SoluLinK
The Conjugation Company™

# How to HRP Label and Purify Your Antibody - No HPLC Needed.

## Introduction

Horseradish peroxidase has long been used as a colorimetric marker for antibody-based antigen detection assays- ELISAs, Westerns, Immunohistochemistry. It may be either directly conjugated to the antibody of interest or linked to a secondary antibody targeting the antibody of interest.

Secondary conjugates are often employed to target a species specific antibody of interest (e.g., mouse, rabbit, goat, etc.). This method may save the researcher precious time in conjugation but secondary antibodies have their drawbacks. Secondary antibodies are known to significantly increase background signal through non-specific binding to antibody/antigen surfaces. For producing maximum specificity, with high signal to noise, it is always best to use direct primary antibody conjugates.

When making a primary antibody-HRP conjugate there are several important factors to consider:

- maintaining the specificity of the antibody for its target
- maintaining high HRP activity to optimize assay signal
- production of high purity conjugate free of contaminating antibody and HRP, either of which compromise assay performance

## Classical Methods for Conjugation of HRP to Antibody

### 1) Reductive amination using Cyanoborohydride- Bad for HRP activity

HRP is a heavily glycosylated enzyme and this method of conjugation activates the polysaccharides through oxidation with periodate converting the sugars to aldehydes. The HRP then has a plethora of available aldehyde groups that can be used to conjugate an antibody of interest. The amine groups of the antibody then form a Schiff base with the aldehydes which are then reduced using sodium cyanoborohydride (Figure 1).

Oxidation of HRP is known to reduce the activity of the enzyme, thus lowering signal in an assay. Even gentle oxidative methods lower HRP activity by as much as 30-50%. Any conjugation method that can activate HRP in a gentler fashion will better maintain HRP activity, leading to higher signals.

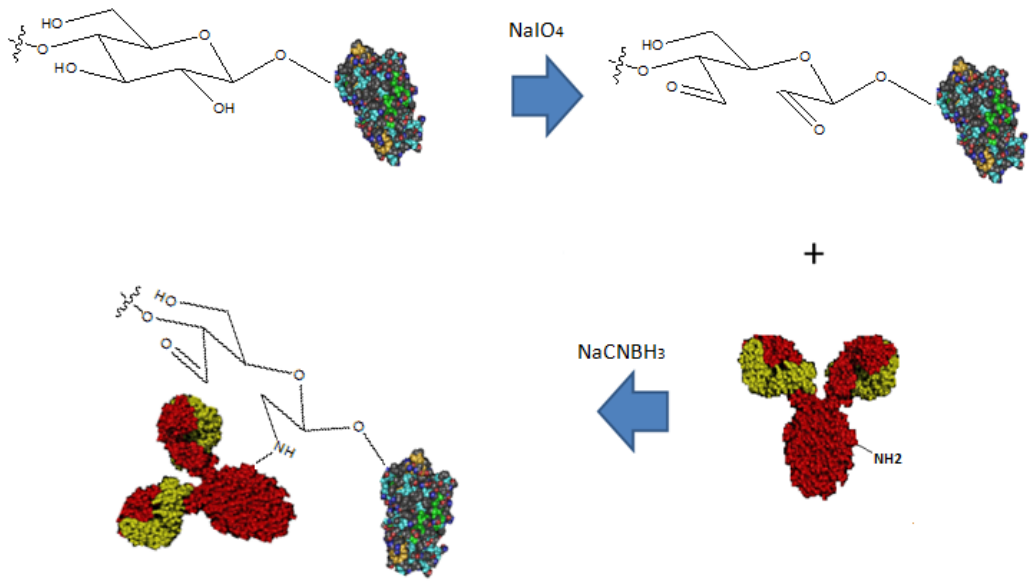



**Figure 1:** HRP-Antibody conjugation using reductive amination. The reductive amination coupling forms secondary amine linkages.

