

# Quantitative and Reproducible Bioconjugation with SoluLINK® Technology

## Introduction

Bioconjugation is the chemical linking of two biomolecules to form a single hybrid that retains the biological activity of each component, yet provides a novel function that is not possible with each individual biomolecule. Most complex biomolecules, such as proteins, exist and function only in aqueous environments. For this reason, the preparation of bioconjugates must be carried out in aqueous solutions, and any suitable bioconjugation chemistry must preserve the biological activity and function of the biomolecules in this type of environment. Conjugates are generally formed through the addition of separate but complementary functional groups to each of the two biomolecules. These functional groups are typically introduced through a process called modification, which consists of attaching linkers to an amine or thiol group present on the biomolecules of interest. The two modified biomolecules are then mixed together to form the desired bioconjugate via the complementary linkers incorporated during modification. Figure 1 presents a typical workflow for this modification and conjugation process.

The conjugation and immobilization of biomolecules has been historically problematic, primarily due to the fact that few covalent bond-forming reactions occurring in water can also be engineered to link biomolecules together in a mild and controllable way. Several methods to conjugate and immobilize biomolecules are described in various publications, but most of these methods are difficult to reproduce, stoichiometrically inefficient, and result in low conjugate yields.

When choosing a conjugation strategy, it is important to keep in mind the characteristics that make a desirable bioconjugation technology. The following list presents the criteria required for development:

- Linkers must be incorporated on biomolecules in a mild, controllable reaction in aqueous solution, in a manner which maintains the biomolecules' inherent function.
- Conjugate bonds should only form between the two complementary linkers incorporated on biomolecules during modification, and not through endogenous functional groups found within the biomolecules themselves.
- Linker incorporation and conjugate formation should be easily quantifiable through simple and non-destructive methods such as spectrophotometry.
- Linker-modified biomolecules and the conjugate linkage itself should be stable under a broad pH range and at elevated temperatures.
- Conjugation reaction kinetics should be fast and stoichiometrically efficient.

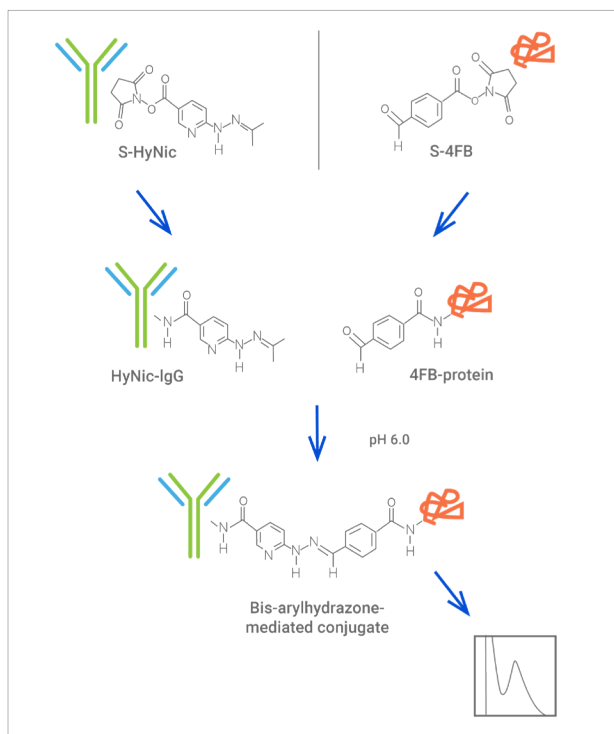


Figure 1: SoluLINK bioconjugation workflow.

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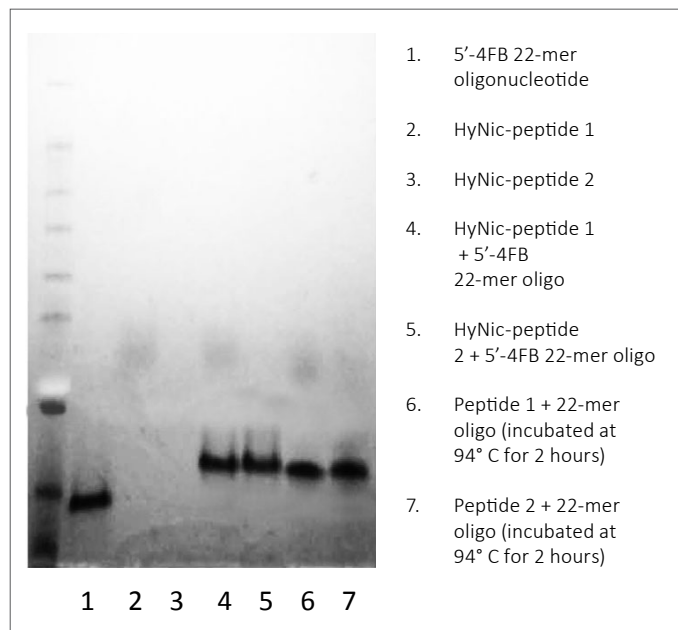
- f. Linkers should be easily incorporated on a variety of biomolecules, including oligos and peptides during solid phase synthesis.
- g. The conjugation reaction should occur directly upon mixing the two modified biomolecules, without the addition of any agent that could disrupt their function such as an oxidant, reductant, or metal.
- h. There should be no undesirable covalent side reactions during modification or conjugation.

