WHITE PAPER

Oligonucleotide-Protein Conjugate Synthesis Made Easy, Efficient, and Reproducible

vector LABORATORIES
Together we breakthrough™

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) and vaccines to increase the adjuvanticity of CpG oligonucleotides(2–5). Liu et al. have used antibody-oligonuceotide conjugates for pretargeting cancer therapeutics (6,7).

Oligonucleotide-protein conjugates have utility in protein detection and quantification in a variety of ways. Protein detection by a technique that merges an ELISA with a polymerase chain reaction (PCR), which is called immuno-PCR, 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 unbound conjugate. This shortcoming has been overcome by the Proximity Ligation Assay (PLA) developed by Fredricksson et al. (9–11) 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 nucleoside triphosphates and ligation enzyme to ligate the two oligonucletides and PCR 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 book ***Bioconjugate Techniques*** (Academic Press) but the methods are difficult to perform, stoichiometrically inefficient and low yielding. Two major problems 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 maleimide/thiol couple has been predominantly used to conjugate oligonucleotides to proteins. This protocol requires the researcher to (1) synthesize a thiol-modified oligonucleotide, (2) incorporate maleimide moieties on the protein, (3) combine the two modified components, and (4) purify by ion exchange chromatography or other chromatographic methods.

While this protocol appears straightforward it is fraught with difficulties, which include:

- The thiol oligonucleotide readily oxidizes to its disulfide, requiring reduction prior to conjugation
- Maleimide-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 conjugate 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. In most cases it is imperative to retain the biological function of the protein 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 oligonucleotide-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-proteins is mandated by its ultimate function, and it would be optimal to be able to control the oligonucleotide to protein molar substitution ratio.

The SoluLINK® Technology Solution

To efficiently prepare oligonucleotide-protein conjugates, SoluLINK technology includes a 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 then conjugated to a HyNic modified antibody (or other protein) by modification of the antibody using S-HyNic, the amine-reactive derivative of 6-hydrazinonicotinic acid. Conjugation is performed by 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 greater than 2 oligonucletotides are targeted and the conjugate yield is 40–60% based on input protein. Conjugation of oligomers of 10–90 nucleotides can be efficiently prepared.