## 2) SMCC activated HRP + 2-MEA activated antibody- Bad for antibodies

2- MEA(2- mercaptoethylamine) reduces the hinge disulfide bonds, which breaks down the antibody creating a piecemeal conjugate with both light and heavy chain intermediates conjugated to HRP, lowering the affinity of the antibody for antigen (Figure 2). This method of antibody activation produces a conjugate but it can significantly reduce the antibody's avidity for cognate antigen.

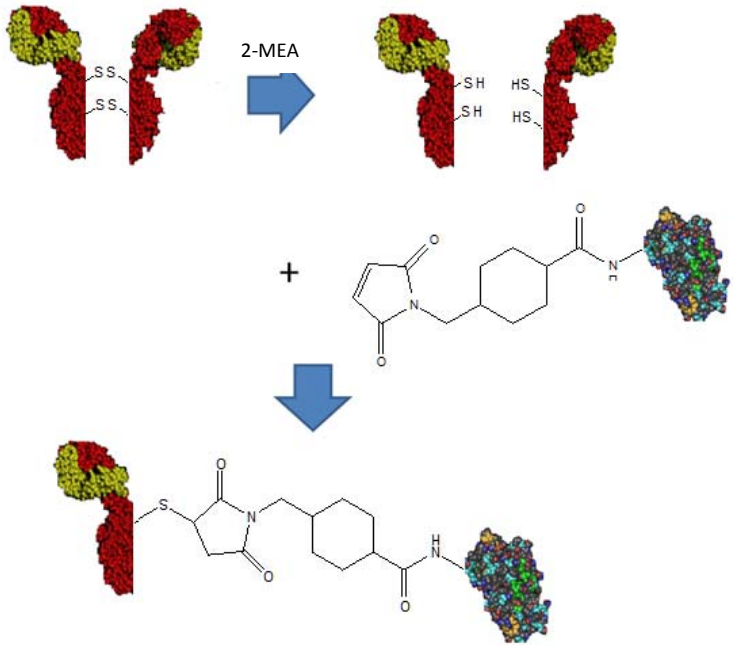



**Figure 2:** Antibody conjugation with SMCC and 2-MEA. Reaction of the reduced antibody with a maleimide-activated enzyme creates a conjugate through thioether bond formation.

**3) SMCC activated HRP + SATA/ SPDP, or Iminothiolane (Trauts reagent) activated Antibody- Bad for antibodies**

This method is by far the best of the classical methods for conjugating antibodies to HRP but it still has serious drawbacks.

**SATA**- this linker requires additional steps to deprotect the thiol using hydroxylamine; a harsh nucleophile that can affect binding affinity of the antibody (Figure 3).

**SPDP**- Requires an additional reducing agent to activate thiols- can reduce disulfides leading to light and heavy chain breakdown products.

**Iminothiolane (Trauts reagent)-** requires a large excess of iminothiolane to ensure sufficient conjugation- can over modify the antibody resulting in loss of affinity. Additionally the iminothiolane activated antibody spontaneously reacts forming undesirable homodimers that can lead to loss of signal.