SoluLINK bioconjugation technology is the only technology that meets these stringent requirements. It mildly, efficiently, and reproducibly conjugates and immobilizes all categories of biomolecules, including proteins, peptides, oligonucleotides, carbohydrates, drugs, and surfaces. This chemistry is based on the reaction of an aromatic hydrazine with an aromatic aldehyde, which forms a stable bis-arylhydrazone conjugate bond (Figure 1). When functionalizing amines, S-HyNic is used to incorporate 6-hydrazinonicotinamide (HyNic) groups. This linker possesses a succinimidyl ester (NHS ester) that readily reacts with amino groups on proteins, other amine-containing biomolecules, and surfaces using standard NHS ester reaction conditions (e.g., phosphate buffer, pH 7.5–8.0). The aromatic aldehyde is incorporated on biomolecules with S-4FB (Figure 1) in the same manner.

For functionalizing biomolecules through free thiol groups, a thiol-reactive maleimide version of HyNic (MHPH; 3-N-Maleimido-6-hydrazinumpyridine hydrochloride) is available to incorporate HyNic linkers. This reagent is especially useful for site-specific conjugation of antibodies through their hinge region after mild reduction with DTT or TCEP to produce antibody-drug conjugates, or for conjugation of biomolecules known to contain a free cysteine or reducible disulfide.

Simple mixing of a HyNic-modified biomolecule with a 4FB-modified biomolecule in a mildly acidic buffer (e.g., phosphate buffer, pH 6.0) yields the desired conjugate. The hydrazine functional group of S-HyNic is protected as its acetone hydrazone, and is in equilibrium with the free reactive hydrazine in aqueous solution. Thus, the HyNic group is free to react with aromatic aldehydes without a separate deprotection step, spontaneously forming the stable bis-arylhydrazone. Unlike aliphatic hydrazones, this bis-arylhydrazone bond has been demonstrated to be both heat and pH stable (95°C for 2 hours; pH 2–10) as shown in Figure 2.

SoluLINK linkers are available in additional formats that further extend the versatility of this technology. These include a cleavable disulfide linker (S-SS-4FB) and water soluble sulfo-NHS ester versions of both HyNic and 4FB. Furthermore, HyNic moieties are readily incorporated on peptides during solid



**Figure 2.** PAGE analysis of peptide-oligonucleotide conjugates prepared with SoluLINK technology. Conjugation of two N-terminus HyNic-modified peptides with a 5'-4FB-modified 22-mer oligo to yield the peptide-oligo conjugates (lanes 4 and 5). Conjugate formation was quantitated using a 1:2 reaction stoichiometry of oligo to peptide. Conjugate bond stability was tested at 95°C in PBS for two hours (Lanes 6 and 7). Visualization by UV backshadowing.

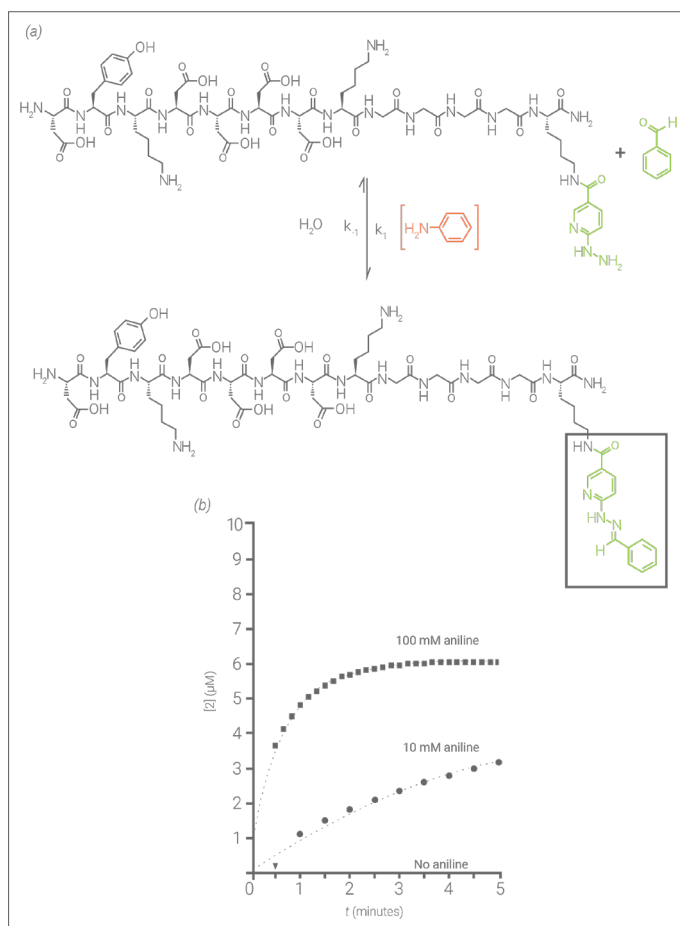
phase peptide synthesis using Boc-HNA. Similarly, 4FB moieties are readily incorporated using A4FB phosphoramidite during solid phase oligonucleotide synthesis.

