Version 07.11.2013

Protein-Oligonucleotide Conjugation Kit

Technical Manual

Catalog # S-9011-1

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Safety Information

WARNING – CHEMICAL HAZARD. Some chemicals used can be potentially hazardous, and can cause injury or illness.

- Read and understand the Material Safety Data Sheets (MSDS) available at Solulink.com before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (e.g. safety glasses, gloves, or clothing). For additional safety guidelines consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's clean-up procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling and disposal.

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I. Introduction

a. Product Description

The Protein-Oligo Conjugation Kit is designed to conjugate a protein with an oligonucleotide. It includes all of the necessary components and protocols for easy and specific crosslinking of any protein with any amino-oligo from 20 to 100 bases in length. This kit is flexible so that researchers with little or no conjugation experience can make their own custom protein-oligo conjugate to suit their needs.

The SoluLink bioconjugation method converts more than 95% of protein to conjugate when four mole equivalents of oligo are added. High conversion rates, coupled with the unique UV traceable bond formed during crosslinking, allows for easy purification and identification of the conjugate from the excess oligo using size exclusion purification methods such as HPLC.

b. The SoluLinK Bioconjugation Method

The Protein-Oligo Conjugation Kit uses SoluLink's superior bioconjugation method to prepare protein-oligonucleotide conjugates in 3 easy-to-perform steps (Figure 1). The first step is the modification of the oligo with our 4FB crosslinker, followed by the formation of the HyNic modified protein. Finally, simple mixing of the two modified biomolecules will result in the formation of a stable, UV-traceable bond formed by the reaction of a HyNic modified protein with a 4FB modified oligonucleotide.

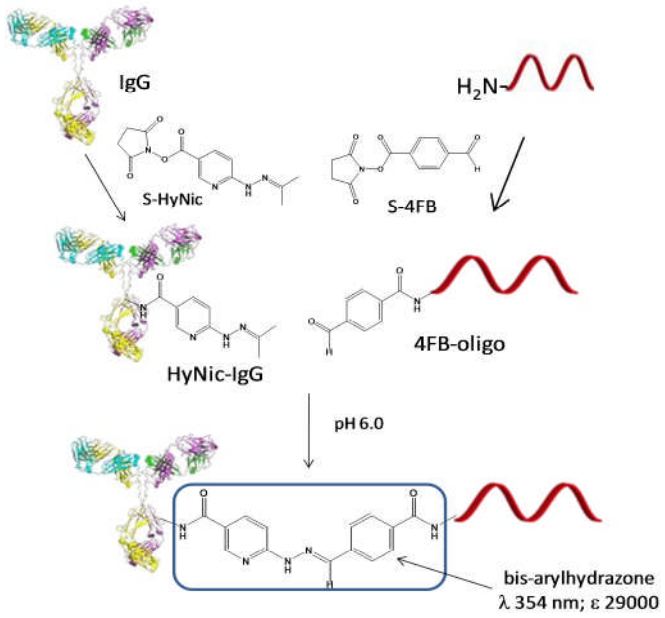


Figure 1: Schematic representation of the three step process to prepare an antibody-oligonucleotide conjugate using SoluLink's Bioconjugation chemistry. Initially an antibody is modified with S-HyNic to incorporate HyNic groups and subsequently the HyNic-modified antibody is reacted with a 4FB-modified oligonucleotide.

This technology has many practical advantages compared to previous crosslinking methods:

1. The reaction is high yielding. Routine yields of conjugate are 50-80% based on starting protein.
2. The reaction is efficient: Only 3-4 mole equivalents of oligo are necessary for the protein, >90% of the protein is conjugated.
3. The conjugate bond is extremely stable: The conjugate bond is stable to 92^{O}C and pH 2.0-10.0.
4. The reaction conditions are mild and do not cause any protein denaturation: Unlike thiol-based conjugation protocols, where reducing reagents are required that can compromise the activity of proteins by cleaving disulfide bonds, the HyNic-4FB conjugation couple leaves disulfide bonds intact. No metals, oxidation or reducing reagents are required.

5. The conjugation is traceable spectrophotometrically. The HyNic-4FB conjugate bond is UV traceable- it absorbs at 354 nm and has a molar extinction coefficient of 29000.

6. The modifications of both the HyNic linker on the protein and the 4FB linker on the oligonucleotide are quantifiable using colorimetric assays. The reproducibility of any reaction is facilitated by accurate characterization of all components. The Molar Substitution Ratio (MSR) of linker groups, *i.e.* the number of HyNic linkers per protein, can be quantified colorimetrically. This kit contains all the reagents necessary to determine the MSRs for both the protein and the oligo.

II. Protein-Oligonucleotide Conjugates: A Review

The diversity and specificity of proteins combined with the specificity of hybridization of oligonucleotides results in unlimited numbers of specific protein detection reagents whose applications are addressed below.

The use of oligo-protein conjugates was initially demonstrated by Sano *et al.*[1] for protein detection by a technique called immuno-PCR (Polymerase Chain Reaction) where a 100mer oligo/antibody conjugate was allowed to bind to its ligand and amplified by PCR demonstrating extremely sensitive protein detection. Since this initial publication there has been a need for a straight forward, efficient and high yielding method for the preparation of these conjugates.

The first generation immuno-PCR protocol was plagued by high background due to non-specific binding of the conjugate and the extreme sensitivity of PCR. This has been overcome by the Proximal Ligation Assay (PLA) developed by Fredriksson and Lundegren.[2] In the PLA assay, two antibodies to different epitopes are conjugated to a 40mer 5'-phosphorylated oligonucleotide through the 3'-end and 60mer oligonucleotide conjugated through its 5'-terminus. The two oligo/antibody conjugates are incubated with the sample, allowed to bind to their respective epitopes, the mixture is washed and then incubated with a 'splint' oligo that hybridizes across the two oligonucleotides that is subsequently ligated. Following ligation, PCR is performed on the ligated oligo generating a quantifiable signal. In subsequent work the oligo/antibody conjugates used by Fredriksson *et al.* and others used conjugates prepared by SoluLink using the HyNic-4FB Conjugation Method. [3-6] Kozlov *et al.* [7] also describe the use of oligonucleotide/antibody conjugates for the sensitive detection of proteins.

