



All-Purpose Crosslinking Kit

Technical Manual

Catalog # S-9002-1

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- 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. Bioconjugation Process

Bioconjugation involves the linking of two biomolecules to form a novel chimeric complex that has the properties of both molecules¹. Solulink's HydraLink™ bioconjugation technology is based on two unique heterobifunctional linkers. These linkers can be used to incorporate either 6-hydrazinonicotinamide (HyNic), an aromatic hydrazine, or 4-formylbenzamide (4FB), an aromatic aldehyde into biomolecules or surfaces containing amino groups. This incorporation process is commonly referred to as 'modification'. Once modified, functionalized biomolecules are desalted to remove excess linker and to exchange the biomolecules into a conjugation-compatible buffer system. The two modified biomolecules can then be mixed together in a process known as conjugation. Conjugation leads to the formation of a bis-aryl hydrazone bond between the two species that is stable even to PCR conditions (Figure 1). No additional reagents are required to stabilize the hydrazone bond that is formed.

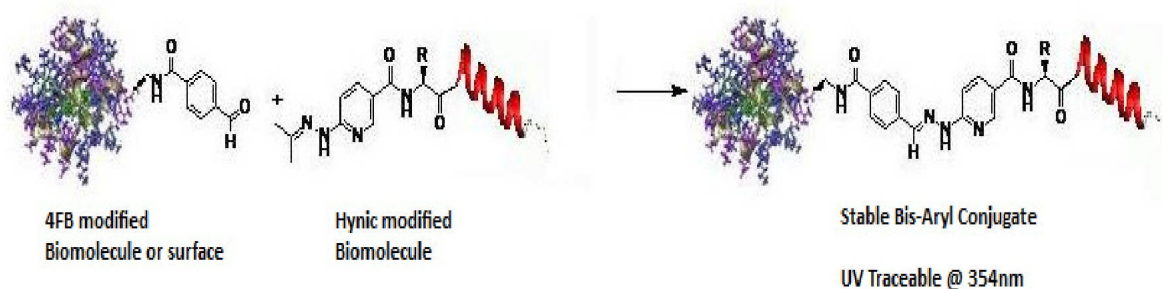


Figure 1: Linking chemistry behind HydraLink™ technology.

This S-HyNic bioconjugation technical manual contains all the comprehensive protocols necessary to make protein-protein bioconjugates. It guides the user with simple to understand and execute instructions. Several successful bioconjugation examples are also illustrated in Appendix IV.

B. Process Overview

Solulink's bioconjugation process is illustrated best using two amine-containing proteins, an antibody and an enzyme (Figure 2).