Another extremely useful characteristic of the SoluLINK bis-arylhydrazone bond is its ability to form a traceable chromophore. This chromophore absorbs maximally at 354 nm with a molar extinction coefficient of 29,000 L·mol<sup>-1</sup>·cm<sup>-1</sup>. This unique feature is exclusive to SoluLINK bioconjugation technology, and it allows for:

1. Facile quantification of the number of linkers incorporated on each biomolecule prior to conjugation.
2. Monitoring of the conjugation reaction in real time to assess its progress.
3. Direct quantification of the number of linkages formed in the conjugate and the precise number of biomolecules or ligands attached.
4. Visualization of the conjugate peak during FPLC or HPLC purification and identification of fractions containing the desired conjugate.
5. Easy and non-destructive quantification.

These properties enable an unprecedented level of control over the linker incorporation ratio on each biomolecule, as well as the degree of conjugation between the biomolecules. Once a certain hydrazone absorbance threshold has been reached in the conjugation process, the reaction may be stopped by quenching with excess sulfolbenzaldehyde to cap any remaining HyNic groups. In this way, a pre-defined level of conjugate formation may be obtained with a high degree of reproducibility from batch-to-batch. This level of control is especially useful during GMP manufacturing.

Fast conjugations are desirable for increasing conjugation efficiency and lowering reagent costs. Dirksen et al.<sup>1,2</sup> showed that aniline significantly accelerates the formation of the bis-arylhydrazone bond (Figure 3, with permission). This striking increase in reaction rate facilitates the conjugation of large biomolecules and the immobilization of biomolecules on surfaces, which has traditionally been very slow and inefficient. As demonstrated in Figure 3, the conjugation of a HyNic-peptide to 4FB proceeds essentially to completion within three minutes in the presence of 100 mM aniline (SoluLINK TurboLINK® Buffer). The high efficiency of the conjugation reaction results in reduced conjugation time, easier purification of the resulting conjugate, significant material cost savings, and better reproducibility.



**Figure 3.** Conjugation of HyNic-peptide with benzaldehyde in the absence and presence of aniline catalyst (a). Product formation was monitored in real time by UV-Vis spectrophotometry of the resulting chromogenic conjugate bond at 340 nm (b).

## Other methods used to prepare covalent conjugates involve azide-alkyne “click” chemistry or maleimide-thiol coupling.

### Disadvantages of Click Chemistry

- The need for heavy metal catalysts that can degrade biomolecules. For example, copper can cause strand breaks in DNA and RNA, as well as oxidation of amino acids in proteins, through formation of reactive oxygen species.
- Non-catalyzed click reactions based on strained cycloalkynes are kinetically slower than copper catalyzed reactions, and are therefore less efficient.
- Certain cyclooctyne linkers can react with cysteine residues on proteins, diminishing the bio-orthogonality of the reaction and possibly degrading biomolecule function.
- Strained cycloalkynes are typically quite hydrophobic, and may lead to biomolecule aggregation or precipitation, as well as loss of function upon coupling of this linker.

### Disadvantages of Maleimide-Thiol

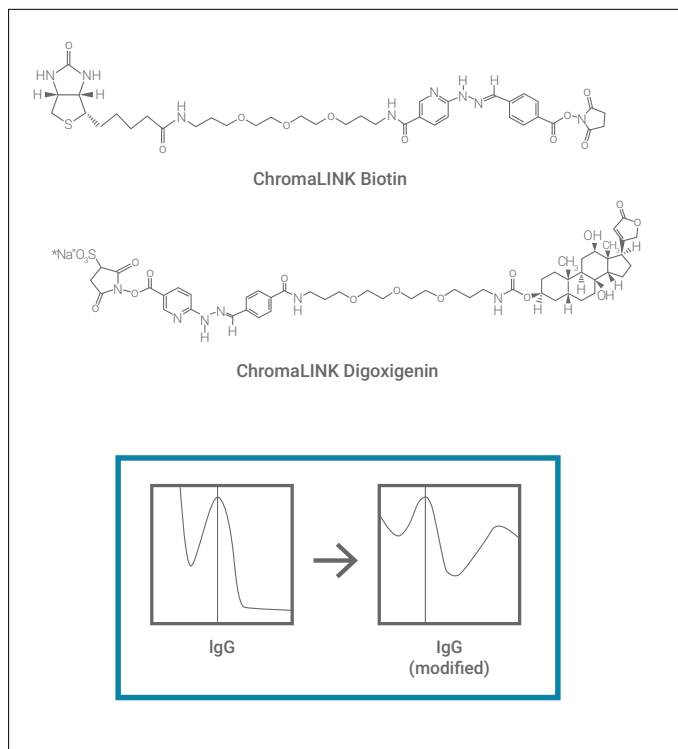
- Aqueous instability (hydrolysis) of maleimide functional groups on biomolecules limits their utility as functionalization agents.
- The need to protect and deprotect thiol groups introduced via heterobifunctional linkers.
- The need to activate certain protected thiol groups by addition of a reducing agent that may compromise biomolecule function.
- Potential for undesirable homo-dimer formation via disulfide bridge formation or disulfide rearrangement.

