Antibody-HRP Conjugation Kit

Description

Antibody-HRP Conjugation Kits (Cat. 1D0570) provide sufficient reagents to perform two antibody-HRP conjugation reactions. Any suitable amount of antibody (100-500 µg) in a fixed volume of 100 µL (1-5 mg/mL) can be conjugated to pre-activated HRP in 60 minutes without the need for purification. Resulting conjugates average > 3.5 HRP per IgG and areready to use in many demanding applications including Westerns, ELISAs, and IHC.

Kit Contents:

Component	Concentration	Amount
Tetrazine-PEG_5-NHS ester M.W. (618.64)	-	2 x 0.5 mg
TCO-modified HRP (270 units/mg)	-	60 µL
DMSO	-	1 mL
BupH™ Saline Buffer Pack	-	1 pack
Zeba™ Spin Columns	-	4

Introduction

Antibody-HRP Conjugation kits provide all the necessary reagents to rapidly conjugate a pre-activated HRP to an antibody (IgG). Conjugation chemistry is based on the fastest, most efficient bioorthogonal reaction described to date, the inverse-electron demand Diels-Alder cycloaddition between tetrazines and *trans*-cyclooctenes (TCO). The extremely fast kinetics of this reaction enables rapid conjugation (30 min or less) at very low concentrations (e.g. 5 µM) in aqueous buffered media. The resulting antibody-HRP conjugate is cross-linked by a stable covalent bond (Figure 1). Significant advantages over maleimide/thiol conjugation chemistry include speed of reaction, conjugation efficiency, and long-term stability of tetrazine/TCO functional groups in aqueous buffered media (months at 4°C).

Important Information

- NHS esters are moisture-sensitive and readily hydrolyze. Avoid moisture condensation by allowing product to come to room temperature before opening. Prepare DMSO working stock solution immediately before use and discard unused portion. Hydrolysis is a competing reaction with primary amines of proteins/peptides.
- For NHS ester reactions, avoid buffers containing primary amines (e.g. Tris, glycine, or carrier proteins such as BSA).
- Reaction between tetrazine-modified antibody and TCO-modified HRP are complete in 1 hour
- Conjugates are ready to use without purification as a result of reaction efficiency.

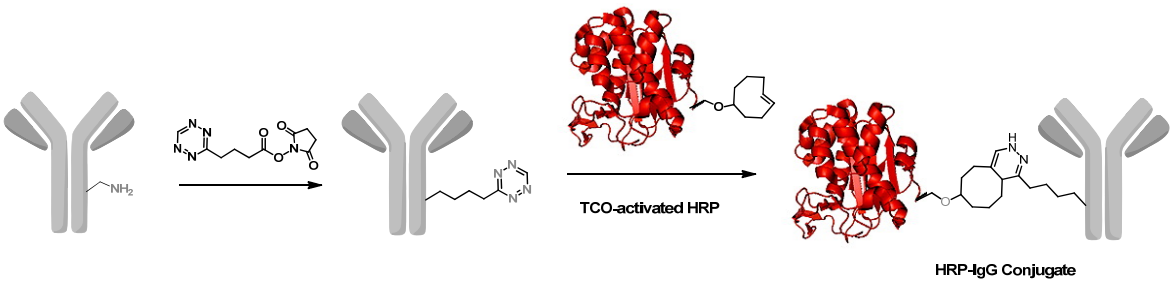


Figure 1. Modification of antibody with tetrazine and conjugation to TCO-activated HRP

Materials Required but Not Provided

- UV-VIS spectrophotometer
- Microcentrifuge capable of handling 1.5 mL tubes
- Quartz semi-micro cuvette (50-100 µL)
- 1.5 mL microfuge tubes
- Pipettes and tips (P-10, P-100, P-1000)
- Ultrapure water (e.g. 18 MΩ-cm)
- 6 N NaOH
- Beaker and stir bar

Material Preparation

A. BupHTM Buffer Preparation

1. Dissolve BupH™ dry-blend buffer pack (provided)into 480 mL ultrapure water. Adjust pH of the solution to 7.5 ± 0.05 with 6N NaOH. Adjust the final volume to 500 mL with ultrapure water. Do not add sodium azide or Proclin 300 preservatives as these reagents interfere with protein determination (A280).