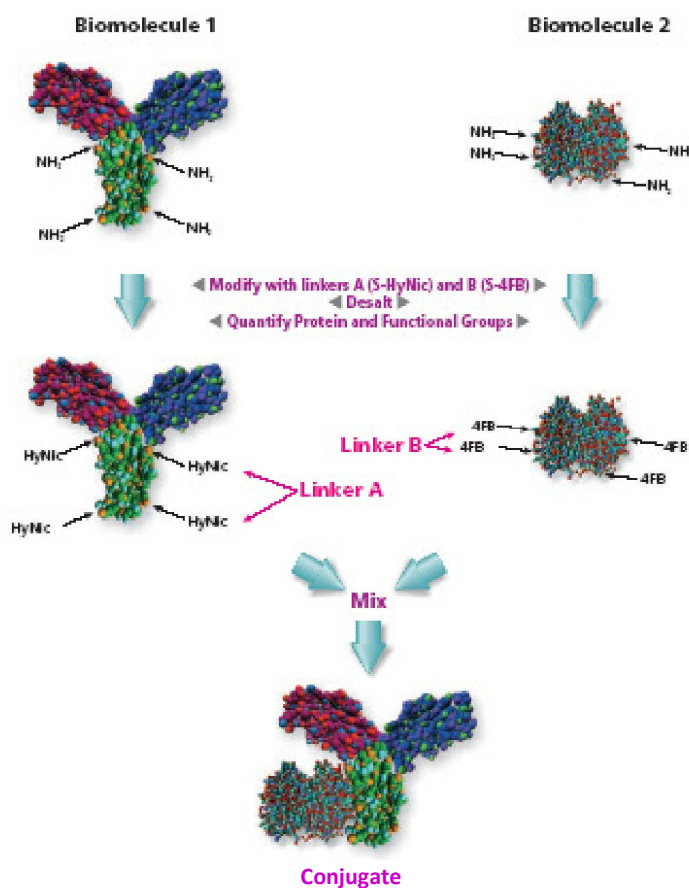


Figure 2. HydraLink bioconjugation process

As illustrated, an antibody is activated with Solulink's heterobifunctional linker S-HyNic to incorporate HyNic followed by activation of a second protein with the heterobifunctional linker S-4FB to incorporate 4FB. Once modified, proteins are desalted and quantified using a BCATM Protein Assay. Subsequently, two simple reactions are performed on small aliquots of each modified protein. These aliquots are used to quantitatively measure the degree of linker incorporation, also known as the molar substitution ratio or MSR. After modification, proteins are simply mixed together to form a stable conjugate. HydraLinkTM technology has been used to conjugate and immobilize many different types of biomolecules including carbohydrates, lipids, oligonucleotides, RNA, DNA, fluorophores and other small molecules. However, **this technical manual is specifically focused on protocols for making protein-protein conjugates.**

C. HydraLink™ Chemistry

HydraLink™ conjugation chemistry is based on the reaction of a HyNic functional group with a 4FB moiety to form a stable bis-aryl hydrazone bond (Figure 3). The bond created is a Schiff base that is both stable and UV-traceable. This unique covalent bond is created when hydrazinonicotinamide (HyNic), incorporated into one type of biomolecule reacts with a formylbenzamide moiety (4FB), incorporated into a second biomolecule. This type of hydrazone bond formed is the only known example of a stable Schiff base, which requires no additional steps (reduction) to stabilize the bond.

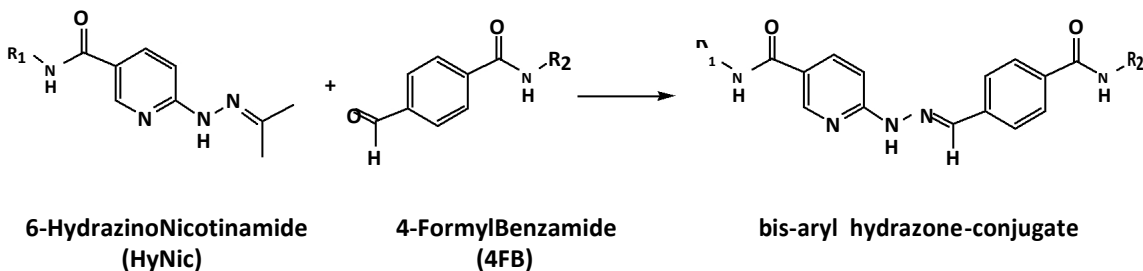


Figure 3. Specific chemical reaction between HyNic and 4-formylbenzamide (4FB) forms the bis-aryl hydrazone conjugate.

S-HyNic and S-4FB were designed and engineered to be ‘complementary’ and thus they react only with each other. S-HyNic was developed to incorporate 6-hydrazinonicotinamide groups into amine-containing biomolecules via an activated N-hydroxysuccinimide ester. This linker possesses a nucleophilic, aromatic hydrazine moiety that is protected as its alkyl (acetone) hydrazone. Acetone protection provides the functional group with long-term stability, either in solid or liquid form. Furthermore, protection helps maintain the aqueous reactive stability of the HyNic group through a dynamic equilibrium established between the acetone-protecting group and the HyNic functionality. No specialized deprotection is required during conjugation since mildly acidic conditions (pH 6.0) slowly remove the protecting group and make HyNic available for reaction with 4FB.

S-4FB (succinimidyl 4-formylbenzoate) is the other linker forming the basis of our HydraLink technology. S-4FB is an aromatic aldehyde that was developed to incorporate 4-formylbenzamide groups into amine-containing biomolecules via an activated N-hydroxysuccinimide ester. Aromatic succinimidyl esters such as S-4FB are more efficient at modifying amines in aqueous buffers than their aliphatic counterparts. Once incorporated, 4FB groups spontaneously react with HyNic-modified biomolecules at room temperature to form stable conjugates. Only aromatic aldehydes form permanently stable covalent hydrazone bonds with aromatic hydrazines or aromatic hydrazides. Aliphatic hydrazines or hydrazides (sold by other vendors) do not form stable and permanent covalent bonds when they react with aliphatic and/or aromatic aldehydes. Only Solulink’s proprietary HydraLink chemistry based on reaction of two aromatic functionalities (HyNic and 4FB) can form a permanent resonance stabilized covalent bond.