SoluLINK bioconjugation technology meets the demanding requirements for production of diagnostic and therapeutic bioconjugates. SoluLINK technology is broadly applicable and allows conjugation of all classes of biomolecules. Additionally, peptides and oligonucleotides may be synthesized with HyNic and 4FB during solid phase synthesis of these important classes of biomolecules. The ability to easily quantify the number of linkers on biomolecules and the resulting conjugate bond enables a level of control over the bioconjugation process not possible with alternative technologies.

## Products and Bioconjugation Examples

### Traceable Biotin and Digoxigenin Labeling with ChromaLINK®

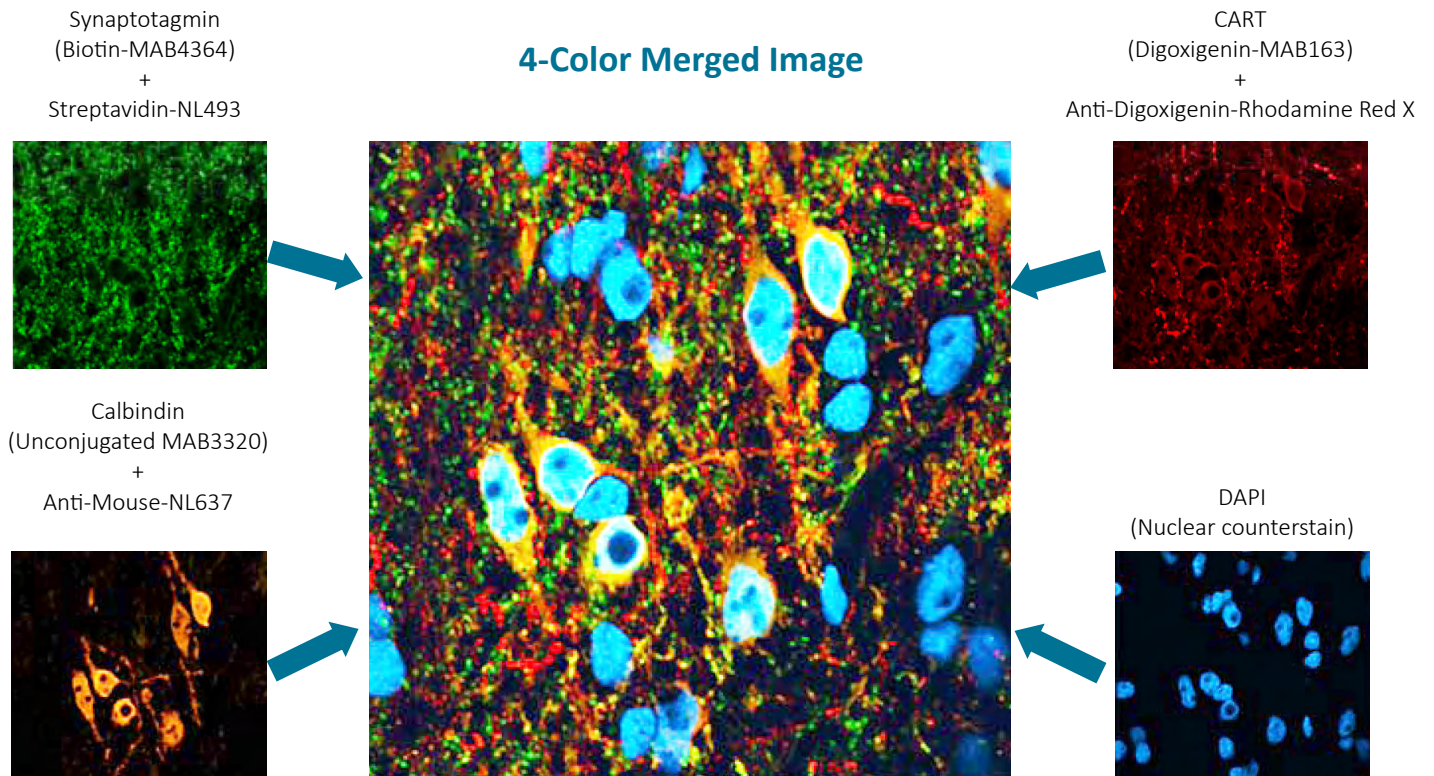
Accurate and controlled incorporation of labels such as biotin and digoxigenin on biomolecules continue to be problematic due to the inherent lack of an internal chromophore or other tracer on these tags. To overcome this problem, ChromaLINK Biotin and Digoxigenin have been engineered to incorporate the SoluLINK UV-traceable bis-arylhydrazone chromophore within the linker arm of each of these molecules. Spectrophotometric quantification of the level of incorporation of these labels is straightforward and highly reproducible. A simple measurement of the 280 nm and 354 nm absorbance values of modified proteins yields both the protein concentration and number of incorporated haptens (Figure 4).



