

Bioconjugation Primer: Why Solulink?

Introduction: Bioconjugation is the linking of two biomolecules to form a hybrid, the bioconjugate, which retains the properties of each individual component, yet yielding a single entity with two complementary functions. Biomolecules exist and function in aqueous environments; therefore, the preparation of bioconjugates is primarily about “chemistry in water.” Any suitable bioconjugate chemistry must be compatible with such an environment, while at the same time preserving the biological activity or function of the biomolecules. Conjugates are generally formed through the addition of separate but reactively complementary functional groups to each of the two biomolecules. The two modified biomolecules are then mixed together to form the desired bioconjugate.

Conjugation and immobilization of biomolecules has historically been very problematic for a variety of reasons; primarily because there are few covalent bond-forming reactions that proceed in water that can be engineered to link biomolecules together. A wide variety of methods to conjugate and immobilize biomolecules are extensively described in [Bioconjugate Techniques](#), but most of these methods are difficult to perform, stoichiometrically inefficient, and result in low conjugate yields.

It is important to understand the desired characteristics of the ideal bioconjugation chemistry. The following list presents many criteria that need to be fulfilled to produce the ideal bioconjugation technology:

- a) linkers must be incorporated on biomolecules in a mild, controllable manner

- b) the inherent biological function of the biomolecules must be unaffected after modification and conjugation
- c) the conjugation reaction occurs directly upon mixing the two modified biomolecules, preferably not requiring addition of an oxidant, reductant, or metal
- d) modified biomolecules are stable over extended periods
- e) formation of the covalent linkage is stable under a broad pH range and at elevated temperatures
- f) quantification of both incorporated linkers and final conjugate is readily performed, *i.e.*, they are spectrophotometrically traceable
- g) conjugation occurs in buffered aqueous solutions, at a physiological pH
- h) stoichiometrically efficient (e.g., 1:1)
- i) fast reaction kinetics
- j) no undesirable covalent side reactions during modification
- k) no electrostatic/hydrophobic interactions
- l) linkers can be incorporated on a variety of biomolecules, including oligonucleotides and peptides, through solid phase synthesis

The classical method used to prepare covalent conjugates makes use of the maleimido/thiol coupling pair. Disadvantages of the maleimido/thiol pair include:

- a) the aqueous instability (hydrolysis) of the linker functional group (*i.e.*, maleimide) on biomolecules during conjugation
- b) the need to protect the thiol group
- c) the slow kinetics of the reaction
- d) the need to activate the protected thiol group with a strong reducing agent (e.g., DTT or TCEP)
- e) the need to activate the protected thiol group by addition of a strong reducing agent (e.g., DTT)
- f) the potential for undesirable homo-dimers via disulfide bridge formation
- g) cleavage and/or partial fragmentation of proteins by reduction of disulfide bonds
- h) reduced or inactive protein function



Bioconjugate Techniques, Greg T. Hermanson, Academic Press, 2008

The Solulink Solution

Solulink has developed a bioconjugate chemistry that more mildly, more efficiently, and more reproducibly conjugates and immobilizes proteins, peptides, oligonucleotides, carbohydrates, and polymers.

Solulink's bioconjugation chemistry is based on the reaction of an **aromatic hydrazine** with an **aromatic aldehyde**, yielding a stable **bis-arylhydrazone** conjugate bond (Figure 1). The aromatic hydrazine is based on 6-hydrazino-nicotinic acid (**HyNic**) and is incorporated on amine-containing biomolecules using S-HyNic (1; Figure 1). S-HyNic possesses a succinimidyl ester (NHS) functionality that readily reacts with amino moieties on proteins and other amine-containing biomolecules, polymers, and surfaces using standard NHS ester reaction conditions, *i.e.*, phosphate buffer, pH 7.2–8.0. The aromatic aldehyde is incorporated on biomolecules with S-4FB (2; Figure 1).

Simple addition of a HyNic-modified biomolecule to a 4FB-modified biomolecule in a mildly acidic buffer, *i.e.*, pH 5.0–6.0, yields the desired conjugate. The hydrazine function is protected as its acetone hydrazone is in equilibrium with the free reactive hydrazine in aqueous solution; therefore, the HyNic group is free to react with aromatic aldehydes without a separate deprotection step, yielding the stable bis-arylhydrazone. The bis-arylhydrazone has been demonstrated to be both heat stable and pH stable (95°C for 2 hr, pH 2–10).