Additionally, oligonucleotide/antibody conjugates have been used for capture of antigens and subsequent addressing to antibody arrays for multiplex detection of proteins as well for cell sorting on the same diagnostic platform.[8,9] Oligonucleotide/protein conjugates have been also been used in vaccines to increase adjuvanticity using CpG oligonucleotide/protein conjugates.[10-12]

III. Accessing 4FB-modified Oligonucleotides

Stable and disulfide-cleavable 4FB oligonucleotides can be obtained in several ways:

1. **5'-4FB oligonucleotide**

   a. **4FB-phosphoramidite:** 4FB-Phosphoramidite (**1;** Figure 2) is available for incorporation of 5'-4FB groups during oligonucleotide solid phase synthesis. Standard coupling protocols are used and the yields are similar to any amino modifier. The 4FB Phosphoramidite may be purchased directly (SoluLink catalog #S-1005) or you may order 5'-4FB oligonucleotides directly from SoluLink.

   b. **5'-amino oligonucleotides**: 5'-amino oligonucleotides may be converted to 5'-4FB modified oligonucleotides in a straight forward high yielding modification step with S-4FB (**2**; Figure 2).

   The Protein-Oligo Conjugation Kit includes S-4FB and all the reagents and materials required to convert a 5'-amino oligonucleotide to a 5'-4FB-oligonucleotide.

2. **3'-4FB oligonucleotide**:
   3'-Amino oligonucleotides are converted to 3'-4FB modified oligonucleotides in an easy, high yielding modification step with S-4FB (**2**; Figure 2).
   The Protein-Oligo Conjugation Kit includes S-4FB and all the reagents and disposables required to convert a 3'-amino oligonucleotide to a 3'-4FB-oligonucleotide.

3. **5'- and 3'-4FB disulfide-cleavable oligonucleotides:** 5'- and 3'-amino oligonucleotides may be converted to disulfide-cleavable oligonucleotides using S-SS-4FB (**3;** Figure 2) in an easy, high yielding modification step. This product with protocol is available separately (SoluLink catalog #S-1037).

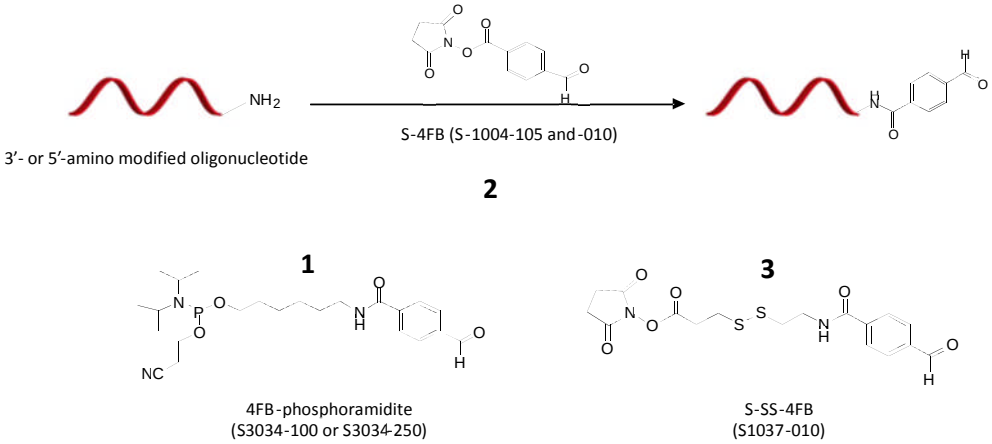


Figure 2: Schematic representation of the conversion of an amino-modified oligonucleotide to a 4FB-oligonucleotide with S-4FB (**2**) (top) and structures of 4FB-phosphoramidite (**1**) and S-SS-4FB (**3**), the reagent used to convert an amino-oligonucleotide to a 4FB-SS-oligonucleotide.

IV. The Keys to Successful Conjugation

The following are three crucial requirements that must be fulfilled for a reproducibly successful preparation of a protein/oligonucleotide conjugate using Solulink's bioconjugation technology:

1. **Desalting:** Prior to modification, the starting protein must be thoroughly desalted, removing all amine contaminants, and exchanged into 1X Modification Buffer.
2. **Protein concentration**: The recommended concentration of the protein (1 – 5mg/ml) must be adhered to in all steps.
3. **Molar substitution ratio:** The Molar ratio of the HyNic on the protein and the 4FB on the oligo must be determined and within the desired range before continuing to the next step.

V. Kit Components

Component	Component #	Size	Storage
S-HyNic	**S-9011-1-01**	**2 X 0.5 mg**	**Desiccated**
S-4FB	**S-9011-1-02**	**2 X 1.0 mg**	**Desiccated**
10X Modification Buffer	**S-9011-1-03**	**1.5 mL**	**4°C**
10X Conjugation Buffer	**S-9011-1-04**	**1.5 mL**	**4°C**
10X TurboLink Catalyst buffer	**S-9011-1-05**	**1.0 mL**	**4°C**
7kDa 0.5 mL Zeba Columns	**S-9011-1-06**	**12**	**4°C**
Anhydrous DMF	**S-9011-1-07**	**1.5 mL**	**Desiccated**
0.5 mM 2-Hydrazinopyridine Reagent	**S-9011-1-09**	**0.5 mL**	**4°C**
0.5 mM 2-Sulfobenzaldehyde Reagent	**S-9011-1-10**	**0.5 mL**	**4°C**
7kDa 2 mL Zeba Columns	**S-9011-1-13**	**2**	**4°C**
10X PBS	**S-9011-1-14**	**1.5 mL**	**4°C**
2.0 mL Collection tubes	**S-9011-1-15**	**12**	**RT**
Oligo Resuspension Solution	**S-9011-1-16**	**1.0 mL**	**4°C**

NOTES:

1) For convenience all kit components can be stored at 4°C
   If precipitate is present in buffers on storage at 4°C redissolve by warming to 37°C before using
2) 10X Modification Buffer: 1.0 M phosphate, 1.5 M NaCl, pH 8.0
3) 10X Conjugation Buffer: 1.0 M phosphate, 1.5 M NaCl, pH 6.0
4) 10X TurboLink Catalyst Buffer: 100 mM aniline, 100 mM phosphate, 150 mM NaCl, pH 6.0

VI. Equipment/Regents Required But Not Provided

Variable-speed bench-top microcentrifuge
Spectrophotometer or Plate Reader
Protein concentration assay reagents such as BCA or Bradford assays

VII. Protocols

Certain strict parameters:

1. Protein molecular weight range 25,000 – 900,000 Daltons
2. Protein concentration range 1.0 – 5.0 mg/ml; Mass of protein to be modified range 50 – 650 µg; protein reaction volume range 50 – 130 µL
3. Oligonucleotide size range 20 – 100 bases
4. Oligonucleotide concentration range 0.3 – 0.6 OD_{260}/µL; Mass of oligo to be modified range 15 – 40 OD_{260}; Oligo reaction volume range 30 – 60 µL