**Figure 4.** Structures of ChromaLINK Biotin and Digoxigenin (top). UV-Vis spectra of unmodified antibody and ChromaLINK Biotin-modified antibody (bottom). The number of ChromaLINK Biotin and Digoxigenin molecules per protein is easily calculated from the 280 nm and 354 nm absorbance values.

For antibody labeling, ChromaLINK Biotin and Digoxigenin One-Shot™ Kits include everything required to label 100 µg of antibody, purify the conjugate, and quantify incorporation of these labels. The ChromaLINK Biotin Protein Labeling Kit is also available for the controlled and traceable biotinylation of any protein from 20-200 kDa, in amounts ranging from 25 micrograms to 1 milligram.

Multiplexed immunodetection techniques such as immunofluorescence have traditionally been hampered by the relatively low number of antibodies available against cellular targets raised in different species. This limits the application of labeled secondary anti-species antibodies in a single tissue or cell sample. Remarkably, with the use of ChromaLINK Biotin and Digoxigenin, same-species primary antibodies may be labeled with haptens and subsequently detected with streptavidin and anti-Dig antibody fluorescent conjugates, respectively, on the same sample without cross-reactivity. Additionally, since the primary antibodies contain multiple haptens for binding of labeled detector molecules, the signal is greatly enhanced when compared to directly-labeled primary antibodies. Figure 5 shows the use of ChromaLINK Biotin and Digoxigenin-modified antibodies in a multiplexed immunofluorescence staining experiment using the same host species primary antibodies (mouse).



**Figure 5.** ChromaLINK Biotin- and Digoxigenin-labeled mouse primary antibodies in a multiplexed immunofluorescence staining experiment. Brain tissue from Sprague Dawley rats was incubated with unconjugated mouse anti-rat/human Calbindin D, followed by detection with donkey anti-mouse secondary antibody conjugated to NorthernLights™ NL-637 (far-red fluorescence). After washing with PBS, the same tissue section was incubated with a mixture of ChromaLINK Biotin-labeled mouse anti-rat Synaptotagmin-1 and ChromaLINK Digoxigenin-labeled mouse anti-rat/human CART. Detection of these mouse primary antibodies was accomplished using a mixture of Streptavidin-NorthernLights NL-493 conjugate (green fluorescence) and mouse anti-digoxigenin conjugated to Rhodamine Red™-X (red fluorescence). DAPI was used as a nuclear counterstain (blue fluorescence).

## Antibody-Oligonucleotide Conjugation

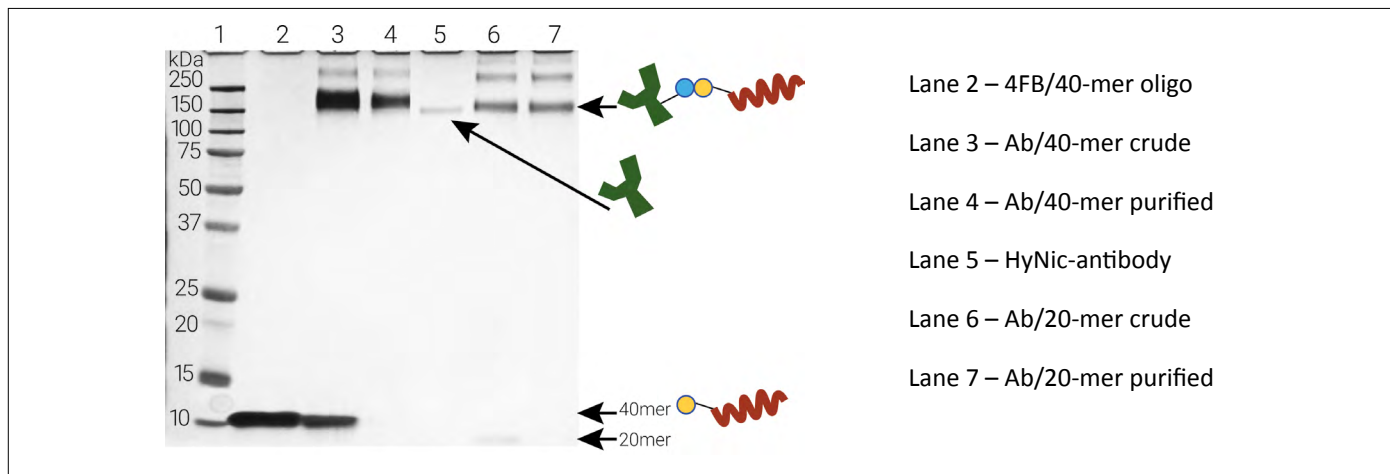
Difficulties in the efficient and reproducible preparation of antibody-oligonucleotide conjugates have limited their exploitation in multiplexed diagnostic assays. The SoluLINK Antibody-Oligonucleotide All-in-One™ Conjugation Kit overcomes these hurdles. This conjugation kit is both stoichiometrically efficient and high yielding, converting >95% of antibody to oligonucleotide conjugate (Figure 6). Furthermore, oligomers of 20–60 nucleotides are conjugated with equally high efficiency. The method is extremely mild, as no metals, reductants, or oxidants are used in the conjugation. Further enhancing the efficiency of conjugation is the use of aniline as a reaction catalyst, which significantly reduces reaction times. The kit is designed to incorporate 2-3 oligos per antibody when 4-5 equivalents of oligo are used.