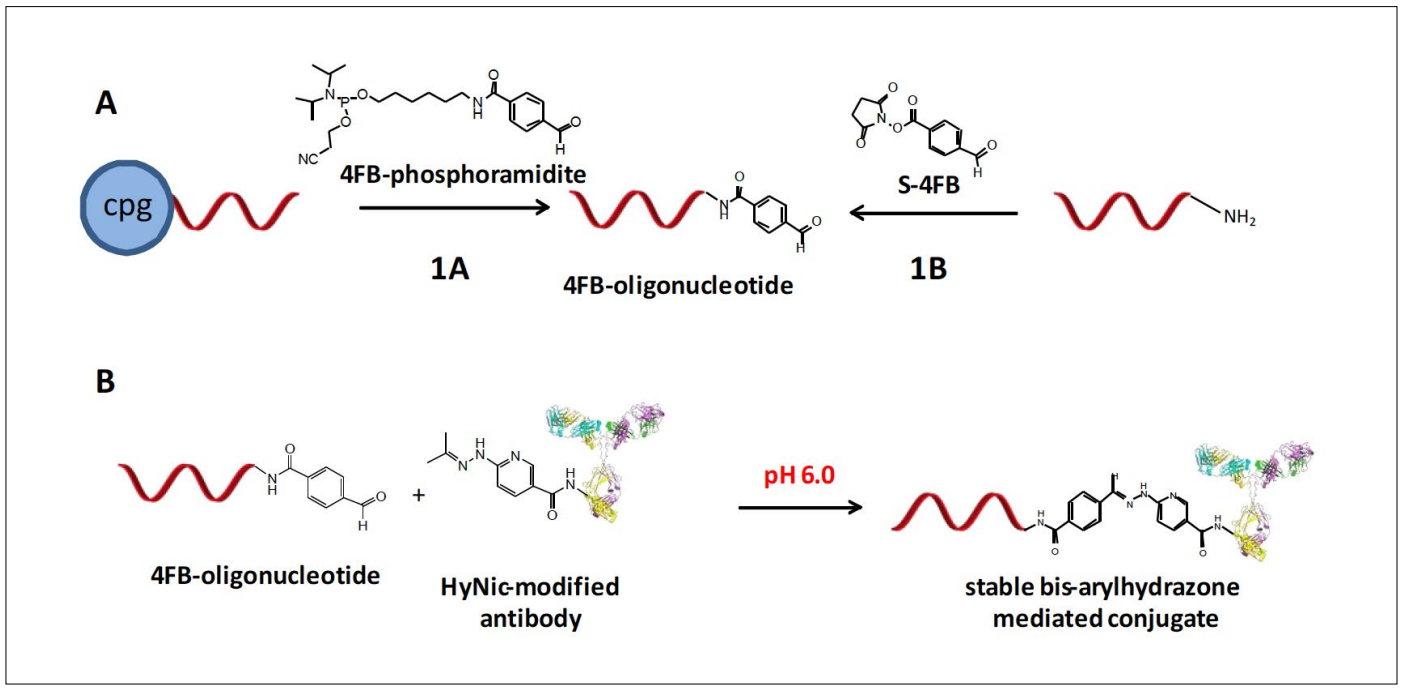

Figure 1. A: *Scheme outlining the incorporation of 4FB moieties on the 5′-terminus of oligonucleotides during oligonucleotide solid phase synthesis using 4FB-phosphoramidite.* B: *Scheme presenting the conjugation of a 4FB-oligonucleotide to a HyNic-modified antibody that yields a bis-aryl hydrazone mediated—oligonucleotide-antibody conjugate.*

Methods

The conjugation of an oligonucleotide to a protein using HyNic/4FB conjugation couple is a 4-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 4FB-phosphoramidite (Figure1), (2) incorporating HyNic moieties on the protein using S-HyNic with 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 a 20mer oligonucleotide-antibody conjugate follows:

- Step 1: 4FB modification of an amino-modified oligonucleotide: A 20mer 5′-amino-modified (MW 6,600; 500 µg) was dissolved in water and washed 3 x 400 µl 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/µl and a one-tenth volume of 10X modification buffer (1.0 M phosphate, 1.5 M NaCl, pH 7.4) was added followed by one-half 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 was added. The reaction mixture is incubated at room temperature for 2 hours. 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) was added. The reaction was incubated at room temperature for 2 hours. The HyNic-modified antibody was desalted using the Thermo Scientific™ 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 hours. If desired, the conjugation can be monitored using a Thermo Scientific NanoDrop™ Spectrophotometer as the bis-aryl hydrazone bond formed on conjugation absorbs at 354 nm (molar extinction coefficient 29,000 M^{-1} cm^{-1}) or by electrophoresis.

- Step 4: Purification: Using the above steps, >95% of the antibody will be converted to conjugate; therefore, only the excess oligonucleotide requires removal. The conjugate was isolated using size exclusion chromatography using a 10 x 300 mm SuperDex® 200 column (Cytiva), eluting with PBS at 1 ml/min using a photodiode 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-aryl hydrazone conjugate bond. Typically the overall yield is >40–60% based on input protein.

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 oligonucleotide-antibody conjugates is the work of Fredriksson et al (9–11). In a series of papers presenting their development of the PLA assay, an improvement upon the immuno-PCR technique. In their initial publication (9) they prepared their 40mer and 60mer oligonucleotide-antibody conjugates using the maleimide/thiol conjugation couple which resulted in very low yield of conjugate following a multiple step purification scheme. Subsequently (10–11) the conjugates were prepared by Solulink using the HyNic/4FB couple, accelerating the development of the PLA assay.

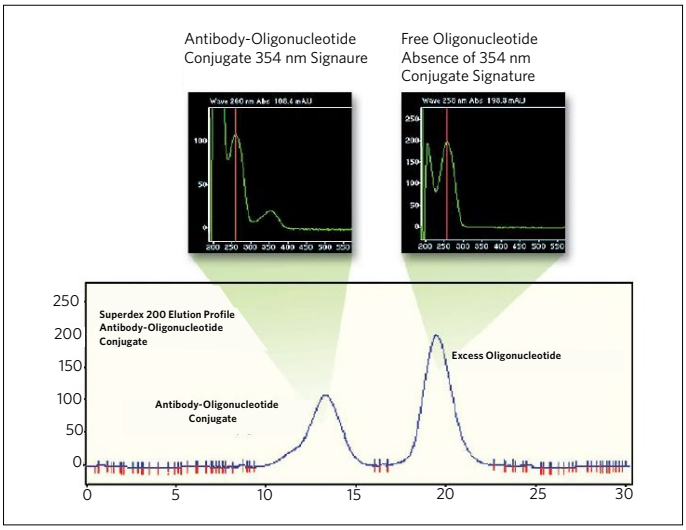

Figure 2 *Lower panel presents the chromatogram profile of the size-exclusion purification of a oligonucleotide-antibody conjugate. The UV spectrum from the photo-diode array detector of the conjugate peak (upper left) shows absorbance at 354 nm, a result of the bis-aryl hydrazone conjugate bond. The UV spectrum of the second peak (upper right) due to the excess 4FB-modified oligonucleotide does not have any absorbance >300 nm.*

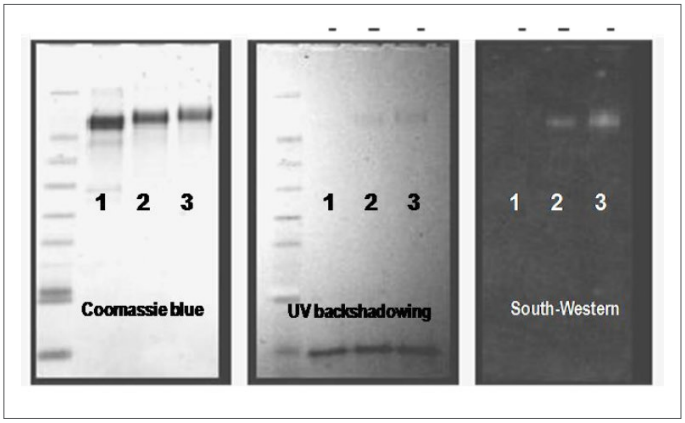

Figure 3 *Lane (1) Control unmodified protein + 5'-4FB-oligo Lane (2) HyNic-modified Ab + 5'-4FB-oligo (4 equivalent) and Lane (3) HyNic-modified protein + 5'-4FB-oligo (8 equivalent).*

Conclusion

The 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).