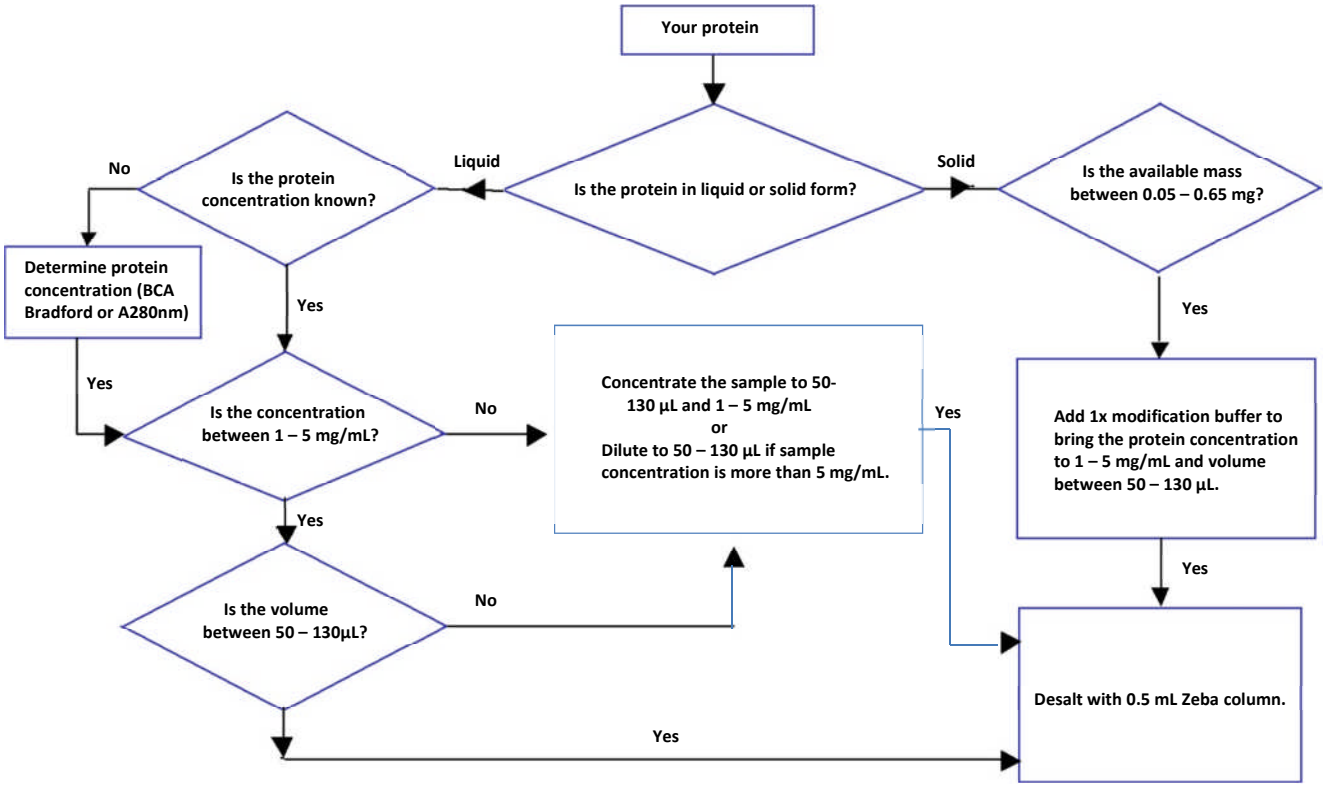


Figure 3. Flow-chart used for guiding a user to the start of the conjugation process.

a. Desalt/Buffer Exchange of the Protein or oligo

Protein and oligo must be completely desalted into 1X Modification Buffer (dilute from 10X Modification Buffer) before they are modified with S-HyNic or S-4FB. SoluLink recommends the use of Zeba™ Desalt Spin Columns (provided) to desalt the protein and oligo. These rapid spin columns are recommended because they do not significantly dilute the sample during desalting and recover 85-90% of the protein or oligo.

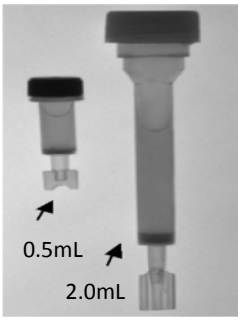


Figure 4. Zeba™ Desalt Spin Columns used to desalt protein and Oligo

Included in this kit are 0.5 mL Zeba Spin Desalt columns (Figure 4) that have a maximum capacity of 130 µL. Therefore up to 0.65 mg of a 5 mg/mL solution of protein or 40 OD of a 0.6 OD/µL solution of oligo can be desalted. As this kit has been designed for two conjugations, ten Zeba columns are included; one to initially desalt the protein with 1x Modification buffer and one to desalt and exchange the modified protein into 1x Conjugation buffer. One to initially desalt the oligo into 1x Modification buffer and two to desalt and exchange the modified oligo into 1x Conjugation buffer. This kit also includes two 0.5 mL or 2 mL Zeba™ desalting columns that have a capacity of 50 – 150 µL or 150-700 µL, respectively, to desalt the final two protein/oligo conjugates into PBS storage buffer after conjugation depending on the final reaction volume.

Zeba Desalting Protocol

0.5mL Zeba™ Spin Column Preparation and Sample Loading

1. Remove spin column's bottom closure and loosen the top cap (do not remove cap).
2. Place spin column in a 2.0 mL microcentrifuge collection tube.
3. Centrifuge at 1,500 x g for 1 minute to remove storage solution.
4. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in the microfuge with the mark facing outward in all subsequent centrifugation steps.
5. Add 300 µL of required buffer to the top of the resin bed. and centrifuge at 1,500 x g for 1 minute; discarding the flow through from the collection tube.
6. Repeat step 5 two additional times, discarding buffer from the collection tube.
7. Column is now ready for sample loading.