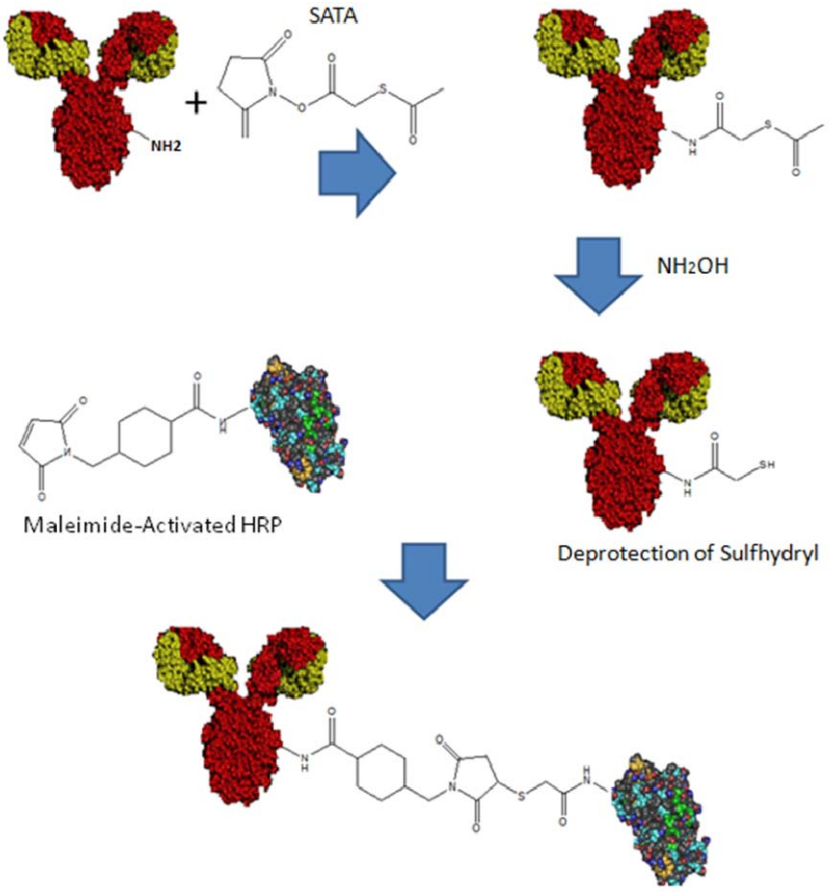



**Figure 3:** SATA activation of the antibody. Reaction of the thiolated antibody with a maleimide-activated enzyme results in thioether cross-links.

None of the three conjugation methods are quantitative in nature and they typically leave unreacted antibody and HRP, making it difficult to purify the final conjugate. Both Method 2 and 3 are based on maleimido-thiol conjugation. Most maleimido conjugation reactions are slow, inefficient and are known to leave a significant amount of unconjugated antibody. Free antibody will preferentially bind antigen, outcompeting binding of the conjugate, lowering signal to noise. Even the presence of 5 or 10% free antibody can lower assay sensitivity by 30-40%!

Excess enzyme is generally used to drive HRP conjugation reactions. Due to the large excess used, the enzyme is often difficult to purify away from conjugate. Many assays have washing steps, but non-specific binding of residual HRP can contribute to higher background. A conjugate free of excess HRP decreases background and leads to higher signal to noise.

## SoluLink HRP Conjugation Products; Solving the problems.

As you can see there are many classical ways of conjugating antibody to HRP, but each has its drawbacks and limitations. SoluLink offers the only comprehensive solution to all these problems in a single "All-in-One" kit. Solving all these problems with a single kit can help you **achieve your ultimate research goals** while saving you time, money, antibody, and other valuable resources.

### SoluLink's Chemistry: non-reducing, gentle method for antibodies.

The HRP Conjugation kit was designed using SoluLinK's proven HydraLink™ chemistry. This chemistry employs the reaction between an aromatic hydrazine and an aromatic aldehyde leading to the formation of stable hydrazone bonds (Figure 4). This bond formation is catalyzed by aniline for rapid, high yielding conjugations. All of the conjugation steps are performed under gentle pH conditions, pH 6.0-7.4, with no harsh chemicals or reducing agents.

