Introduction: Oligonucleotide-protein and oligonucleotide-polymer conjugates have been used widely in therapeutics and diagnostics. Researchers and assay developers continue to design new oligonucleotide conjugates in inventive ways for novel uses. The great diversity of antibodies and oligonucleotides make their conjugates especially powerful.

Oligonucleotide-protein conjugates have been used in therapeutics for siRNA-protein delivery\(^1\), vaccines to increase vaccine adjuvanticity of CpG oligonucleotides.\(^2^5\) Liu et al. have used antibody-oligonucleotides conjugates for pretargeting cancer therapeutics.\(^6^7\)

Oligonucleotide-protein conjugates have found utility in protein detection and quantification in a variety of ways. Protein detection by a technique called immuno-PCR (Polymerase Chain Reaction) was initially demonstrated by Cantor et al.\(^8\) and shown to be the most sensitive method for protein detection and quantification. This original method was hampered by high background due to inefficient removal of non-bound conjugate. This shortcoming has been overcome by the Proximity Ligation Assay developed by Fredricksson et al.\(^9^11\) a second generation immuno-PCR assay wherein two antibody-oligonucleotide conjugates directed to different epitopes on the same protein are treated with a 'splint' oligonucleotide that hybridizes across the two oligonucleotides. This is followed by the addition of a nucleoside triphosphate and ligation enzyme to ligate the two oligonucleotides followed by a PCR reaction across the ligation site. Kattah et al.\(^9\) have recently developed a multiplex protein assay based on PCR amplified oligonucleotide-Fab fragment or monomeric streptavidin conjugates.\(^12\)

The Problem: It has been easier to design ways to use oligonucleotide-protein conjugates than it has been to prepare them. Their synthesis has been problematic for a variety of reasons. Many methods to conjugate oligonucleotides to proteins are described in Hermanson’s extensively cataloged Bioconjugate Techniques book (Academic Press, 2008) but all the methods are difficult to perform, stioichiometrically inefficient and low yielding. Two major problems facing scientists include activation of the oligonucleotide and its reactive conjugate partner, e.g. an antibody or enzyme, and efficiency of conjugation resulting in extensive purification and low yields.

Historically the use of the maleimido/thiol couple has been predominantly used to conjugate oligonucleotides to proteins. This protocol requires (1) synthesizing a thiol-modified oligonucleotide, (2) incorporate maleimido moieties on the protein, (3) combine the two modified components and (4) purify by ion exchange chromatography or other chromatographic method. While this protocol appears straightforward it is fraught with difficulties including:

- the thiol oligonucleotides readily oxidizes to its disulfide requiring reduction prior to conjugation
- maleimido-modified biomolecules must be prepared and used immediately
- it is difficult to monitor each stage of the conjugation
- the efficiency of conjugation is poor requiring difficult purification schemes to isolate a pure conjugate
How can these problems be solved?

1) **Have a chemistry wherein a stable reactive moiety on the oligonucleotide can be incorporated during its solid phase oligonucleotide synthesis.**

Being able to simply order a ready-to-add stable reactive oligonucleotide would allow the researcher to simply add the oligonucleotide to the modified protein to produce the desired conjugate.

2) **Have a chemistry that can be controlled so the inherent reactivity of the protein is retained following modification.**

It is most cases it is imperative that the biological function of the protein be retained following modification and conjugation. To do this one must be able to control the modification of the protein.

3) **Have an efficient conjugation chemistry in which all the modified protein is converted to conjugate.**

To prepare an oligo/protein conjugate in high efficiency a conjugation technology must lead to complete conversion of all protein to conjugate using a reasonable excess of oligo with respect to protein. In many cases the number of oligonucleotides/protein is mandated by its ultimate function and it would be optimal to be able to control the oligonucleotide/protein substitution ratio.

**The Solulink Solution** To efficiently prepare oligonucleotide/protein conjugates Solulink has engineered its HyNic/4FB conjugation couple (Figure 1) to accomplish this task. The 4FB moiety is incorporated on the 5’-terminus of an oligonucleotide by modifying a 5’-amino oligonucleotide with S-4FB or direct incorporation of a 4FB-moiety using 4FB-phosphoramidite 1 (Figure 1). A 3’-4FB oligonucleotide is prepared from a 3’-amino-modified oligonucleotide using S-4FB. Both methods are efficient and high yielding.

The 4FB-modified oligonucleotide is conjugated to HyNic modified antibody (or other protein) by modification of the antibody using S-HyNic, the

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**Figure 1:** A: Scheme outlining the incorporation of 4FB moieties on the 5’-terminus of oligonucleotides during oligonucleotides solid phase synthesis using Solulink’s 4FB-phosphoramidite 1. B: Scheme presenting the conjugation of a 4FB-oligonucleotide to a HyNic-modified antibody yield a bis-arylhydrazone mediated oligonucleotides/antibody conjugate.
amine reactive derivative of 6-hydradzinonicotinic acid. Conjugation is performed by simply adding the 4FB-oligonucleotide to the HyNic-modified antibody. The efficiency and control of the conjugation is demonstrated by the fact that for each oligonucleotide required to be conjugated only 1.5-2.0 equivalents of 4FB-oligonucleotide are added. Furthermore it is routinely found that >95% of the antibody is converted to conjugate when >2 oligonucleotides are targeted and the conjugate yield is 40-60% based on input protein. Conjugation of oligomers of 10-90 nucleotides can be efficiently prepared.