Sample Loading

1. Place column in **a new 2.0 mL microcentrifuge tube**, remove cap and slowly apply 50-130µL of sample to the center of the compacted resin bed.
2. Centrifuge column at 1,500 x g for 2 minutes to collect desalted sample. Discard column after use.
3. Confirm protein or oligo concentration and now ready for next step.

b. Amino Oligo modification Protocol with S-4FB ***(Calculator Worksheet 1)***

A. Enter Amino-Oligo Information into Conjugation Calculator

1. Enter the following amino-oligo parameters directly from the Oligo vendor's Certificate of Analysis into the **Protein-Oligonucleotide Conjugation Calculator** worksheet 1 - amine-oligonucleotide modification calculator on section I.

   a) Oligonucleotide name
   b) OD_{260} units supplied by vendor
   c) Oligonucleotide molar extinction coefficient (liter cm^{-1} mol^{-1})
   d) Oligonucleotide molecular weight (Daltons)
   e) Nanomoles per OD_{260} as listed on the product data sheet

   Note: If the certificate of analysis is not available, the necessary information may be obtained by entering the oligo sequence into the Oligo Analyzer tool on IDT's website at the following address:

   http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx#

B. Resuspend Amino-Oligo

1. **Ensure at least 15 OD_{260} units of oligo are available for modification—this information can be found on the oligo product data sheet supplied by the vendor.**

2. Place the vial containing lyophilized oligonucleotide in a microcentrifuge and centrifuge at 15,000 x g for 15 seconds to pellet the lyophilizate at the bottom of the tube. If the tube contains between 15 and 25 OD_{260} units of oligo, add 50 µL of **Oligo Resuspension Solution** to the tube. If the tube contains more than 25 OD_{260} units of oligo, add a sufficient volume of Oligo Resuspension Solution to create a 0.5 OD_{260}/µL solution (example: if there are 30 OD_{260} units of oligo, add 60 µL of Oligo Resuspension Solution to create a 0.5 OD_{260}/µL solution).

   Note- **30 - 60 µL of oligo solution is using to modify with S-4FB**

3. Allow the pellet to re-hydrate for 1 minute, then vortex the solution on medium speed for 10 seconds to assist dissolution. This process will need to be repeated several times until no un-dissolved lyophilizate remains. Briefly spin the tube to ensure the entire solution is pooled together at the bottom, and then proceed to step C to measure the amino-oligo concentration.

C. Measure Amino-Oligo Concentration on a Spectrophotometer

The actual amino-oligo concentration can be measured either on a conventional spectrophotometer or micro-volume UV-VIS scanning spectrophotometer (e.g.,NanoDrop™ spectrophotometer). When using a conventional spectrophotometer, a quartz or UV-transparent plastic cuvette is required.

NanoDrop™ Spectrophotometer (models ND-1000 and ND-2000)

1. Determine the concentration (OD_{260}/µL) of the resuspended amino-oligo on a NanoDrop™ as follows:

   Note - ***The definition of the OD_{260}/µL of oligo is the A_{260} value of 1 µL of oligo solution in 1000µL of H2O at 1 cm path length.***

   a) In a microcentrifuge tube, prepare a 1:200 dilution of the dissolved amino-oligo by transferring 2 µL oligo with a calibrated P-2 pipette into 398µL molecular grade H_2O. Vortex well to mix.

   b) Select the "Nucleic Acid" menu option on the NanoDrop and initialize the instrument using molecular grade water (NanoDrop 1000 only).

   c) Clean the sample pedestal and blank the instrument with 2 µL molecular grade H_2O.

   d) Measure the A_{260} of the 1:200 amino-oligo solution as displayed in the **10 mm path length window.** Record the A_{260} value.

   e) **Divide this number by 5** (convert to 1:1000 dilution) to calculate the OD_{260}/µL concentration of the stock oligo solution.

2. Multiply the OD_{260}/µL value calculated in (e) above by the volume of initial oligo resuspension solution using to dissolve oligo pellet (step B2) and to determine the total OD_{260} units available after dissolution (do not enter this information into the calculator at this point).

3. **Important: If less than 15 OD_{260} units are recovered after resuspension (from 2 above), obtain additional amino-oligo. 40 OD_{260} is the maximum units of amino-oligo to be modified.**

Conventional UV-VIS Spectrophotometer

1. Determine the concentration (OD_{260}/µL) of the resuspended amino-oligo using a quartz or UV-transparent cuvette and a spectrophotometer as follows:

   a) In a microcentrifuge tube, prepare a 1:500 dilution of the dissolved amino-oligo by transferring 2 µL oligo with a calibrated P-2 pipette into 998µL molecular grade H_2O. Vortex well to mix.

   b) Blank the spectrophotometer at 260 nm using 1mL molecular grade H_2O.

   c) Measure the A_{260} of the 1:500 amino-oligo. Record the A_{260} value.

   d) **Divide this number by 2** (convert to 1:1000 dilution) to calculate the OD_{260}/µL concentration of the stock oligo solution.

2. Multiply the OD_{260}/µL value calculated in (d) above by the volume of initial oligo resuspension solution using to dissolve oligo pellet (step B2) and to determine the total OD_{260} units available after dissolution (do not enter this information into the calculator at this point).

3. **Important: If less than 15 OD_{260} units are recovered after resuspension, obtain additional amino-oligo. 40 OD_{260} is the maximum units of amino-oligo to be modified.**

D. Buffer Exchange Amino-Oligo

1. Prepare a oligo desalting spin column by using 0.5mL Zeba™ column with **1x Modification buffer** (dilute 10X stock buffer 1/10 with water) following the Zeba desalting procedure on page 9.

2. Remove the spin column from the collection tube (discard the collection tube containing excess buffer) and place the column in **a new microcentrifuge tube**. Slowly and carefully pipet exactly 30 - 60 µL of oligo solution into the center of the resin bed. Be careful not to let the oligo solution contact the tube wall; it must channel down through the resin itself. Replace the cap and loosen one-quarter turn.

3. Centrifuge the column again at **1,500 x *g* for 2 minutes** to collect the desalted oligo and measure the volume with appropriate pipet. Enter this volume (µL) of desalted amino-oligo recovered into the oligo modification calculator (Section II).

4. Vortex the oligo solution to mix thoroughly. Repeat the concentration determination as described in section C above. Enter the calculated OD_{260}/µL stock oligo concentration into the Oligo Modification Calculator (Section II).

   Note: Excess un-desalted amino oligo from Part D may be stored at -20°C or lower indefinitely.

E. Dissolve S-4FB Reagent

1. Briefly centrifuge the tube of S-4FB reagent at 15,000 x *g* to ensure that all material is at the bottom of the tube. Add 40µL Anhydrous DMF to the 1.0 mg vial of S-4FB reagent and vortex for 20 seconds to re-suspend. Continue to periodically vortex until the pellet is completely dissolved. **The sample may need to be pipetted up and down several times to dissolve completely**. Briefly spin the completely dissolved reagent to the bottom of the tube.

F. Modify Amino-Oligo with S-4FB Reagent

1. After entering the volume of oligo recovered (step D3) and its concentration (step D4), the calculator will determine the volume of DMF to add, as well as the volume of S-4FB in DMF to add to modify the oligo (output on section III of the oligo modification calculator).

2. First, add the indicated volume (µL) of DMF to the oligo solution and briefly vortex to mix. Next, add the volume of dissolved S-4FB in DMF indicated to the amino-oligo and vortex vigorously to mix. Do not centrifuge the reaction mixture after the S-4FB reagent is added.