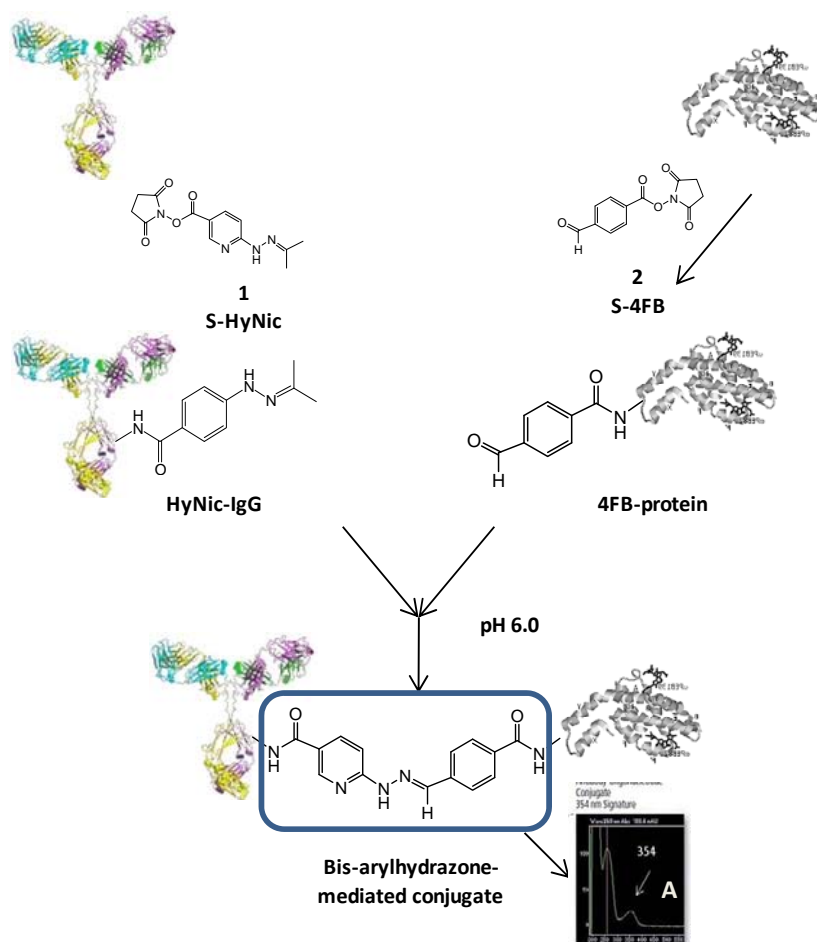


Figure 1. Scheme presenting the chemistry involved in conjugating two biomolecules using Solulink's HyNic/4FB conjugation couple. The bis-arylhydrazone bond is UV-traceable, absorbing at 354 nm. Inset A is the UV spectrum of an HyNic-4FB hydrazone-mediated antibody-oligonucleotide

Solulink has also synthesized a related family of HyNic and 4FB linkers that further extend the versatility of this technology; including linkers that react with thiol moieties, contain extended hydrophilic linkers (PEG4), or are cleavable as disulfide linkers. Furthermore, HyNic moieties are readily incorporated on peptides during solid phase peptide synthesis and 4FB moieties are readily incorporated on oligonucleotides during solid phase oligonucleotide synthesis using a phosphoramidite.

Another extremely useful characteristic of the bis-arylhydrazone bond is that it forms a traceable chromophore. This chromophore absorbs at 354 nm

with a molar extinction coefficient of 29,000 (Figure 1 inset). This unique property allows the researcher to (1) follow the linking or labeling reactions in real time on a spectrophotometer, (2) directly quantify the number of linkages formed in the conjugate (e.g., 1:1, 1:2, 1:3), and (3) visualize or trace conjugate fractions during FPLC or HPLC purification.

Further increasing the versatility of the HyNic/4FB couple for the preparation of bioconjugates is the discovery by Dirksen *et al.*^{1,2} that aniline significantly catalyzes the formation of the bis-arylhydrazone bond (Figure 2, with permission). The striking increase in conjugate reaction rate is particularly important for the conjugation of two proteins. As demonstrated in Figure 3, the conjugation of HyNic-IgG (1 mol. equiv.) to 4FB-BSA (1.5 mol. equiv.)