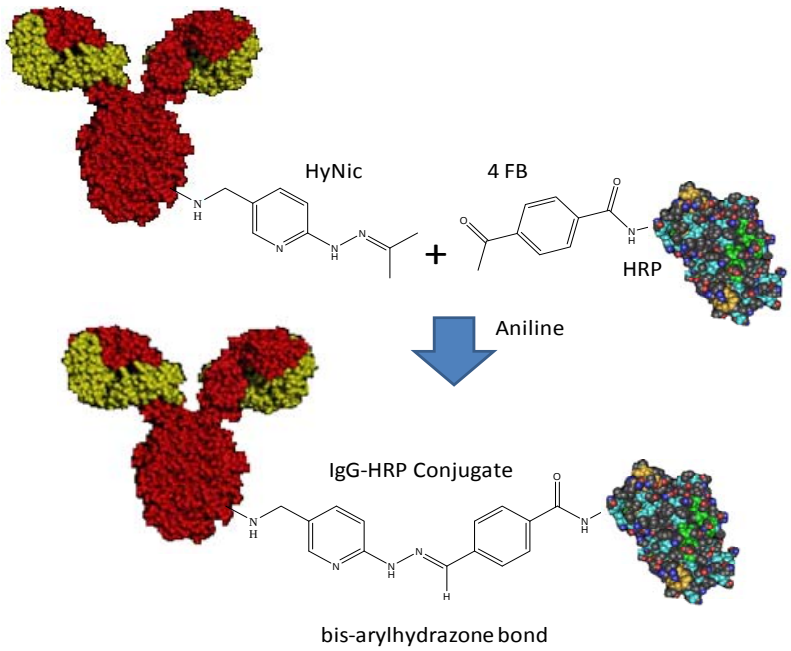



**Figure 4:** SoluLink HydraLink™ method for conjugating for conjugating HyNic-modified antibody with pre-activated 4FB-HRP.

## SoluLink's Pre-Activated HRP: maintains high enzyme activity

The SoluLink method pre-activates high activity HRP (> 250U/mg) with a stable 4FB linker (4-formylbenzamide). The 4FB group is reactive only with hydrazine groups and therefore will not react with any other functional groups on the enzyme. 4FB is not susceptible to hydrolysis- no freeze drying or lyophilization is necessary- thus providing a high solution-stable pre-activated HRP enzyme and subsequently a high activity conjugate. (Figure 5).

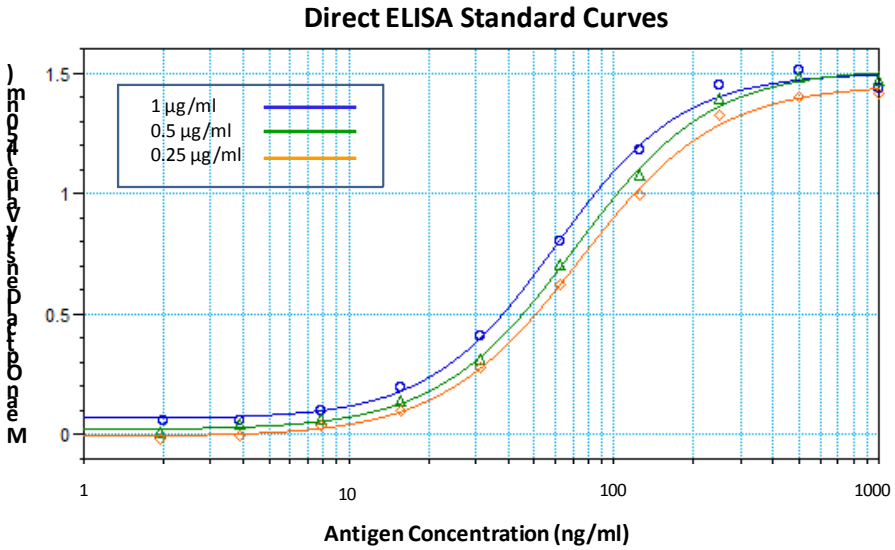



**Figure 5:** Direct *ELISA* curves generated using an HRP conjugate made with the All-in-One kit. Immobilized antigen was then detected at 3 different conjugate concentrations (1 µg/ml. 0.5 µg/ml. 0.25 µg/ml) using TMB substrate (20 minutes @ 450 nm) on a Molecular Devices plate reader

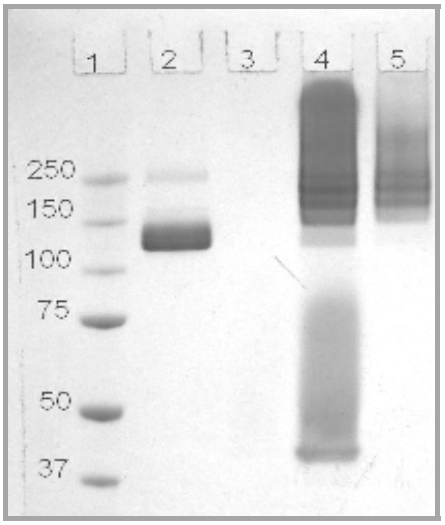
**SoluLink's Catalyst: efficiently drives the antibody reaction to completion**

