WHITE PAPER

How to Prepare Quantifiable HRP Conjugates

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Introduction

Horseradish peroxidase (HRP) has long been used as a colorimetric marker for antibody-based antigen detection assays such as ELISAs, western blots, and immunohistochemistry (IHC). 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.

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 purity to optimize assay signal
- Production of high-purity conjugate free of antibody and HRP contaminants, both 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.

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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 to create a piecemeal conjugate with both light- and heavy-chain intermediates conjugated to HRP and lowers the affinity of the antibody for antigens (Figure 2). This method of antibody activation produces a conjugate, but it can significantly reduce the antibody's avidity for antigens.

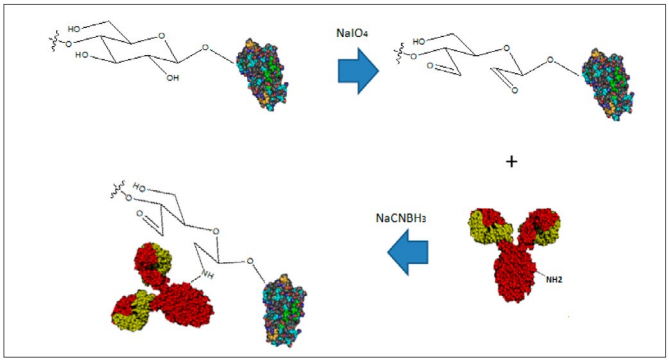


Figure 1 *HRP-Antibody conjugation using reductive amination. The reductive amination coupling forms secondary amine linkages.*

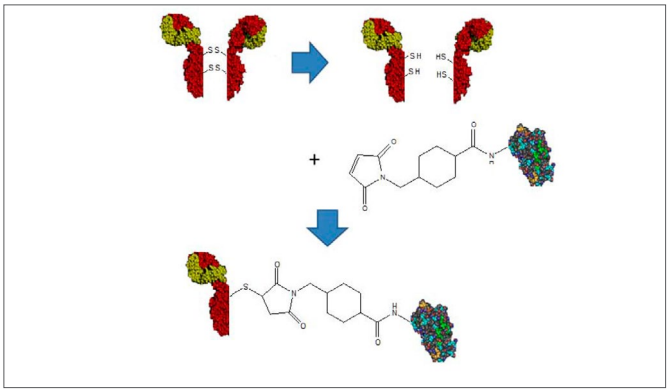


Figure 2 *Antibody conjugation with SMCC and 2-MEA. Reaction of the reduced antibody with a maleimide-activated enzyme creates a conjugate through thioether bond formation.*

3. SMCC activated HRP + SATA/SPDP, or iminothiolane (Traut's 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 (Traut's 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.

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 methods 2 and 3 are based on maleimide-thiol conjugation. Most maleimide conjugation reactions are slow, inefficient, and known to leave a significant amount of unconjugated antibody. Free antibody will preferentially bind antigens, outcompeting binding of the conjugate, and reducing the signal-to-noise ratio. Even the presence of 5–10% 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.

HRP conjugation methods solve the problems.

As you can see there are many classical ways of conjugating antibody to HRP, but each has its drawbacks and limitations. SoluLINK® bioconjugation technology offers a comprehensive solution to all these problems and is available in a single "All-in-One" kit (purification included). 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 Bioconjugation Technology—a non-reducing, gentle method for antibodies.

The HRP-Antibody All-in-One™ Conjugation Kit with TurboLINK™ catalyst was designed using SoluLINK bioconjugation technology. This technology 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 TurboLINK catalyst buffer 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.

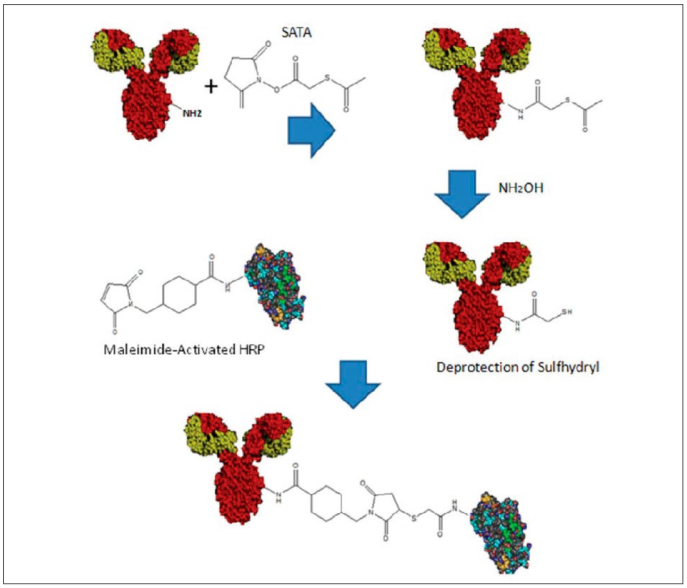


Figure 3 *SATA activation of the antibody. Reaction of the thiolated antibody with a maleimide-activated enzyme results in thioether cross-links.*