Both linkers (S-HyNic and S-4FB) are readily used to modify proteins, surfaces, or various other biomolecules. The reaction between HyNic and 4FB is acid catalyzed with optimal reaction kinetics occurring at pH 4.7, although conjugation reactions are typically performed using milder acid conditions (e.g. pH 6.0) that better preserve the biological activity of most proteins.

4FB-modified proteins are extremely stable to long-term storage, even more so than HyNic-modified proteins. Once incorporated, 4FB remains reactive to HyNic groups for up to a year when stored at -80°C in aqueous/glycerol solutions or even months at 4°C . This reactive stability provides 4FB and HyNic with greater flexibility and convenience as compared to other conjugation chemistries. For example, proteins and other biomolecules modified with HyNic and 4FB can be stored at 4°C (or lower) for several weeks until the conjugation reaction is ready to be performed. Reactive stability permits exquisite control and reproducibility of the conjugation process since modified proteins remain stable and reactive. Other conjugation chemistries (e.g. maleimide/thiol) are more susceptible to hydrolysis and homo-polymerization, making these older technologies more difficult to control and reproduce.

By contrast, 4FB and HyNic groups are highly inert to cross-reaction with other protein functional groups (i.e. $-\text{COOH}$, $-\text{NH}_2$, $-\text{SH}$) or with themselves (i.e. HyNic-HyNic or 4FB-4FB interactions). Once a protein is modified, HyNic and 4FB functional groups remain inert to all other functional groups except each other.

A wide variety of protein-protein and other biomolecules have been conjugated using Solulink's HydraLink™ chemistry. These include:

- protein-protein
- protein-peptide
- protein-oxidized glycoprotein
- oligonucleotide-oligonucleotide
- protein-oligonucleotide
- protein-carbohydrate
- oligonucleotide-peptide
- biomolecule-fluorophore

Solulink also offers maleimide-activated analogs of S-HyNic and S-4FB if amino groups are not available for reaction. These analogs modify proteins through thiol groups when primary amino groups are not accessible. We refer to these linkers as M-HyNic and M-4FB. They are currently sold in our catalog under the trade names MHPH and MTFB, respectively.

D. Linker and Conjugate Stability

There are two types of stability associated with the conjugation process. Reactive stability refers to the time-dependent hydrolysis (loss) of reactive functionalities after incorporation into a protein (e.g. HyNic or 4-FB). Bond stability refers to the stability of the covalent bond linking the biomolecules together.

Resistance of functional groups to hydrolysis (reactive stability) is critical for obtaining high conjugation yields in a reproducible manner. Figure 4 presents the reactive stability of HyNic-modified IgG stored in aqueous media.

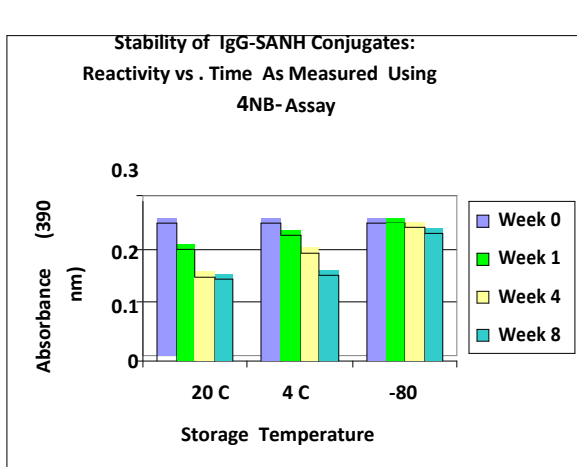


Figure 4. Reactive stability of HyNic functional groups conjugated to IgG. Stability was monitored by reaction of 4-nitrobenzaldehyde* (4-NB, a colorimetric reagent) with HyNic-modified IgG to form a UV-traceable hydrazone. This absorbance signature was used to track the continued reactivity of HyNic-modified IgG stored at various temperatures through time. The stability of the protected HyNic functional group can be clearly seen. This reactive stability enables researchers to modify proteins days or even weeks before they are actually conjugated. (Note that we have switched to 2-sulfobenzaldehyde in lieu of 4-nitrobenzaldehyde for solubility reasons.)