In a second breakthrough for antibody-oligonucleotide conjugation, a method was developed to purify the conjugate by adsorption onto a proprietary magnetic affinity matrix, which allows for the removal of excess oligonucleotide. This is followed by gentle elution of the highly purified conjugate. Overall yield of the antibody-oligonucleotide conjugate is 30–50% based on antibody recovery from the purification

step. The conjugate is >95% free from unconjugated antibody and oligonucleotide, making the conjugates prepared with this kit suitable for the most demanding of applications. Multiple conjugates can easily be prepared simultaneously, satisfying the requirement for multiple antibody-oligonucleotide conjugates for highly multiplexed detection schemes.

## Protein-Protein Conjugation

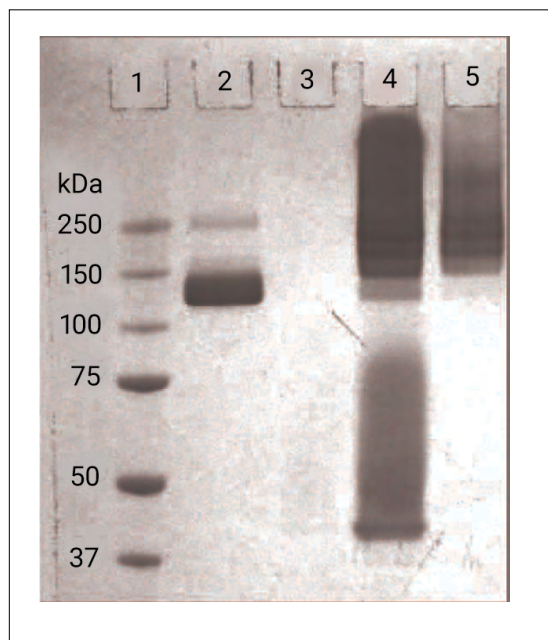
SoluLINK All-in-One Conjugation Kits exploit the efficiency of the hydrazone couple to produce high quality antibody conjugates with high yield. These kits allow preparation of protein-protein conjugates, such as HRP-antibody and PE-antibody, for the most demanding of applications. Each kit includes a convenient purification spin column to remove excess label for the highest signal-to-noise ratio and sensitivity without tedious chromatographic separation. Figure 7 presents gel results of the conjugation of HyNic-antibody to 4FB-HRP using the HRP-Antibody All-in-One Conjugation Kit. Each kit completely converts antibody to conjugate, free of unconjugated label. The SoluLINK Protein-Protein Conjugation Kit enables simple and rapid conjugation of any protein with a molecular weight between 25-950 kDa.



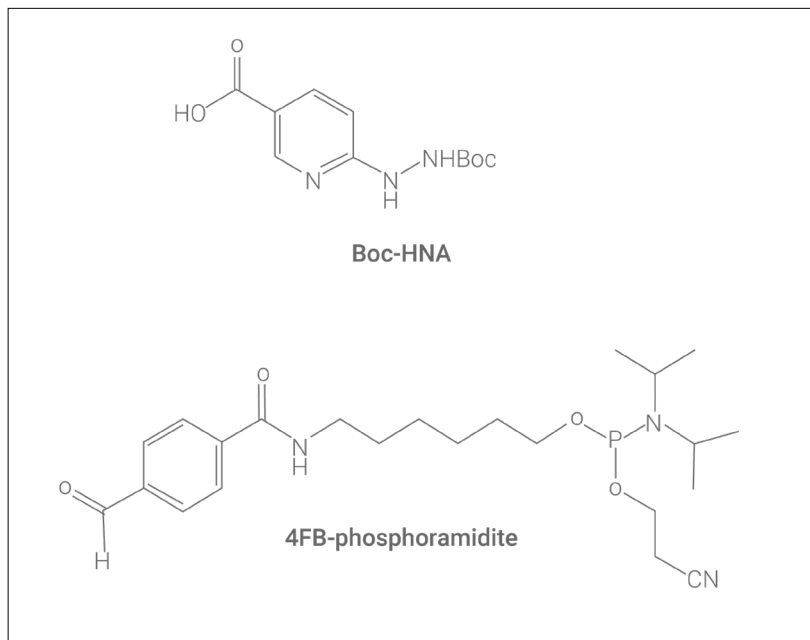
**Figure 6.** Antibody-Oligonucleotide conjugation. DNA silver stained non-reducing SDS-PAGE gel demonstrating efficient conjugation of a 40-mer (Lanes 2-4) and 20-mer (Lanes 6 and 7) oligonucleotide to antibody. Virtually no unconjugated antibody remains after the conjugation reaction in both oligonucleotide samples (lanes 3 and 6). Purified conjugates (lanes 4 and 7) are free of excess unconjugated oligonucleotide.