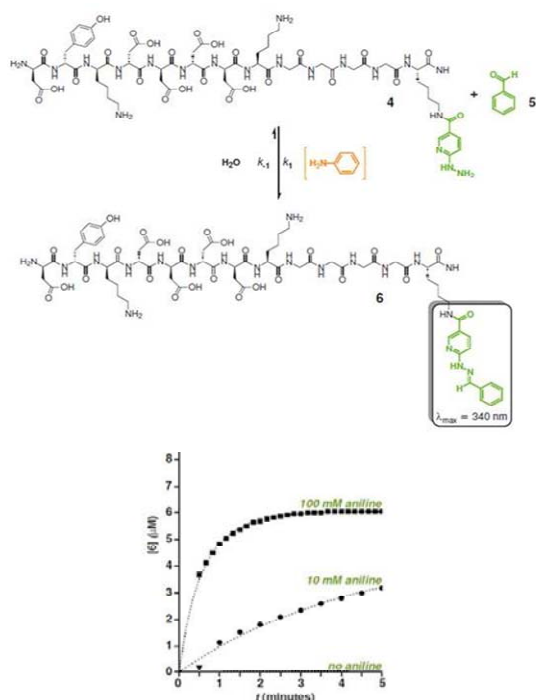


Figure 2. The HyNic/4FB conjugation couple. Ligation of HyNic and 4FB with aniline-catalyzed hydrazone ligation between 6-hydrazinopyridyl (HyNic)-functionalized peptide **4** and benzaldehyde **5** (left) followed by UV-VIS spectroscopy (340 nm).

proceeds to nearly complete conversion to conjugate in 2 hr in the presence of 10 mM aniline (Solulink's TurboLink Buffer). Maleimido/thiol-based chemistry converts only approximately 50% of IgG to conjugate when a 1:4 IgG:BSA ratio is used.

The efficiency of the HyNic/4FB couple results in easier purification, significant cost savings, and better reproducibility. Also to be noted is that the efficiency of conjugation of proteins using maleimido/thiol chemistry is also dependent on the molecular weights of the two proteins. For example, conjugation of IgG (MW 150 kDa) to HRP (MW 45 kDa) proceeds more efficiently than conjugation of IgG to phycoerythrin (R-PE; MW 250 kDa). Any two proteins, regardless of molecular weights, can be efficiently conjugated using the HyNic/4FB couple in the presence of 10 mM aniline.

HyNic/4FB Conjugation Couple Advantages

The many significant advantages to Solulink's HyNic/4FB-based conjugation technology include:

- an efficient conjugation chemistry
- no metals, reducing, or oxidizing agents are required
- fast conjugation kinetics catalyzed by aniline
- conjugate bond is stable to extremes of heat and pH
- conjugate bond forms a chromophore that can be used to follow reactions in real time and quantify the number of linkages in a conjugate
- versatile: proteins, oligos, and peptides can be rapidly conjugated to each other and to surfaces
- direct conjugate bond formation under mild buffer conditions
- excellent retention of inherent biological function of biomolecules following controlled modification and conjugation
- 4FB linker can be incorporated on oligonucleotides during solid phase synthesis

- j) HyNic linker can be incorporated on peptides during solid phase synthesis

Conjugation Examples

To demonstrate the versatility and breadth of utility of Solulink's HyNic/4FB conjugation couple, we present examples of the preparation of protein-protein, peptide-oligonucleotide, and protein-oligonucleotide conjugates (Figures 3, 4, and 5) respectively. More detailed descriptions for preparing these types of conjugates, as well as others, can be found in a series of Solulink white papers that can be downloaded from <http://www.solulink.com/support/white-papers>.

Protein-Protein Conjugation

Solulink has taken advantage of the efficiency of conjugation of two proteins using the HyNic/4FB couple in the presence of aniline to develop an All-in-One Conjugation kit that allows preparation of IgG-HRP, IgG-AlkPhos, and IgG-R-PE conjugates without the need for chromatographic purification.