A second type of stability is bond stability. To illustrate hydrazone bond stability, two different 15-mer HyNic-modified peptides were synthesized using a solid phase peptide synthesizer and a HyNic-analog called (6-Boc-HNA). This reagent incorporates a HyNic moiety on the NH₂ - terminus of any peptide. As seen in Figure 5, reaction of two HyNic-modified peptides with a 5'-4FB-modified oligonucleotide produced two peptide-oligonucleotide conjugates. Bond stability was confirmed by heat stressing the peptide-oligonucleotide hydrazone bond at 94°C for 2 hours.

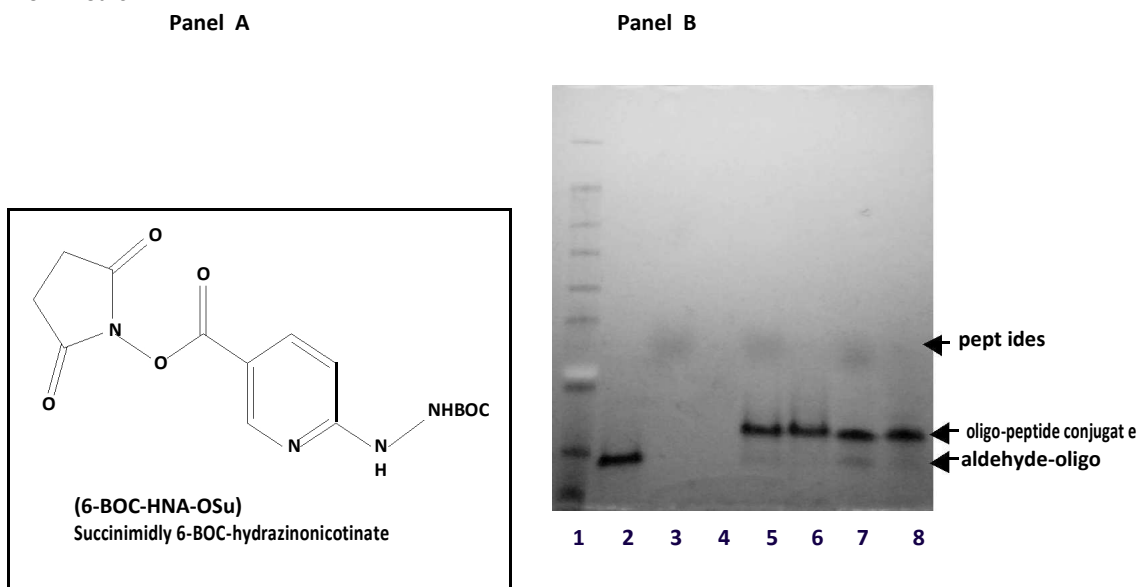


Figure 5. 6-Boc-HNA reagent (Panel A) was used to modify the NH₂ -terminus of two 15-mer synthetic peptides with a HyNic moiety. SDS-PAGE was used to analyze conjugation products. Reaction of the HyNic-modified peptides with a 4FB-modified oligonucleotide (lane 2) formed conjugates (lanes 5, 6). Bond

stability was tested by heat stressing the peptide-oligo-conjugates at 94 °C for 2 hours (lanes 7, 8). Lane 1 is the protein molecular weight marker.

E. Linker and Conjugate Traceability

HydraLink chemistry is the only conjugation chemistry that permits direct spectral monitoring of the modification and conjugation process. In order to monitor the modification process, Solulink engineered two colorimetric reagents (2-HP and 2-SBA) that readily form a hydrazone bond on reaction with 4FB-modified and HyNic-modified proteins. These two reagents rapidly react with modified proteins to generate a UV –traceable absorbance signatures at 350 and 390 nm, respectively.

Linker traceability allows the molar substitution ratio or MSR to be determined. The MSR is the number of functional groups (either HyNic or 4FB) incorporated per protein molecule. The

MSR is important for two reasons, first it ensures batch-to-batch consistency between conjugations, and secondly it is used to monitor the stability (over time) of HyNic and 4FB modified proteins or other biomolecules. These simple colorimetric reagents make conjugation of proteins and other biomolecules nearly foolproof.