## Peptide-Oligonucleotide Conjugation

Preparation of peptide-oligonucleotide conjugates using traditional maleimide-thiol chemistry is difficult to perform and reproduce, and it results in low yields. SoluLINK technology greatly simplifies the process, as complementary linkers can be incorporated onto their respective sequences during solid phase synthesis. For example, HyNic can be incorporated internally or on the N- or C-terminus during solid phase peptide synthesis using Boc-HNA (Figure 8). A 4FB group can be incorporated on the 5'-terminus of an oligonucleotide during synthesis using A4FB-phosphoramidite (Figure 8). Alternatively, a 4FB group can be added to the 5'- or 3'-terminus of an oligonucleotide by a simple, high-yielding conversion of an amino oligo using S-4FB. Simple mixing and incubation of the 4FB-oligonucleotide with a 2 or 3-fold excess of the HyNic-peptide, followed by purification using diafiltration, affords the conjugate in nearly quantitative yield.



**Figure 7.** Protein-Protein conjugation. Coomassie blue stained gel of antibody-HRP conjugate. Gel loading: protein molecular weight ladder (lane 1), HyNic-modified antibody (lane 2), unpurified conjugation reaction (lane 4), and purified antibody-HRP conjugate (lane 5). Polymeric antibody-HRP conjugates produced with the HRP-Antibody All-in-One Conjugation Kit deliver increased sensitivity for applications such as ELISA and immunohistochemistry versus conjugates with a lower HRP to antibody ratio.

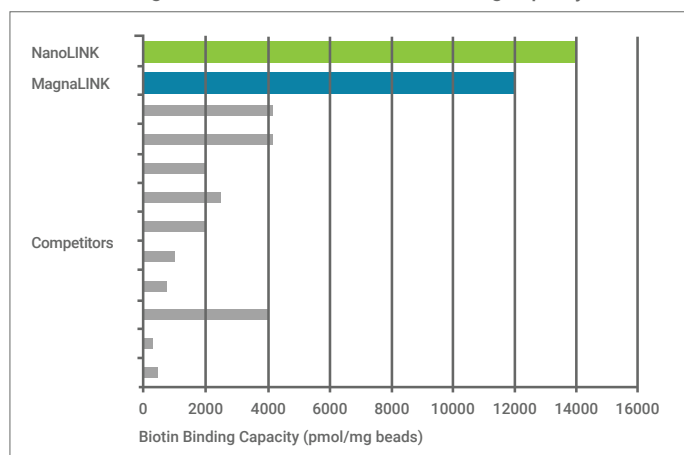


**Figure 8.** Structures of Boc-HNA and 4FB-phosphoramidite. Boc-HNA is used to incorporate HyNic on the N- and C-termini of peptides during solid phase peptide synthesis. 4FB-phosphoramidite incorporates 4FB on the 5'-terminus of oligonucleotides during solid phase oligo synthesis.

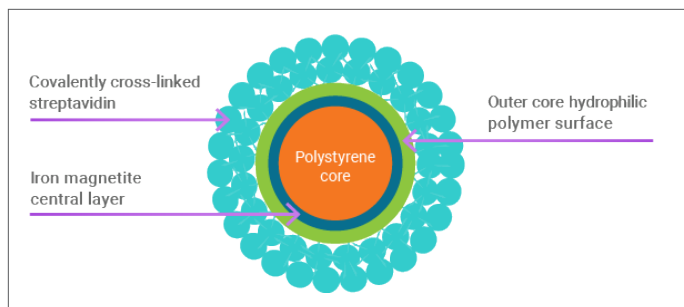
## Streptavidin Magnetic Beads and Agarose

NanoLINK® Streptavidin Magnetic Beads (1 µm) and MagnaLINK® Streptavidin Magnetic Beads (2.8 µm) possess up to 15-times greater biotin binding capacity than other commercially available products (Figure 9). This is accomplished by immobilizing HyNic-labeled streptavidin onto a 4FB-activated bead surface using SoluLINK bioconjugation technology as shown in Figure 10. The extraordinarily high coupling efficiency of SoluLINK technology yields much higher streptavidin loading levels when compared to competing products. Higher binding capacity permits the use of less solid phase (beads) to capture a given amount of a biotinylated target protein or other biomolecule, resulting in proportionally lower backgrounds and cost.