3. Incubate at **room temperature** for **2 hours** to allow the reagent to react with the amino-oligo.

G. Removal of excess S-4FB

Five minutes prior to the end of the 4FB/oligo modification reaction, prepare two desalting columns as follows:

1. Prepare two oligo desalting spin columns by using 0.5mL Zeba™ column with **1x Conjugation buffer** (dilute 10X stock buffer 1/10 with water) following the Zeba desalting procedure on page 9.

2. Remove the spin columns from the collection tubes (discard the collection tubes containing excess buffer) and place the columns in **new microcentrifuge tubes** and ready for using.

3. Before load to column, centrifuge the oligo-modification reaction from *Part F* at **15,000 x *g*** for 2 minutes to pellet any insoluble reaction by-products *(Please note the increased spin speed of 15,000 x g, rather than 1,500 x g that is used during the desalting process).* In the next step, use only the clear supernatant (which contains the 4FB oligo) in the desalting process, leaving the pellet (if any) in the tube.

4. Slowly and carefully pipet the entire modification reaction (except for precipitate, if any) onto the center of only one of the spin columns. Be careful not to let the oligo solution contact the tube wall; it must channel down through the resin itself. Replace the cap and loosen one-quarter turn. Leave the other column on the bench top during the next step.

5. Centrifuge the column containing the modified oligo at **1,500 x *g* (not 15,000 x *g*) for 2 minutes** to recover the desalted oligo into the microcentrifuge tube.

6. Immediately transfer the entire elute from step 5 to the other spin column and repeat the desalting process by centrifuging at **1,500 x *g* for 2 minutes to a new microcentrifuge tube.** This "double-desalting" will ensure that all traces of un-incorporated 4FB are removed from the oligo.

7. Measure the volume with a P-200 pipette. Vortex the solution to mix thoroughly before proceeding to Part H.

H. Measure 4FB-Oligo Concentration

Measure the 4FB-Oligo concentration as follows:

Using a NanoDrop™ Spectrophotometer (models ND-1000 and ND-2000)

1. In a microcentrifuge tube prepare a 1:50 dilution of the 4FB-oligo by transferring 2 µL oligo with a calibrated P-2 pipette into 98 µL molecular grade H_2O. Vortex well to mix.
2. Select the "Nucleic Acid" menu option on the NanoDrop and initialize the instrument using molecular grade water (ND-1000 only).

3. Clean the sample pedestal and blank the instrument with 2µL molecular grade H_2O.
4. Clean the sample pedestal and measure the A_{260} of the 1:50 4FB-oligo solution as displayed in the **10 mm path length window.** Record the A_{260} value.
5. **Divide this number by 20** (convert to 1:1000 dilution) to calculate the OD_{260}/µL concentration of the stock 4FB-oligo solution. Then enter this calculated OD_{260}/µL concentration of 4FB-oligonucleotide into the Modification Calculator (Section IV).

Using a conventional 1 cm pathlength spectrophotometer

1. In a microcentrifuge tube prepare a 1:500 dilution of the 4FB-oligo by transferring 2 µL oligo with a calibrated P-2 pipette into 998µL molecular grade H_2O.
2. Blank the spectrophotometer at 260 nm using 1mL molecular grade H_2O.
3. Measure the A_{260} of the 1:500 4FB-oligo. Record the A_{260} value.
4. **Divide this number by 2** (convert to 1:1000 dilution) to calculate the OD_{260}/µL concentration of the stock 4FB-oligo solution. Then enter this calculated OD_{260}/µL concentration of 4FB-oligonucleotide into the Modification Calculator (Section IV).

I. Quantify 4FB Molar Substitution Ratio

The following 4FB Molar Substitution Assay quantifies the amount of 4FB attached to the oligonucleotide. The assay is performed by reaction of an aliquot of 4FB-oligo with 2-HP reagent at **37°C for 60 minutes**, after which the A_{360} of the sample is measured on a spectrophotometer. This assay ensures that the oligo is both 4FB-modified and properly buffer exchanged. Use the appropriate instructions below depending on the specific type of spectrophotometer available to you (NanoDrop™ or conventional).

Assay Protocol

1. Prepare a 2-HP blank solution by adding exactly 2 µL water to exactly 18 µL 2-HP Reagent in a clean reaction tube (not provided); label '2-HP Blank'.

2. Prepare a 4FB-oligo MSR sample by adding exactly 2 µL 4FB-modified oligo to exactly 18 µL 2-HP reagent in a clean reaction tube (not provided); label '4FB-Oligo'. Vortex both solutions well to mix, and then briefly spin the tubes to pool the entire contents to the bottom of each tube.

3. Incubate the 2-HP blank and 4FB-Oligo reactions at 37°C for **60 minutes**. After the incubation period, centrifuge both tubes at 1,500 x *g* for 15 seconds to collect condensation at the bottom of the tube. Gently vortex to thoroughly mix.

NanoDrop 4FB Molar Substitution Ratio Assay

1. Launch the NanoDrop™ software and select the **UV-VIS menu** option. Initialize the instrument with 2 µL molecular grade water (NanoDrop ND-1000 only).

2. Blank the NanoDrop™ with 2 µL 2-HP blank solution and clean the pedestal. Set the λ1 absorbance wavelength to read at **360 nm**.

3. Place a 2 µL aliquot of the 4FB-Oligo MSR reaction on the pedestal and click the 'Measure' icon. The 1.0 mm A_{360} absorbance will appear. Enter this value directly into the MSR calculator (Section V) using **Protein-Oligonucleotide Conjugation Calculator** on calculator worksheet -1 to obtain MSR.

Conventional Spectrophotometer 4FB Molar Substitution Ratio Assay (100µL micro-cuvette method)

1. Prepare a 1:10 dilution of the **2-HP blank** solution by adding 180 µL of molecular grade H_2O into the blank tube.

2. Prepare a 1:10 dilution of the **4FB-oligo** MSR reaction solution by adding 180 µL of molecular grade H_2O into the blank tube. Vortex both solutions well to mix well.

3. In a 1 cm, 100 µL quartz micro-cuvette, blank the spectrophotometer at λ = 360 nm with 100 µL of 1:10 diluted 2-HP blank.