After protein modification, HyNic and 4FB moieties are quantified using two simple spectrophotometric assays (see Figure 6). These assays require small aliquots (i.e. 20ug) of either a HyNic-modified or 4FB-modified protein. HyNic groups are measured using Solulink's 2-sulfobenzaldehyde (2-SBA, Cat. # S-2005) while 4FB groups are measured using 2-hydrazinopyridine.2HCl (2-HP, Cat. # S-2002).

Reaction between 2-SBA reagent and HyNic-modified proteins leads to the formation of a traceable absorbance signature @ 345 nm with a molar extinction coefficient at ϵ_{345} -28,500 l-mol⁻¹-cm⁻¹. Reaction between 2-HP reagent and 4FB-modified proteins leads to the formation of a traceable absorbance signature @ 350nm with a molar extinction coefficient at ϵ_{350} - 24,500 l-mol⁻¹-cm⁻¹. These signatures are used in conjunction with our [Modification Calculators](#). Together, they accurately quantify the degree of linker incorporation for each modified protein.

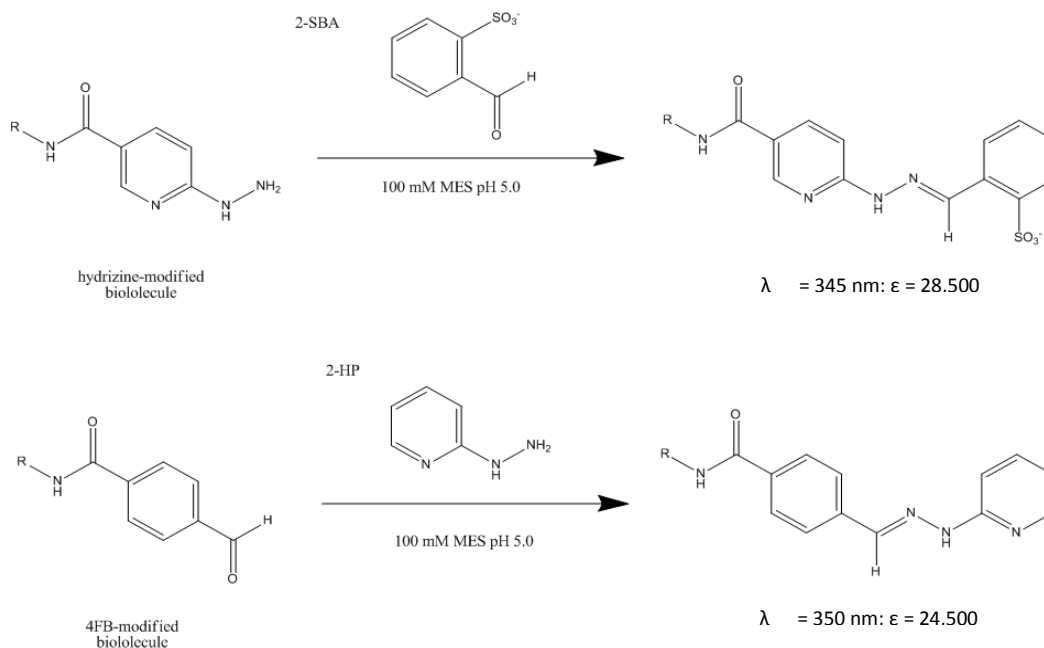


Figure 6: Colorimetric reactions used for quantifying HyNic and 4FB functional groups.

After conjugation and during purification, conjugates can also be traced through the hydrazone bond that links the two biomolecules together. UV absorbance signatures at 354nm are routinely used to track and monitor conjugate peaks. This conjugate tracking signature is unique to HydraLink chemistry.