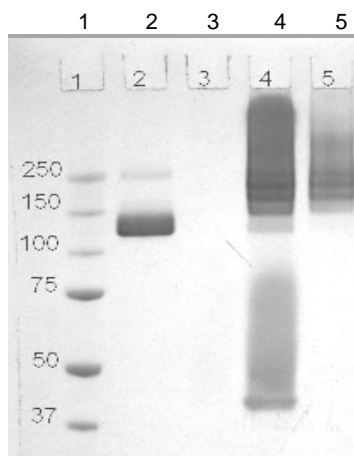


Figure 3. Protein-protein conjugation. Coomassie blue stained gel of the conjugation of HyNic-antibody to 4FB-HRP showing protein ladder (Lane 1), HyNic-IgG (Lane 2), crude conjugation reaction (Lane 4) and purified IgG-HRP conjugate (Lane 5). It is to be that a polymeric IgG-HRP as produced here is the desired construct for these conjugates for optimal sensitivity.

Figure 3 presents gel results of the conjugation of HyNic-IgG to 4FB-HRP. Note the near complete conversion of IgG to conjugate (lane 4). A simple centrifugation step removes excess enzyme, and yields purified conjugate.

Peptide-Oligonucleotide Conjugation

The preparation of these conjugates using maleimido/thiol chemistry is difficult to perform, difficult to reproduce, and is low yielding. The

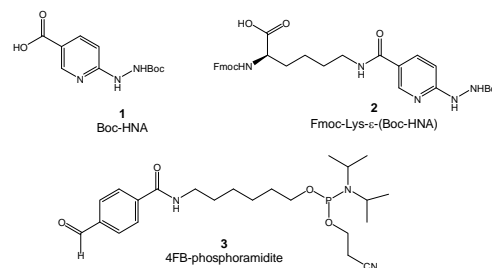


Figure 5. Structures of Boc-HNA (1) and Fmoc-Lys-e-(Boc-HNA). These reagents are used to incorporate HyNic groups on the N- and C-termini of peptides, respectively. 4FB-phosphoramidite is used to incorporate 4FB groups on the 5'-terminus of oligonucleotides.

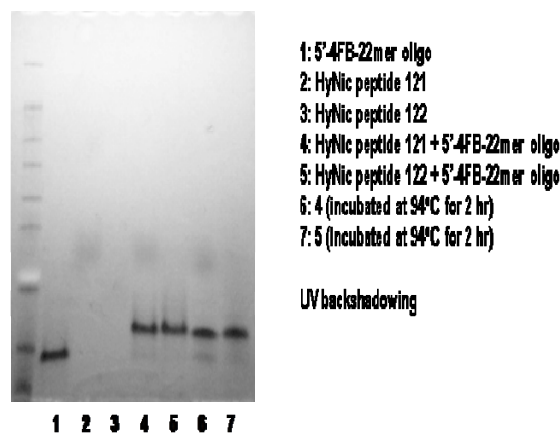


Figure 4. Peptide-oligonucleotide conjugation. SDS-PAGE gel of conjugates visualized with UV back-shadow. Conjugation of two N-terminal HyNic-modified peptides 121 and 122 (Lanes 2 and 3) reacted with a 5'-4FB modified 19-mer oligo (Lane 1) to yield the peptide-oligo conjugates (Lanes 4 and 5). As seen in lanes 4 and 5, conjugate formation was efficient at a 1:3 reaction stoichiometry (oligo:peptide). Conjugate bond stability was tested at 95°C in PBS for two hr (Lanes 6 and 7).

HyNic/4FB couple greatly simplifies the process as both linkers can be incorporated onto their respective sequences during their solid phase syntheses. For example, the HyNic peptide reagents can be incorporated on the N- or C-terminus, or internally during peptide solid phase synthesis (1 and 2; Figure 4). The 4FB group can be incorporated on the 5'- or 3'-terminus of an oligonucleotide by a simple, very high-yielding conversion of a 3'- or 5'-amino oligo using sulfo-S-4FB. Alternatively, a 4FB-phosphoramidite for direct incorporation on the 5'-terminus can be used. Simple mixing and incubation of the 4FB-oligonucleotide with a 2–3 fold excess of the HyNic-peptide, followed by purification by diafiltration, produces the conjugate in near quantitative yield.

Protein-Oligonucleotide Conjugation The ability to efficiently and reproducibly prepare antibody-oligonucleotide conjugates has limited their exploitation in multiplexed diagnostic assays.