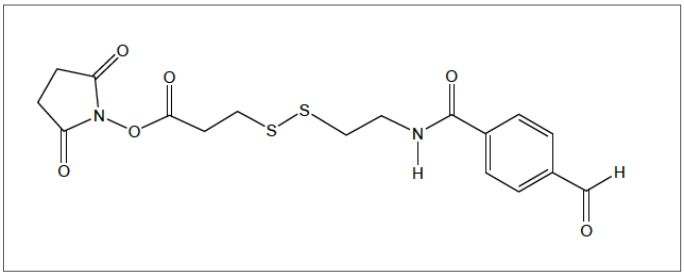

Figure 4 *Structure of disulfide cleavable linker S-SS-4FB.*

Published References

1. Jeong JH, et al. 2009. siRNA, Conjugate Delivery Systems. *Bioconjugate Chemistry.*

2. Datta SK, et al. 2004. Antigen-Immunostimulatory Oligonucleotide Conjugates: Mechanisms and Applications. *Immunological Reviews.*

3. Spiegelberg HL, et al. 2002. Allergen-Immunostimulatory Oligodeoxynucleotide Conjugate: A Novel Allergoid for Immunotherapy. Current Opinion in *Allergy and Clinical Immunology.*

4. Horner AA, et al, 2001. Immunostimulatory DNA-Based Vaccines Elicit Multifaceted Immune Responses Against HIV at Systemic and Mucosal Sites. *The Journal of Immunology.*

5. Higgins BAD, et al. 2006. Modulation of Immunogenicity and Allergenicity by Controlling the Number of Immunostimulatory Oligonucleotides Linked to Amb a 1. *The Journal of Allergy and Clinical Immunology.*

6. Liu G, et al. 2008. An Experimental and Theoretical Evaluation of the Influence of Pretargeting Antibody on the Tumor Accumulation of Effector. *Molecular Cancer Therapeutics.*

7. Liu G, et al. 2007. A Novel Pretargeting Method for Measuring Antibody Internalization in Tumor Cells. *Cancer Biotherapy & Radiopharmaceuticals.*

8. Sano T, et al. 1992. Immuno-PCR: Very Sensitive Antigen Detection by Means of Specific Antibody-DNA Conjugates. *Science.*

9. Gustafsdottir SM, et al. 2005. Proximity Ligation Assays for Sensitive and Specific Protein Analyses. *Analytical Biochemistry.*

10. Fredriksson S, et al. 2007. Multiplexed Protein Detection by Proximity Ligation for Cancer Biomarker Validation. *Nature Methods.*

11. Fredriksson S, et al. 2008. Multiplexed Proximity Ligation Assays to Profile Putative Plasma Biomarkers Relevant to Pancreatic and Ovarian Cancer. *Clinical Chemistry.*

12. Kattah MG, et al. 2008. HIT: A Versatile Proteomics Platform for Multianalyte Phenotyping of Cytokines, Intracellular Proteins and Surface Molecules. *Nature Medicine.*

13. Bailey RC, et al. 2007. DNA-Encoded Antibody Libraries: A Unified Platform for Multiplexed Cell Sorting and Detection of Genes and Proteins. *Journal of the American Chemical Society.*

14. Fan R, et al. 2008. Integrated Barcode Chips for Rapid, Multiplexed Analysis of Proteins in Microliter Quantities of Blood. *Nature Biotechnology.*

SoluLINK Recommended Products

- Amino-oligonucleotide modification calculator
- Protein modification calculator
- Protein/oligo conjugation calculator

Product	Note	Catalog No.	Quantity
Protein–Oligonucleotide Conjugation Kit		S-9011-1	Kit
Antibody–Oligonucleotide All-in-One™ Conjugation Kit		A-9202-001	Kit
S-HyNic Linker (DMF Soluble)	Amino-reactive HyNic	S-1002-105	5 x 1.0 mg
		S-1002-010	10 mg
MHPH (Maleimide HyNic) Linker	Thiol-reactive HyNic	S-1009-010	10 mg
S-4FB Linker (DMF Soluble)	Amino-reactive 4FB	S-1004-105	5 x 1.0 mg
		S-1004-010	10 mg

For more information please visit

vectorlabs.com/solulink

LIT3030. Rev.01

vector LABORATORIES