F. Kit Components

| Component | Component # | Quantity |
|--|-------------|----------|
| S-HyNic | S-9002-1-01 | 10 mg |
| S-4FB | S-9002-1-02 | 10 mg |
| 2-Hydrazinopyridine.2HCl | S-9002-1-03 | 25 mg |
| 2-Sulfobenzaldehyde | S-9002-1-04 | 25 mg |
| Anhydrous DMF | S-9002-1-05 | 1.5 mL |
| 10X Conjugation Buffer | S-9002-1-08 | 1.5 mL |
| 10X Modification Buffer | S-9002-1-07 | 1.5 mL |
| 5K MWCO Vivaspin Diafiltration Filter | S-9002-1-10 | 4 each |

H. Additional Components needed

Conjugation

BCA Protein Reagents (Reagent A and B) (Pierce Chemical #23223 and 23224) Albumin Protein Standard (or similar, Pierce Chemical # #23209)

40°C water bath

37°C heat block

Zeba™ Desalting Spin Columns (0.5 ml and 2 ml) (Pierce Chemical, Cat. # 89883,89889)

Molecular grade water 1L (Ambion, Cat. # 9932)

1.5 ml microfuge tubes (polypropylene)

96-well polystyrene plates

96-well plate reader (absorbance @ 562 nm) Vortex mixer

High speed fixed angle microcentrifuge (1.5 ml tubes) Low speed fixed angle centrifuge (5-15 ml tubes)

Calibrated Rainin pipetman or similar (P-10, P-100, P-1000)

Analytical balance (Mettler) or similar

Quartz cuvette (1 ml) UV-spectrophotometer

Analysis and Purification

Gel rig (e.g. Invitrogen Xcell SureLock System) or similar

Electrophoresis power supply (250V)

4-12% Bis-Tris Protein Gradient Gels and Buffers (Invitrogen, Carlsbad, CA) NuPage™

LDS Sample Buffer (Invitrogen, NP0007)

Molecular weight markers (Bio-Rad, Hercules, CA, Cat. # 161-0373)

Beckman System Gold Chromatography Workstation w/Scanning Array Detector

Superdex™ 200 Prep Grade filtration media (GE HealthCare, Cat. #17-1-43-01) Q-

Sepharose HP (GE HealthCare, Cat. #17-1014-01)

GE HealthCare Low pressure Chromatography Column (XK-16/40 type)

II. Bioconjugation

A.) Getting Started

Over the years, Solulink scientists have accumulated extensive protein-protein conjugation experience. Based on this experience, Solulink has developed and optimized conjugation protocols that work well. Experience has taught us that certain strict limitations need to be placed on initial buffer composition, starting mass (mg) and concentrations (mg/ml). As a consequence, before starting a conjugation project we recommend the use of the flow chart outlined in Figure 7. To use the chart, simply start at the box labeled 'your protein' and proceed to answer the questions in the flow chart. The chart eventually guides the user to the first step in the HyNic Bioconjugation protocol.