**Method:** The conjugation of an oligonucleotide to a protein using Solulink’s HyNic/4FB conjugation couple is a four step process: (1) incorporation of a 4FB-moiety on an oligonucleotide by modifying a 3’ or 5’-amino-modified oligo with S-4FB or incorporation of a 4FB moiety on the 5’-terminus using Solulink’s 4FB-phosphoramidite (Figure 1, 1), (2) incorporating HyNic moieties on the protein using S-HyNic using standard protein modification procedures for NHS-esters, (3) conjugating the two modified biomolecules by simply mixing and incubating at room temperature for several hours and (4) purification by size exclusion chromatography.

An example protocol for the preparation of an 20mer oligonucleotide/antibody conjugate follows:

**Step 1: 4FB modification of an amino-modified oligonucleotide:** A 20mer 5-amino-modified (MW 6600; (500 ug; umol)) was dissolved in water and washed 3 X 400 uL with nuclease free water using a 5000 MWCO VivaSpin diafiltration apparatus (Sartorius). The concentration of the amino-modified oligonucleotide was adjusted to 0.2-0.5 OD/uL and a 1/10 volume of 10X modification buffer (1.0 M phosphate, 1.5 M NaCl, pH 7.4) was added followed by ½ volume of DMF. A 20 mg/mL stock solution of S-4FB in DMF was prepared and an aliquot containing 20 equivalents of S-4FB is added and the reaction mixture is incubated at room temperature for 2 h. The 4FB-modified oligonucleotide is purified by desalting using a second VivaSpin diafiltration apparatus.

**Step 2: HyNic-modification of an antibody:** To a 2 mg/mL solution of a desalted antibody in modification buffer (100 mM phosphate, 150 mM NaCl, pH 7.4) was added a 20 mg/mL solution of S-HyNic (20 equivalents) and the reaction was incubated at room temperature for 2 hours. The HyNic-modified oligonucleotide was desalted using a Pierce Zeba desalting column.

**Step 3: Conjugation:** To the HyNic-modified antibody (1 equivalent) was added the 4FB-modified oligonucleotide (4 equivalents). The reaction mixture was incubated at room temperature for 4-16 h. If desired the conjugation can be monitored using a NanoDrop spectrophotometer as the bis-arylhydrazone bond formed on conjugation absorbs at 354nm (molar extinction coefficient 29000) or by electrophoresis.

**Step 4: Purification:** Using the above protocols >95% of the antibody will be converted to conjugate therefore only the excess oligonucleotide requires removal. The conjugate was isolated using by size exclusion chromatography using a 10 X 300 mm SuperDex200 column (GE Healthcare) eluting with PBS at 1 mL/min using a photo-diode array detector. Figure 2 presents the results routinely achieved. The initial higher molecular weight peak is the conjugate that is further characterized by the signature 354 nm absorption of the bis-arylhydrazone conjugate bond. Routinely the overall yield is >40-60% based on input protein.
**The Results:** The Coomassie Blue stained gel in Figure 3 presents the results of a typical 4FB-oligonucleotide/HyNic antibody conjugation demonstrating that >95% of the antibody is converted to conjugate. The oligonucleotide is visualized by UV-backshadowing and the retention of functionality of the oligonucleotide is demonstrated performing a SouthWestern analysis wherein a fluorescein-labeled complementary oligonucleotide is hybridized to the conjugate on the gel.

Demonstrating the significant technological advance that the 4FB/HyNic couple offers to the preparation of antibody oligonucleotide conjugates is the work of Fredriksson et al. 9-11 in a series of papers presenting their development of the Proximal Ligation Assay (PLA), a second generation immunoPCR technique. In their initial publication they prepared their 40mer and 60mer oligonucleotide/antibody conjugates using the maleimido/thiol conjugation couple that resulted in very low yield of conjugate following a multiple step purification scheme. Subsequently the conjugates were prepared by Solulink using the HyNic/4FB couple accelerating the development of the PLA assay.

![Figure 3: Lane (1) Control unmodified protein + 5'-4FB-oligo Lane (2) HyNic-modified Ab + 5'-4FB-oligo (4 equiv) and Lane (3) HyNic-modified protein + 5'-4FB-oligo (8 equiv)](image)

**Conclusion:** Solulink’s HyNic/4FB bioconjugation couple has been engineered to prepare oligonucleotide/antibody conjugates efficiently, reproducibly and in excellent yields. 4FB-modified oligonucleotides as long as 90 bases have been successfully conjugated. Furthermore this method has been used to conjugate siRNA duplexes to antibodies using a disulfide cleavable linker, *i.e.* S-SS-4FB linker (Figure 4).

![Figure 4: Structure of disulfide cleavable linker S-SS-4FB.](image)

**References**


### Protocols

- Amino-oligonucleotide modification calculator/spreadsheet: [Link](#)
- Protein modification spreadsheet/calculator: [Link](#)
- Protein/oligo conjugation calculator/spreadsheet: [Link](#)

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