4. Measure the A_{360} of the 1:10 4FB-Oligo MSR sample from step 2 above in the cuvette. Record the A_{360} and enter this value directly into the MSR calculator (Section V) using **Protein-Oligonucleotide Conjugation Calculator** on calculator worksheet 1 to obtain MSR.

c. *Protein Modification Protocol with S-HyNic* ***(Calculator Worksheet 2)***

Recommended Guidelines for Modifying Proteins with S-HyNic

The modification process is a critical element of any conjugation project. For this reason, we have included a more detailed discussion of this important step. For example, the number of functional groups incorporated per protein molecule is commonly referred to as the molar substitution ratio (MSR). The final MSR obtained after a modification reaction with S-HyNic is a function of several variables that include protein concentration, number of available amino-groups on the protein (often related to M.W.), excess linker equivalents added (e.g. 5X, 10X or 20X) and reaction pH. Table 1 presents the results of a study to determine the level of HyNic incorporation on an antibody adding 5X, 10X and 20X equivalents of S-HyNic at 1.0, 2.5 and 5.0 mg/mL of antibody concentration in 1X modification buffer.

Protein concentration	5X HyNic	10X HyNic	20X HyNic
1.0 mg/mL	1.0	1.4	3.0
2.5 mg/mL	3.2	5.3	7.9
5.0 mg/mL	4.9	6.6	7.8

Table 1: MSR of Modifying Bovine IgG with S-HyNic

Note - It is recommend that the MSR of HyNic-proteins is 4 - 8 for proteins greater than 100,000 Daltons, and 2-4 for proteins equal to or less than 100,000 Daltons.

In general, as the protein concentration and number of linker equivalents are increased, the molar substitution ratio increases. Caution is recommended since over-modification can dramatically change the isoelectric point of the protein and result in precipitation of the protein or loss of biological activity. This is especially critical with proteins <50 kD MW.

S-HyNic-Protein Modification Protocol (Calculator Worksheet 2)

1. Desalt protein to be modified with S-HyNic into **1X Modification Buffer** (dilute 10X stock buffer 1/10 with water) following the Zeba desalting procedure on page 9. Between 50 - 130 µL of protein solution at a concentration between 1 - 5 mg/mL may be used.

2. On the S-HyNic Modification Calculator input the name, molecular weight, protein concentration, volume of protein to be modified, and the mole equivalents of HyNic used to modify protein in the green fields. Add the required volume of anhydrous DMF to a 0.5 mg vial of pre-weighed S-HyNic reagent. Pipette the solution up and down to dissolve the pellet. The volume of DMF will be output in the pink field. If the volume of DMF output is ***more than 700 µL***, two or more times dilution of the HyNic reagent may be required.

   For example if the volume of DMF output is 5857 µL, then three times dilutions are required:

   (1) Make a 58.57X stock solution by dissolving S-HyNic pellet in 100 µL of DMF (5857µL/100µL = 58.57).

   (2) Next, make a 5.857X S-HyNic solution by adding 10 µL of 58.57X S-HyNic solution into 90 µL of DMF and mix well (1:10 dilution).

   (3) Third, make 1X S-HyNic solution by adding 10 µL of 5.857X S-HyNic solution into 48.57 µL of DMF and mix well (1:5.857 dilution).

3. If protein molecular weight is greater than 50,000 Daltons, add 2.0 µL of S-HyNic reagent into the desalted protein. If protein molecular weight is equal or less than 50,000 Daltons, add 3.0 µL of S-HyNic reagent into the desalted protein as calculated using the **Protein-Oligonucleotide Conjugation Calculator** on calculator worksheet 2. Briefly vortex to mix.
4. Incubate the reaction at room temperature for 2.5 hours.
5. Proceed to desalt the HyNic-modified protein using 0.5 mL **Zeba™** column equilibrated with 1X Conjugation buffer (dilute 10X stock buffer 1/10 with water) using the Zeba desalting protocol on page 9.

Determining the HyNic Molar Substitution Ratio (MSR)

After desalting with a Zeba™ spin column to remove excess HyNic from the modification reaction, the protein concentration is determined by using either BCA or Bradford assay (the protein concentration must *not* be determined by reading A_{280} on a spectrophotometer at this step because of the contribution at A_{280} of HyNic itself). After the HyNic-protein concentration is determined proceed to MSR assay.

Assay Protocol

1. Transfer 2 µL of HyNic-modified (desalted) protein solution (1-5 mg/mL in 1X Conjugation Buffer) to a new 1.5 mL microfuge tube containing 18 µL of SBA working solution. Prepare another reaction tube (blank) containing 2 µL of 1X Conjugation Buffer and 18 µL of 2-SBA working solution.
2. Vortex to mix well, then incubate both reaction tubes at 37^{O}C for 1 hour.
3. Remove the reaction tubes from 37^{O}C and briefly centrifuge at 10,000 x g to collect any condensate. Vortex both samples thoroughly before reading the A_{348} by one of the following methods:

Method A: NanoDrop™ Method

1. Launch the NanoDrop™ software and select the UV-VIS menu option. Initialize the instrument with 2 µL water (NanoDrop™ ND-1000 only).
2. Blank the NanoDrop™ with 2 µL blank (Conjugation Buffer + SBA) solution and clean the pedestal.
3. Set the λ1 wavelength to 348nm. Place 2 µL of the HyNic-protein MSR reaction on the pedestal and click the "Measure" icon. The 1.0 mm A348 absorbance will appear. **Multiply this number by 10** (converts from 1 mm to 1 cm) and then enter this value into the HyNic MSR Calculator **Protein-Oligonucleotide Conjugation Calculator** on calculator worksheet 2 to obtain MSR.

Method B: Cuvette Spectrophotometer (100 µL, 1-cm micro-cuvette)

1. Prepare a 1:10 dilution of blank (Conjugation Buffer + SBA) solution by adding 180 µL of deionized water into the blank sample tube and mix well.
2. Prepare a 1:10 dilution of HyNic-protein MSR reaction solution by adding 180 µL of deionized water into the HyNic-protein MSR reaction tube and mix well.
3. In a 1 cm, 100 µL quartz micro-cuvette, blank the spectrophotometer at 348nm with 100 µL of the blank solution prepared in step 1 above.
4. Remove the blank solution and add 100 µL of the HyNic-protein MSR sample solution from step 2 above to the cuvette.
5. Record the 348nm absorbance value of the HyNic-protein MSR sample. **Multiply this number by 10** (to account for the 10-fold dilution) and then enter this value into the HyNic MSR Calculator **Protein-Oligonucleotide Conjugation Calculator** on calculator worksheet 2 to obtain MSR.

d. HyNic-Protein – 4FB-Oligo Conjugation Protocol (Calculator Worksheet 3)

1. Using the **Protein-Oligonucleotide Conjugation Calculator** on calculator Worksheet 3**.** On the HyNic-Protein/4FB-Oligo conjugation Calculator, input the name, molecular weight,

concentration and mass of HyNic- protein to be modified, and the name, molecular weight, concentration, MSR of 4FB-oligo and Extinction coefficient of oligo in the green fields.

