH_2O.
4. To start the reaction, add a 4-fold molar excess (an equal weight ratio) of HRP-M to the IgG-SH pool and mix thoroughly in a polypropylene tube. Combine HRP-M with the protein/IgG as quickly as possible.
5. If desired, the reaction mixture can be concentrated to > 5-10 mg/mL using simple centrifuge concentration methods.
6. Incubate overnight in the dark at RT with gentle agitation on a rotator platform.
7. If not done earlier, concentrate the reaction mixture to > 5-10 mg/mL.

FIGURE 4: HRP CONJUGATE USED IN WESTERN BLOT

Conjugated antibodies are used in Western blots to generate the signal.

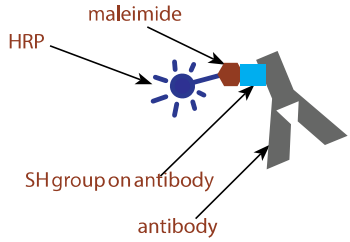


D. Termination of Maleimide Conjugation Reaction

1. Prepare 5 mL of 25 mg/mL L-Cysteine in PBS, pH 7.3.
2. Add a 5% v/v ratio of the L-Cysteine solution to the reaction mixture and mix in the dark for 15 minutes at RT. Recalculate the new reaction volume. For example, add 1 mL L-Cysteine to 19 mL reaction mixture yielding a total volume of 20 mL.
3. Prepare 2 mL of 50 mg/mL Iodoacetamide in PBS.
4. Based on the new reaction volume, add an 8% v/v ratio of the 50 mg/mL Iodoacetamide solution. Recalculate the new reaction volume. For example, add 1.6 mL 50 mg/mL Iodoacetamide to 20 mL reaction mixture yielding a total volume of 21.6 mL.
5. Prepare a solution of 1 M boric acid adjusted with 10N NaOH to pH 9.7.
6. To improve the Iodoacetamide reactivity, increase the pH by adding a 2% v/v ratio of 1 M boric acid, pH 9.7 and let it mix for 15 minutes at RT in the dark. Recalculate the new reaction volume. For example, add 0.43 mL 1 M boric acid to 21.6 mL reaction mixture yielding a total volume of 22 mL.
7. Concentrate the reaction mixture to a small volume appropriate for step 8.
8. Remove any free HRP enzyme that may not have bound to the IgG protein by performing a size exclusion gel filtration purification. Alternatively, to remove only the salts and components from the malemide reaction termination (but not the free HRP), dialyze the newly prepared HRP conjugate against PBS, pH 7.3.
9. Always store HRP conjugates at 2-8°C, protected from light. Alternatively, store as a concentrated conjugate stock in an appropriate HRP conjugate stabilizer (such as ICT's HRP Conjugate Stock Stabilizer, 5X, catalog #667).

FIGURE 5: HRP-CONJUGATED ANTIBODY

HRP-M makes it easy to create detection conjugates. The maleimide is linked to the HRP, which facilitates a stable bond with sulfhydryl groups on the target antibody (shown) or protein.



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