Solulink's HyNic/4FB bioconjugation couple, as applied to the conjugation of oligonucleotides with antibodies, is stoichiometrically efficient and high yielding, converting >95% antibody to antibody-oligonucleotide conjugate (Figure 5). Furthermore, conjugations of oligomers of 20–60 nucleotides are conjugated with equal efficiency. The method is extremely mild, as no metals, reductants, or oxidants are used in the conjugation step. Further enhancing the efficiency of conjugation is the use of aniline as a reaction catalyst. In a standard conjugation protocol, 5 equivalents of 4FB-

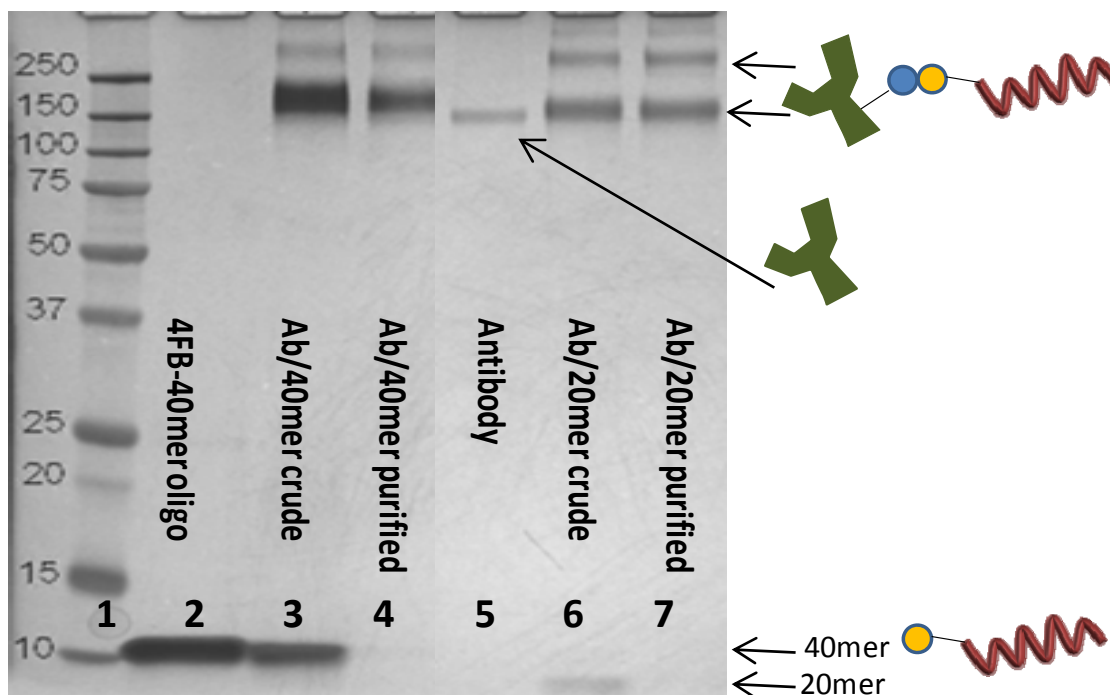


Figure 5. Antibody-oligonucleotide conjugation. The silver stained SDS-PAGE gel presents data for the conjugation and purification of a 40-mer (Lanes 2 and 3) and a 20-mer (Lanes 6 and 7) 4FB-oligonucleotides to HyNic-modified antibodies. In the case of the 40-mer oligonucleotide-antibody conjugate, it is evident that there is virtually no free antibody in the conjugate. In both purified conjugates, there is no visible free oligonucleotide. The 'broad' conjugate bands are due to a distribution of 2–4 oligonucleotides conjugated to each antibody.

oligonucleotide are used, resulting in the conjugation of 2–3 oligonucleotides per antibody.

In a second breakthrough for antibody-oligonucleotide conjugation, we developed a method to purify the conjugate by adsorption to a proprietary magnetic affinity matrix that allows removal of excess 4FB-oligonucleotide, followed by elution of the purified conjugate using mild elution buffers. The overall yield of the antibody-oligonucleotide conjugate is 30–50% based on antibody recovery. The conjugate is >95% free from unconjugated HyNic-antibody and 4FB-oligonucleotide. Multiple conjugates can be prepared simultaneously, satisfying the requirement for the use of this protocol to prepare multiple antibody-oligonucleotide conjugates for highly multiplexed detection of antigens. Refer to Solulink's white paper, **Antibody-Oligonucleotide Conjugate Preparation**: <http://www.solulink.com/support/white-papers>.