2. The calculator will determine the volumes of HyNic-protein to combine with 4FB-oligo and the volume of 10X TurboLink Catalyst Buffer required to add into the conjugation solution in the blue field. **If the volume of 4FB-oligo output is less than 1 µL, the 4FB-oligo (OD_{260}/µL) can be diluted 10 fold with 1X Conjugation buffer. Be sure to change OD_{260}/µL by divided by 10.**
3. Incubate at room temperature for 2 -3 h or 4°C for overnight.
4. The conjugation reaction is now ready for purification by column chromatography, if required, or desalting into storage buffer.
5. The TurboLink™ Catalyst should be removed soon after the conjugation reaction is complete. The reaction solution may either be purified by using chromatography immediately, or may be desalted into storage 1X PBS buffer using either a 0.5 or 2 mL Zeba™ column based on the conjugation reaction volume (0.5 ml Zeba™ column has a capacity of 50 – 150 µL; 2 ml Zeba™ column has a capacity of 150 – 700 µL).
6. If using a 0.5 mL Zeba, proceed to buffer exchange the oligo/protein conjugated into **1X PBS Buffer** as described on page 9. For using the 2 mL Zeba™ column, please see the protocol below.
7. Determine final protein concentration using BCA or Bradford assay. Add bacteriostat (e.g. 0.05% sodium azide) and/or a protein stabilizer (e.g. 0.5% BSA) if necessary, then store at 4°C.

Zeba™ Column Desalting Protocol

2 mL Zeba™ Spin Column Preparation and Sample Loading (150 – 700 µL)

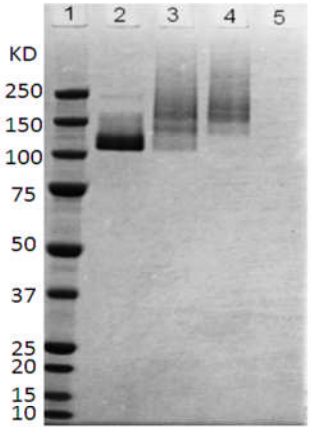
1. Remove spin column bottom closure and loosen the top cap (do not remove cap).
2. Place spin column in a 15 mL conical collection tube.
3. Centrifuge at 1,000 x g for 2 minutes. Discard the flow through from the collection tube.
4. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in the 15 mL conical collection tube with the mark facing outward and away from the center of the rotor in all subsequent centrifugation steps.
5. Add 1 mL of **1X PBS Buffer** to the top of the resin bed and centrifuge at 1,000 x g for 2 minutes. Discard the flow through from the collection tube.
6. Repeat step 5 an additional two times, discarding the flow through from the collection tube.
7. Place the Zeba™ column in a new 15 mL conical collection tube, remove cap, and slowly apply sample onto the center of the compacted resin bed (150–700 µL).
   Note- For sample volumes less than 350 µL, apply a 40 µL 1X PBS Buffer stacker to top of the resin bed after the sample has fully absorbed to ensure maximal protein recovery. Avoid contact with the sides of the column when loading.
8. Centrifuge column at 1,000 x g for 2 minutes to collect desalted sample.
9. Determine final protein concentration using BCA or Bradford assay. Add bacteriostat (e.g. 0.05% sodium azide) and/or a protein stabilizer (e.g. 0.5% BSA) if necessary, then store at 4°C.

VIII. Analysis of the conjugated protein with Oligo

After completion of the conjugation reaction, a small aliquot of the crude reaction mixture is often analyzed using a 10-12% Bis-Tris gel in an SDS (non-reducing) running buffer system. The amount of crude sample loaded on these gels depends on the type and sensitivity of staining method being used. For example, Coomassie blue stain can easily detect 2-4 micrograms of crude conjugate per lane; DNA silver stain can detect 250-500ng of conjugate per lane. An appropriate protein marker is loaded side by side, as well as HyNic-protein and 4FB-modified oligo as references. The protein/oligo conjugate may migrate slowly in the gel and is visualized as slightly higher molecular weight species.

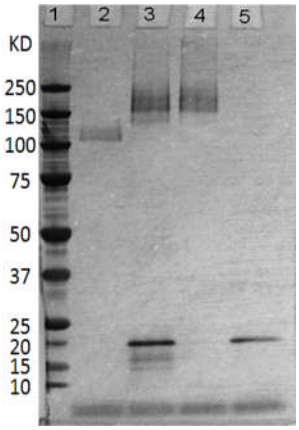
Protein-Oligo conjugation sample:

Monoclonal mouse anti-human IgG1 (3mg/ml) was modified with 20 mole equivalents of S-HyNic. Oligo (75 bp) was modified with 20 mole equivalents of S-4FB. Then HyNic-mouse anti-human IgG1 was conjugated with 3 moles equivalents of 4FB-oligo (75 bp) and purified with HPLC (SuperDex 200, 60min program). Respectively, loading 1μg or 450ng of conjugated on 10% SDS-PAGE, followed by coomassie or DNA silver stain.



Coomassie Stain:

Lane 1. MW marker
Lane 2. Mouse anti-human IgG1 (2 μg)
Lane 3. Crude HyNic-mouse anti-human IgG1/4FB-oligo
Lane 4. Purified HyNic-mouse anti-human IgG1/4FB-oligo
Lane 5. Oligo (75 bp)



DNA Silver Stain:

Lane 1. MW marker
Lane 2. Mouse anti-human IgG1
Lane 3. Crude HyNic-mouse anti-human IgG1/4FB-oligo
Lane 4. Purified HyNic-mouse anti-human IgG1/4FB-oligo
Lane 5. Oligo (75 bp)

Figure 5. Sample of HyNic-protein/4FB-Oligo conjugation

IX. Purification

All conjugation reactions will be a mixture of reaction products consisting of the desired conjugate along with some un-conjugated 4FB-modified oligo. For this reason, the conjugation reactions are purified using chromatographic methods to remove excess 4FB-modified oligo. Various FPLC or HPLC chromatography workstations are available for this purpose (Figure 6). Solulink routinely purifies conjugates using size exclusion. Other media such as metal chelate affinity chromatography or protein A/G columns may be used where appropriate.