B. Antibody Preparation

1. If the antibody (100-500 µg) is lyophilized and free of exogenous amines (e.g. glycine or Tris), resuspend in 100 µL BupH™ buffer (pH 7.5) to obtain a1-5 mg/mL solution. Proceed to Tetrazine Labeling of Antibody in Section E.
2. If the antibody (100-500 µg) is already in solution (e.g.100 µL PBS at 1-5 mg/mL), proceed to Tetrazine Labeling of Antibody in Section E.
3. If the antibody (100-500 µg) is already in a Tris or glycine containing buffer (100 µL), we recommend buffer exchange prior to Tetrazine labeling. Buffer exchange the antibody as described in Section C and D below.

C. Spin Column Equilibration into BupH™ (pH 7.5)

1. Twist off the column's bottom closure and loosen the cap (red). Place each column into a clean 1.5 mL microfuge tube.
2. Centrifuge column at 1,500 x g for 2 minutes to remove storage solution. Place a pen mark on theside of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing away from the center of the rotor in all subsequent centrifugation steps.Note-resin willappear white in color and compacted after centrifugation.

3. Add 0.3 mL BupH™ buffer (pH 7.5) to the top of spin column, replace the cap and loosen.
4. Centrifuge at 1500 x g for 2 minutes to remove buffer.
5. Repeat steps 3 and 4 two additional times, discard buffer from collection tube after each spin.
6. Transfer the now equilibrated spin column (resin appears white and dry) into a clean 1.5 mL microfugetube and proceed to buffer exchange of antibody as describe in Step D below.

D. Buffer Exchange of Antibody

1. Buffer exchange antibody into BupH™ equilibrated spin column by slowly applying 100 µL antibody solution (1-5 mg/mL) to the center of spin column resin bed.
2. Centrifuge at 1,500 x g for 2 minutes. Retain the eluate (100µL) at bottom of 1.5 mL collection tube.
3. Antibody (100 µL) is now buffer exchanged into BupH™ (pH 7.5).

E. Tetrazine Labeling of Antibody

1. Select the volume DMSO required to dissolve Tetrazine-PEG_5-NHS reagent according to Table 1.

Table 1.

Staring amount of antibody (µg)	Volume of DMSO required to dissolve Tetrazine-PEG5-NHS Reagent (µL)
100	300
150	225
200	150
250	125
300	100
350	87.5
400	75
450	67.5
500	60

2. Add the indicated volume DMSO to tetrazine reagent, vortex for 1 minute to fully dissolve.
3. Start the antibody labeling reaction by adding 5 µL of dissolved tetrazine reagent to 100 µL antibody in BupH™ (or PBS).
4. Allow reaction to proceedfor 60 minutes at room temperature.
5. Remove excess labeling reagent from the reaction with a new spin column as described in Sections and D.
6. Tetrazine-modified antibody (100 µL) is now ready for conjugation to HRP-TCO.

F. Antibody-HRP Conjugation

1. Select the volume of TCO-activated HRP required for conjugation to the available amount of antibody according to Table 2.

Table 2.

Starting amount of antibody (µg)	Volume TCO-modified HRP required for conjugation to antibody (µL)
100	6
150	9
200	12
250	15
300	18
350	21
400	24
450	27
500	30

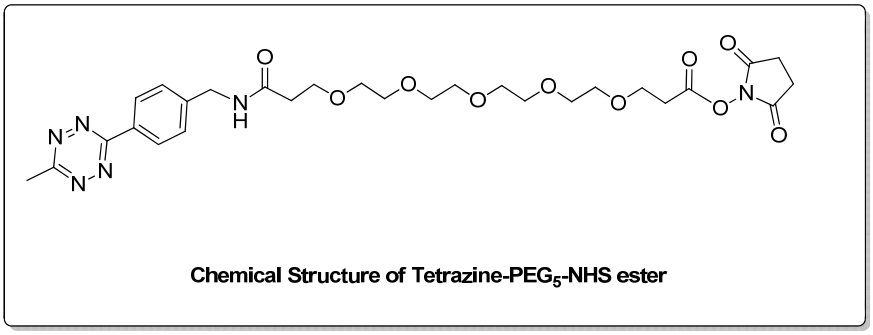
2. Add required volume TCO-HRP (provided) to tetrazine-modified antibody according to Table 2.
3. Allow conjugation reaction to proceed for 60 minutes at room temperature.
4. Conjugate is now ready for use. Determine conjugate concentration as follows:
   a) Transfer a small aliquot of conjugate(5-10 µL) into suitable volume BupH™ (1:10 dilution).
   b) Place diluted conjugate into semi-micro cuvette, measure A280on spectrophotometer.
   c) Calculate conjugate concentration (mg/mL):A280 x dilution factor x 1.5 = mg/mL
5. Store antibody-HRP conjugate at 4°C. For long-term storage, dilute the conjugate into a suitable HRP stabilizer such as Stabilzyme™ HRP stabilizer (Trademark of Surmodics, Inc.).