Traceable Biotin and Digoxigenin Labeling of Biomolecules with ChromaLink™ Linkers

Accurate and controlled incorporation of haptens such as biotin and digoxigenin on biomolecules continues to be problematic due to the inherent lack of an internal chromophore on these tags. To overcome this problem, Solulink incorporated its UV-traceable (354 nm) bis-arylhydrazone chromophore into the linker arm in both biotin and digoxigenin modification reagents, the ChromaLink™ Biotin and ChromaLink™ Digoxigenin (Figure 7). Spectrophotometric quantification of the incorporation of these chromophoric haptens is straightforward and highly reproducible. Simply measuring the A_{280} and A_{354} absorbance of the modified proteins yields both the protein concentration and number of incorporated labels (Figure 6).

For antibody labeling, Solulink designed and produced the ChromaLink™ Biotin and ChromaLink™ Digoxigenin One-Shot Kits, which include everything required to label, purify, and quantify incorporation of these labels from a single 100 µg quantity of antibody. Solulink has also developed the more flexible ChromaLink™ Biotin Protein Labeling Kit, used in the controlled traceable biotinylation of any protein ranging from 20 to 200 kDa in size, in concentrations ranging from 0.25 to 10 mg/mL.

Figure 7 shows the use of biotin- and digoxigenin-modified antibodies in a multiplex immunohistochemistry staining experiment.

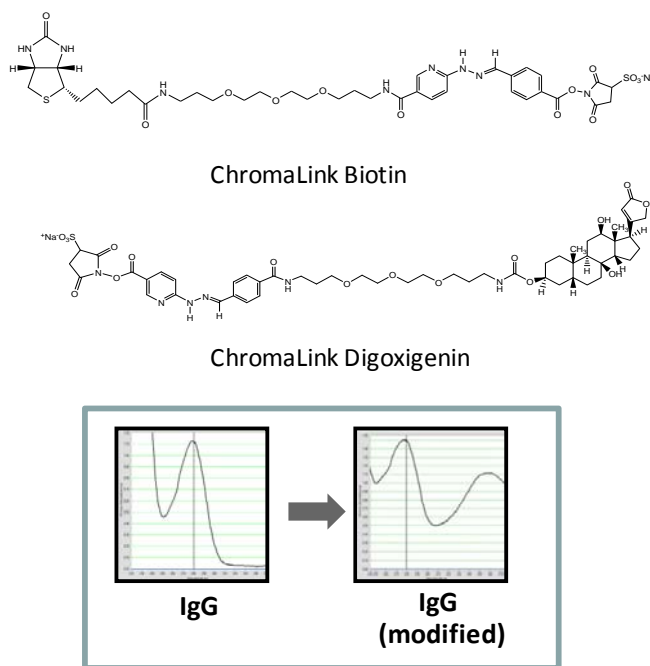


Figure 6. Structures of ChromaLink™ Biotin and ChromaLink™ Digoxigenin reagents. UV spectra of unmodified IgG and ChromaLink Biotin-modified IgG.