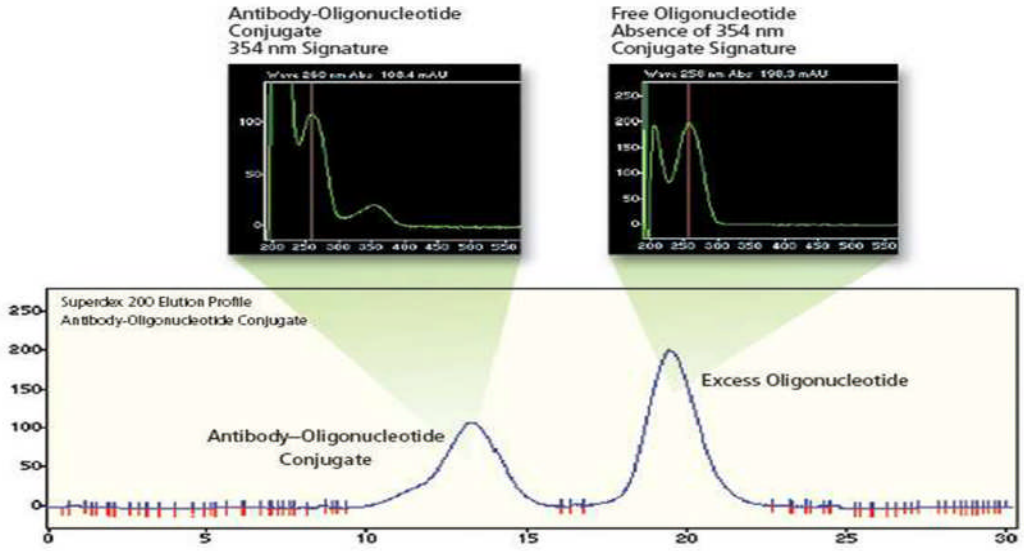


Figure 6: Purification of an antibody-20mer oligo conjugate by size exclusion HPLC (SuperDex 200; GE HealthCare). The initial peak is the desired conjugate followed by unconjugated oligonucleotide. Above each peak its UV spectrum- note the 354 nm absorbance of the conjugate peak due to the formation of the conjugate bond.

X. Kit Stability

Component	Storage	Stability
Unopened Kit	**4°C**	**Refer to Certificate of Analysis**
S-HyNic / S-4FB in DMF	**4°C**	**1 day**
4FB-oligonucleotide	**< -20°C**	**> 1 year**
HyNic-modified protein	**-**	**Use immediately**
Purified protein-oligo conjugate with 0.05% azide	**4°C**	**6 months**

XI. Troubleshooting

Problem	Possible Cause	Recommended Action
Poor or undetectable conjugate yield	Amino-oligo may not be sufficiently 4FB-modified	Verify 4FB-Oligo MSR to ensure proper conjugation
	Quality or purity of starting oligo poor	Concentrate 4FB-oligo into the require range (0.3-0.5 OD260/μL)
	Quality or purity of starting protein poor	Confirm the purity and quantity of the starting protein by using BCA/Bradford and SDS-PAGE analysis
Poor HyNic modification of protein	Amine contaminant, e.g. Tris or glycine buffer,	Change buffer by using desalting column before modification
	Presence of carrier protein such as BSA or gelatin	Remove carrier protein or get new protein without carrier
	Initial protein concentration is too low	Concentrate protein using a diafiltration filter, use an initial 1-5 mg/mL for efficient labeling of proteins
	Improper storage of S-HyNic reagent can lead to hydrolysis of the NHS ester	Keep and store the S-HyNic reagent sealed in the pouch provided
Precipitaion of protein on modification	Over modification of the protein	Spin down precipitate and try to recover any protein left in solution. May require modifying new batch of protein with fewer equivalents
Protein precipitates during conjugation reaction	Conjugation reaction pH may be close to the isoelectric point of the conjugate being formed	Conjugate at a different pH (e.g. pH 5 or 7 or higher)
Protein-DNA conjugates are degraded	Conjugation reaction contains either single or double stranded nucleases	Use only molecular grade water (DNase-free) when conjugating DNA to proteins

XII. References

1) Sano T, Smith CL, Cantor CR. Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates. Science. 1992 **258**, 120-2.
2) Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gústafsdóttir SM, Ostman A, Landegren U, Nat Biotechnol.. Protein detection using proximity-dependent DNA ligation assays. 2002,**20**, 473-7.
3) Fredriksson S, Horecka J, Brustugun OT, Schlingemann J, Koong AC, Tibshirani R and Davis RW, Multiplexed Proximity Ligation Assays to Profile Putative Plasma Biomarkers Relevant to Pancreatic and Ovarian Cancer, Clinical Chemistry, 2008, **54,** 582-589.
4) Fredriksson S, Dixon W, Ji H, Koong AC, Mindrinos M and Davis RW, Multiplexed protein detection by proximity ligation for cancer biomarker validation , Nature Methods 2007, **4**, 327.
5) Jarvius M, Paulsson J, Weibrecht I, Leuchowius K-J, Andersson A-C, Wählby, Mats Gullberg C, Botling J, Sjöblom T, Markova B, Östman A, Landegren U and Söderberg O, *In situ* detection of phosphorylated PDGF receptor β using a generalized proximity ligation method, Mol. And Cell. Proteomics 2007, 1500-9
6) Schallmeiner E, Oksanen E, Ericsson O, Spångberg L, Eriksson S, Stenman U-H, Pettersson K and Landegren U, Sensitive protein detection via triple-binder proximity ligation assays, Nature Methods 2007, **4**, 135 – 137.
7) Kozlov IA, Melnyk PC, Stromsborg KE, Chee MS, Barker DL, Zhao C, Efficient strategies for the conjugation of oligonucleotides to antibodies enabling highly sensitive protein detection, Biopolymers 2004, **73**, 621.
8) Bailey RC, Kwong G, Radu CG, Witte ON, and Heath JR, DNA-Encoded Antibody Libraries: A Unified Platform for Multiplexed Cell Sorting and Detection of Genes and Proteins, J. Amer. Chem. Soc. 2007, 129, 1959-1967.
9) Kattah MG, Coller J, Cheung RK, Oshidary N, Utz PJ, HIT: a versatile proteomics platform for multianalyte phenotyping of cytokines, intracellular proteins and surface molecules. Nat Med. 2008 Nov;14, 1284-9.
10) Datta SK, Cho HJ, Takabayashi K, Horner AA, Raz E., Antigen- immunostimulatory oligo-nucleotide conjugates: mechanisms and applications., Immunol Rev. 2004 Jun;199:217-26.
11) Allergen-immunostimulatory oligodeoxynucleotide conjugate: a novel allergoid for immunotherapy., Curr Opin Allergy Clin Immunol. 2002 **2**, 547-51.
12) Horner AA, Datta SK, Takabayashi K, Belyakov IM, Hayashi T, Cinman N, Nguyen MD, Van Uden JH, Berzofsky JA, Richman DD, Raz E., Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites., J Immunol. 2001, **167**, 1584-91.