Troubleshooting

Problem	Possible Cause	Solution
Little or no conjugate is formed	Antibody concentration is incorrect.	Confirmpurity and proper concentration of antibody prior totetrazine labeling.Buffer exchange antibody into BupH(pH 7.5) if necessary.
	Tetrazine-PEG5-NHS-ester is hydrolyzed	Allow product to equilibrate to room temperature before opening. Avoid exposure to moist conditions.
		Avoid buffers containing primary amines such as Tris and glycine. Buffer exchange antibody before labeling if necessary.
	Excess labeling reagent improperly removed.	Remove excess un-reacted tetrazine by spin column procedure.

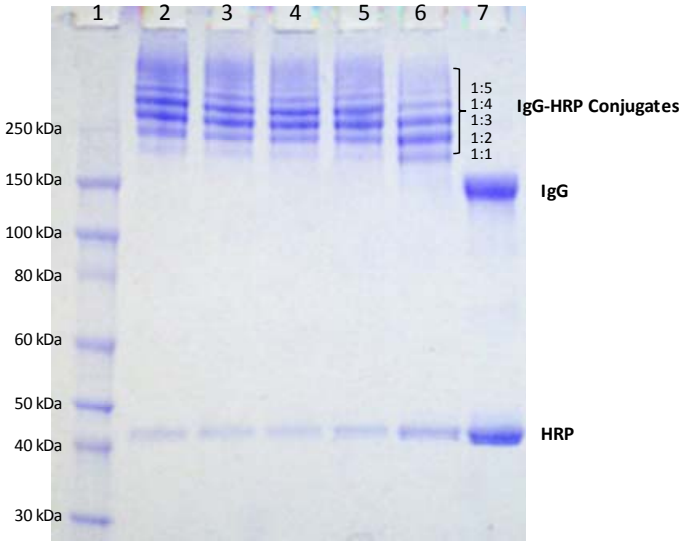
References

1. Blackman, M. L., *et. al.* (2008). "Tetrazine Ligation: Fast Bioconjugation Based on Inverse- Electron-Demand Diels-Alder Reactivity." *J. Am. Chem. Soc*., **130**:13518-13519.
2. Devaraj, N. K., *et. al.* (2008) "Tetrazine-Based Cycloadditions: Application to Pretargeted Live Cell Imaging." *Bioconjugate Chem.,* **19(12)**: 2297–2299.
3. Devaraj, N. K., *et. al.* (2009) "Fast and Sensitive Pre-Targeted Labeling of Cancer Cells through a Tetrazine/*trans*-Cyclooctene Cycloaddition." *Angew. Chem. Int. Ed.,* **48(38):** 7013-7016.



Chemical Structure of Tetrazine-PEG_5-NHS ester

Example #1: Five Goat IgG samples (100 µL at 5mg/mL, 4 mg/mL, 3 mg/mL, 2 mg/mL and 1 mg/mL)were labeled as described with tetrazine reagent. After 60 minutes, excess reagent was removed and the antibody conjugated to HRP-TCO for 60 minutes. Aliquots of each reaction were then analyzed by SDS-PAGE



1. Protein M.W. Markers
2. GIgG-TET @ 5 mg/mL + HRP-TCO (5 µg final rxn @ 60 minutes)
3. GIgG-TET @4 mg/mL + HRP-TCO (5 µg final rxn @ 60 minutes)
4. GIgG-TET @ 3 mg/mL + HRP-TCO (5 µg final rxn @ 60 minutes)
5. GIgG-TET @ 2 mg/mL + HRP-TCO (5 µg final rxn @ 60 minutes)
6. GIgG-TET @ 1 mg/mL + HRP-TCO (5 µg final rxn @ 60 minutes)
7. GIgG @ 3 mg/mL + HRP-TCO (5 µg final rxn @ 60 minutes) Neg. Control