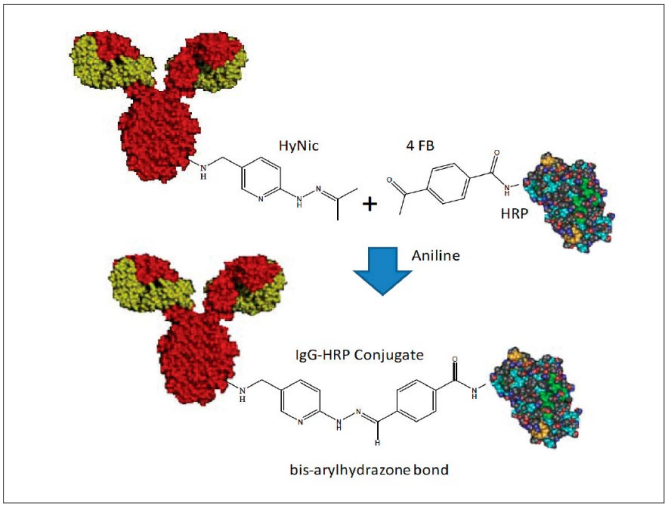


Figure 4 *SoluLINK bioconjugation technology for conjugating HyNic-modified conjugating antibody with pre-activated 4FB-HRP.*

Pre-activated HRP maintains high enzyme activity.

This method pre-activates high-activity HRP (>250U/mg) with a stable 4-formylbenzamide (4FB). 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 and no freeze drying or lyophilization is necessary, thus providing a highly solution-stable, pre-activated HRP enzyme and, subsequently, a high-activity conjugate. (Figure 5).

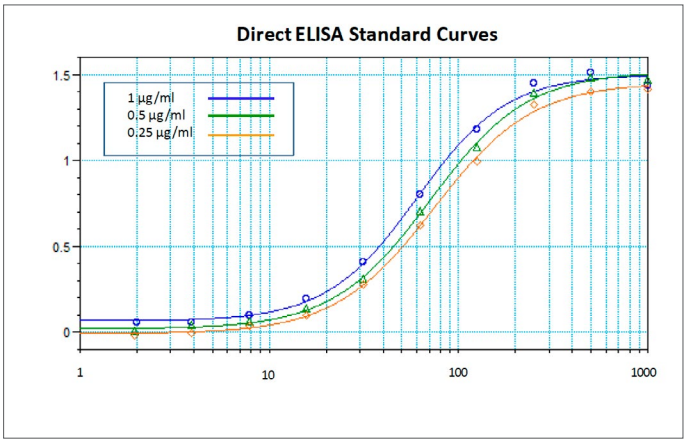


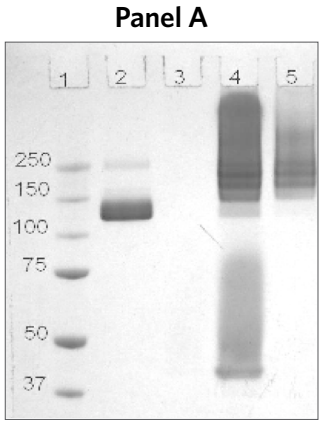
Figure 5 *Direct ELISA curves generated using an HRP conjugate made with the All-in-One kit. Immobilized antigen was then detected at 3 different conjugate concentrations (1 µg/ml, 0.5 µg/ml, 0.25 µg/ml) using TMB substrate (20 min at 450 nm) on a Molecular Devices plate reader.*

TurboLink Catalyst Buffer efficiently drives the antibody reaction to completion.

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

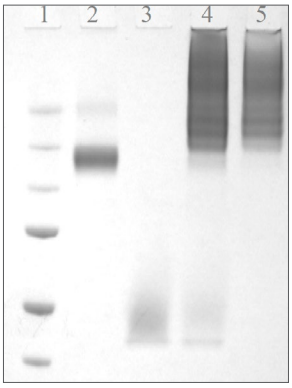
Purification process—rapid spin columns remove all unreacted HRP from crude conjugate reactions.

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



Panel A: Mouse Anti-FITC-HRP Conjugate

1. Protein MWt 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 MWt 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 bioconjugation technology.*

Conclusion

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

Recommended Products

- [A-9002-001] HRP-Antibody All-in-One™ Conjugation Kit
- [S-1002-105] S-HyNic Linker (DMF Soluble)

Published References

1. Dirksen A, et al. 2007. Nucleophilic Catalysis of Oxime and Hydrazone Reactions by Aniline. *ACS*
2. Dirksen A, et al. 2006. Nucleophilic Catalysis of Oxime Ligations. *Angewandte Chemie International Edition.*
3. Dirksen A, et al. 2006. Nucleophilic Catalysis of Hydrazone Formation and Transimination: Implications for Dynamic Covalent Chemistry. *Journal of the American Chemical Society.*

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