NanoLINK & MagnaLINK vs. Others - Free Biotin Binding Capacity



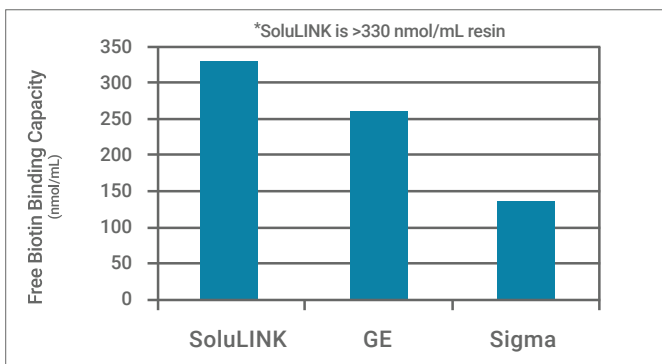
**Figure 9.** Comparison of biotin binding capacity of NanoLINK and MagnaLINK Streptavidin Magnetic Beads versus competitors.



**Figure 10.** Illustration showing cross section of NanoLINK and MagnaLINK Streptavidin Magnetic Beads.

SoluLINK Streptavidin Agarose Ultra Performance™ was developed with several key parameters in mind—high biotin binding capacity and low non-specific binding, on a highly crosslinked 6% agarose support. This results in high flow rates with consistent permeation, as well as the highest biotin binding capacity of any agarose bead on the market (Figure 11). Streptavidin is immobilized onto the beaded agarose resin using SoluLINK technology, resulting in high density and highly stable immobilizations with minimal protein leaching. Moreover, unlike other conjugation strategies such as cyanogen bromide, SoluLINK conjugation leaves the agarose with no net charge, which could otherwise interact with biomolecules to cause unwanted non-specific binding and background. The core agarose is specially formulated to be of unparalleled uniformity, with a relatively small mean diameter of 35µm. These properties, combined with a high level of bead crosslinking, create a resin that is tolerant of high pressures and centrifugal forces often experienced in high-throughput screening and purification applications.

Comparison of Free Biotin Binding Capacity\*



**Figure 11.** Comparison of Streptavidin Agarose Ultra Performance biotin binding capacity versus competitors.

## Summary

SoluLINK bioconjugation technology has been engineered to efficiently and easily prepare biomolecule conjugates. This technology is superior to classical methods, and it better satisfies the stringent requirements demanded by diagnostic and therapeutic applications. The ability to easily quantify linker incorporation and conjugate formation allows for an unparalleled level of reproducibility in conjugate formation. SoluLINK technology has been cited in hundreds of research articles, and is used by many top biotechnology companies worldwide to produce bioconjugates for diagnostic and therapeutic applications.

## Product Selection Guide

Product	Catalog No.
<b>Biotin and Digoxigenin Labeling</b>	
ChromaLINK One-Shot Antibody Biotinylation Kit	<a href="#">B-9007</a>
ChromaLINK Biotin Protein Labeling Kit	<a href="#">B-9007-105K</a>
ChromaLINK Digoxigenin One-Shot Antibody Labeling Kit	<a href="#">B-9014</a>
<b>Magnetic Bead Immobilization</b>	
NanoLINK Streptavidin Magnetic Beads (1.0 µm)	<a href="#">M-1002</a>
MagnaLINK Streptavidin Magnetic Beads (2.8 µm)	<a href="#">M-1003</a>
MagnaLINK 4FB Magnetic Beads (2.8 µm)	<a href="#">M-1004</a>
<b>Agarose Bead Immobilization</b>	
Streptavidin Agarose Ultra Performance	<a href="#">N-1000</a>
<b>Oligo Conjugation: Antibody-Oligo &amp; Protein-Oligo</b>	
Antibody-Oligonucleotide All-in-One Conjugation Kit	<a href="#">A-9202</a>
Protein-Oligo Conjugation Kit	<a href="#">S-9011</a>
<b>Antibody Labeling: Fluorophores and HRP</b>	
Fluorescein One-Shot Antibody Labeling Kit	<a href="#">F-9001</a>
HRP-Antibody All-in-One Conjugation Kit	<a href="#">A-9002</a>
R-PE Antibody Conjugation Kit	<a href="#">P-9002</a>
<b>Protein-Protein Conjugation</b>	
Protein-Protein Conjugation Kit	<a href="#">S-9010</a>

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1. Dirksen, A., & Dawson, P.E. (2008). *Rapid Oxime and Hydrazone Ligations with Aromatic Aldehydes for Biomolecular Labeling*. *Bioconjugate Chem.*, 19(12), 2543–2548. doi:10.1021/bc800310p
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