HydraLinK™ is the only catalyzed conjugation chemistry capable of quantitatively converting 100% of an antibody to its conjugate form. Improvements to HydraLink™ chemistry now include the discovery that the aromatic compound, aniline, catalyzes the reaction between aromatic aldehydes and aromatic hydrazines (1, 2, 3). Aniline increases both the rate and efficiency of conjugate formation under mild reaction conditions; leading to quantitative conversion of free antibody to HRP.

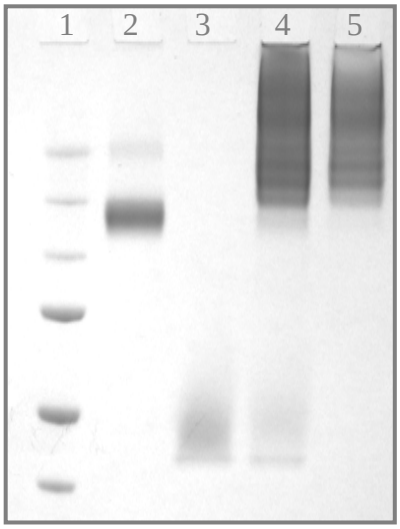
**SoluLink's Purification: rapid spin columns remove all unreacted HRP from crude conjugate reactions**

Quantitative conversion of the antibody to conjugate greatly simplifies conjugate purification. SoluLink has developed a novel spin column that quantitatively remove excess HRP to provide high purity, ready-to use conjugate (Figure 6).

Panel A




Panel B




**Panel A**- Mouse Anti-FITC-HRP Conjugate

1. Protein m.w. marker
2. HyNic-modified Mouse Anti-FITC Monoclonal (5µg)
3. 4FB-modified HRP (5 µg)
4. All-in-One mouse Anti-FITC-HRP conjugation reaction (crude) (10 µg)
5. All-in-One spin column purified mouse Anti-FITC-HRP conjugate (crude reaction purified ~5 µg)

**Panel B**- Bovine IgG-HRP Conjugate

1. Protein m.w. marker
2. HyNic-modified Bovine IgG (5µg)
3. 4FB-modified HRP (5 µg)
4. All-in-One Bovine IgG-HRP conjugation reaction (crude) (10 µg)
5. All-in-One spin column purified Bovine IgG-HRP conjugate (crude reaction purified ~5 µg)

**Figure 6:** Coomassie-stained (4-12% SDS-PAGE) gels illustrate typical conjugation results using the SoluLink conjugation method.

**Conclusion:**

Primary antibody conjugations *can* be easier to perform reliably without harming your antibody or enzyme reporter. SoluLink's HRP-Antibody All-in-One conjugation Kit will allow you to easily make and purify your high quality conjugate, leaving you more time and resources to focus on your research.

**Recommended Products:**

[A-9002-002] HRP-Antibody All-in-One Conjugation Kit
[S-9002-1] S-HyNic Conjugation Kit
[S-1002-105] S-HyNic Conjugation Reagent

**References:**


**1.** ***Dirksen, A., Hackeng, T., Dawson, P.,(2007).*** *Nucleophilic Catalysis of Oxime and Hydrazone Reactions by Aniline. ACS Poster*

**2.** ***Dirksen, A., Hackeng, T., Dawson, P., (2006).*** *Nucleophilic Catalysis of Oxime Ligations. Angew. Chem. Int. Ed. 45, 7581-7584*

**3.** ***Dirksen, A., Dirksen, S., Hackeng, T., Dawson, P (2006).*** *Nucleophilic Catalysis of Hydrazone Formation and Transimination: Implications for Dynamic Covalent Chemistry. JIAICIS Communications.*