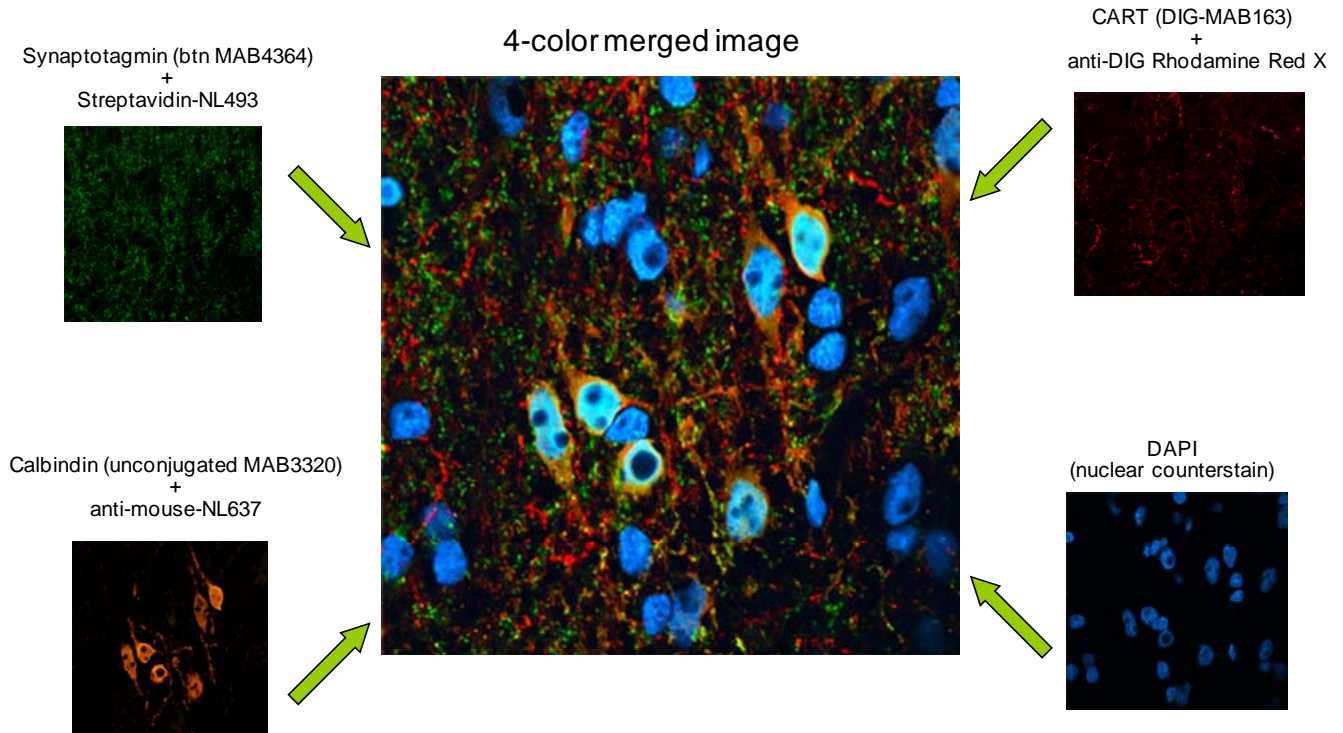


Figure 7. Biotin- and digoxigenin-modified antibodies in a multiplex immunohistochemistry staining experiment. Brain tissue from rats (Sprague Dawley, 200 g) were initially incubated with unconjugated primary antibody against the first target protein followed by detection with anti-primary secondary antibody conjugated to NorthernLights™ NL-493 tag (green fluorescence). Subsequently, the same tissue sections were incubated with a mixture of same species biotin-labeled primary antibody (against the second target protein) and DIG-labeled primary antibody (against the third target protein) followed by detection using a mixture of Streptavidin NorthernLights™ NL-557 tag (red fluorescence) and anti-DIG secondary antibody conjugated to a Cy5™ tag (fluorescence in the far red spectral region).

Biomolecule Immobilization

Immobilization of proteins, especially antibodies, is critically important in many biological applications; including immunoprecipitation, cell enrichment, antigen purification, and immunodetection. For this reason, researchers often require that proteins be immobilized on either a magnetic or nonmagnetic surface.

There are two main methods used to immobilize proteins: 1) immobilization of biotinylated proteins on streptavidin-modified surfaces or 2) direct covalent immobilization on activated surfaces. Solulink has engineered its conjugation chemistry to

offer both alternatives within the NanoLink™ and MagnaLink™ family of products.

Streptavidin Immobilization

NanoLink™ Streptavidin Magnetic Beads (0.8 μm) and MagnaLink™ Streptavidin Magnetic Beads (2.8 μm) possess 3 to 15 times greater biotin binding capacity than any other commercially available magnetic bead (Figures 8–9). Solulink accomplished this by ‘polymerizing’ streptavidin on an activated bead surface using its proprietary HyNic/4FB chemistry. Higher binding capacity permits the use of less solid phase (beads) to capture a given amount of a biotinylated target protein or

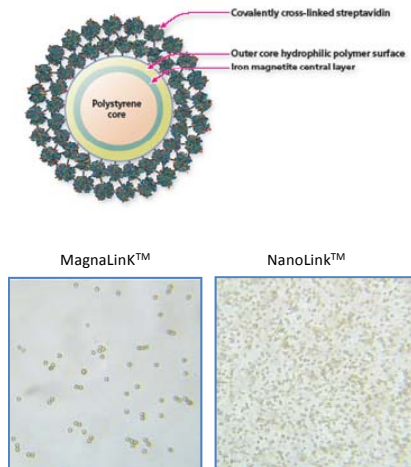


Figure 8. Comparison of magnetic beads. MagnaLink™ and NanoLink™ Streptavidin magnetic beads and their corresponding solution phase image (light microscope 400x).

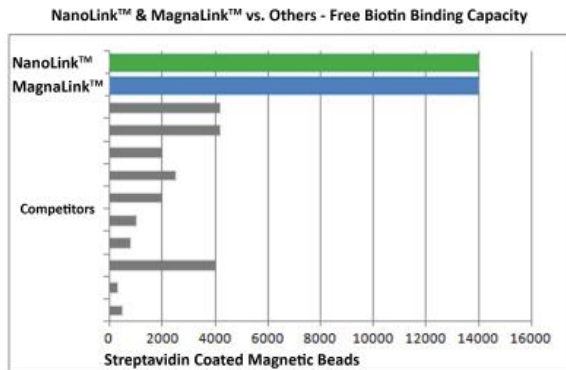


Figure 9. Binding capacity of magnetic beads. Biotin binding capacity of MagnaLink™ and NanoLink™ Streptavidin magnetic beads versus the competition (pmol/mg).

biomolecule, resulting in proportionally lower backgrounds and cost.

Direct Immobilization

Current methods to covalently immobilize proteins on magnetic and nonmagnetic surfaces are similar in that chemically reactive surfaces such as epoxy, tosyl, or NHS functional groups are mixed with biomolecules in anticipation that lysine groups located on the protein surface will covalently bind to the activated surface. There are several problems associated with this paradigm, as immobilization which is directed to lysine groups on proteins is

uncontrolled, leading to over-modification and loss of protein function. In addition, for each protein to be immobilized, pH and protein levels must be

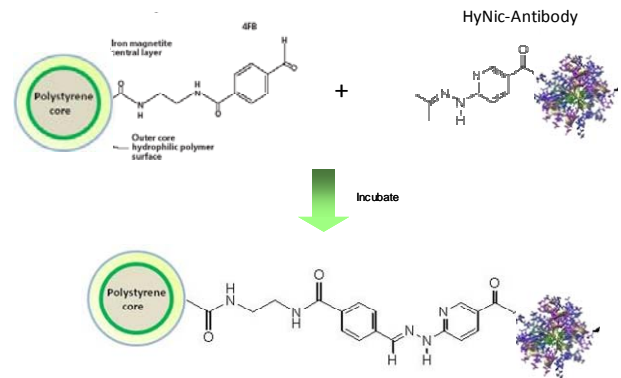


Figure 10. Scheme presenting the chemistry used to covalently immobilize HyNic-proteins on 4FB beads.

optimized on a case-by-case basis to ensure optimal functionality. It is well known that protein immobilization efficiency using these chemistries is often low and can be accompanied by significant amounts of nonspecific binding and/or leaching. These limitations lead to suboptimal assay performance and reproducibility.

Solulink has overcome many of these problems by offering an immobilization technology that is highly efficient and reproducible. Solulink's protocols differ (Figure 10) in that the protein to be immobilized is first controllably and reproducibly modified with HyNic groups, then the HyNic-modified protein (or peptide) is simply mixed with a 4FB-activated surface. In this way, protein immobilization efficiency consistently exceeds 75%, with unmatched batch-to-batch reproducibility.

Summary

Solulink's HyNic/4FB-based bioconjugation technology has been engineered to efficiently prepare all types of biomolecular conjugates. In most cases, Solulink's conjugation technology is far superior to classical methods and better satisfies the stringent requirements demanded by diagnostic and therapeutic products. As a case in point, Solulink's oligonucleotide-antibody conjugation technology was used during the development of the PCR-based Proximity Ligation Assay^{8, 17, 20}, that is recognized as the most sensitive protein detection assay.

Citations Solulink's bioconjugation technology has been used by researchers worldwide.

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