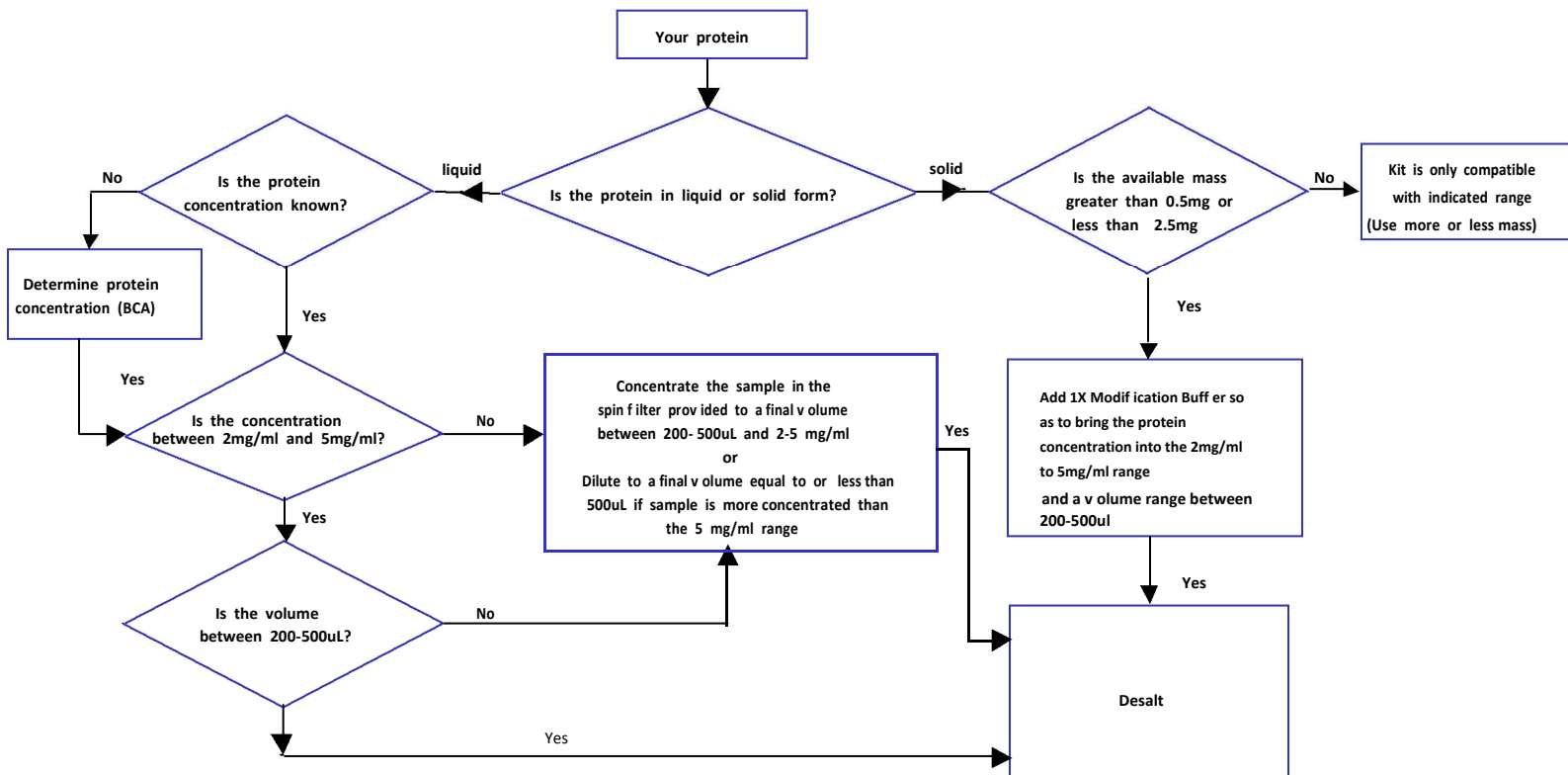


Figure 7. Flow-chart used for guiding a user to the start of the conjugation process.

Summary of Conjugation Steps

After a user has determined that they have sufficient quantities of both proteins, they can proceed to conjugate them. Successful protein-protein conjugation involves the execution of 10 sequential steps, as summarized in Figure 8. This summary is included to insure that the user is intimately familiar with the complete conjugation process.

| Step | Time (minutes) | |
|----------------------------------|----------------|----------|
| | Total | Hands-On |
| 1 Desalt | 15-30 | 15-30 |
| 2 Protein Determination | 30 | 10 |
| 3 Concentration (if necessary) | 30 | 5 |
| 4 Modification | 120 | 15 |
| 5 Desalt | 15-30 | 15-30 |
| 6 Protein Determination | 30 | 10 |
| 7 Molar Substitution Ratio (MSR) | 45 | 15 |
| 8 Conjugation | 480-960 | 15 |
| 9 Purification | 60 | 30-60 |
| 10 Analysis | 180 | 60 |

Figure 8. Sequential steps for producing a protein-protein conjugate.

Step 1

Proteins must be desalted before they are modified with HydraLink linkers (S-HyNic/S-4FB). Desalting insures that potential (and often unexpected) amine contaminants do not interfere with the modification process

Step 2

After desalting, the protein concentration must be determined using a protein determination assay. Accurate concentrations are an important part of any protein-protein conjugation. Protein concentrations are used to calculate the number of linker equivalents (S-HyNic and S-4FB) needed for the modification reaction.

Step 3

Modification reactions are most efficient within defined protein concentrations (e.g. 2-5 mg/ml) and defined volumes (typically 200-500ul). For this reason, proteins sometimes need to be concentrated before they are modified. Concentration filters are provided in the kit for this purpose.

Step 4

Desalted proteins (2-5 mg/ml range) are modified with S-4FB and S-HyNic linkers in a process known as modification. These linkers are used to incorporate 4FB and HyNic moieties into proteins.

Step 5

After modification, modified proteins are desalted and buffer exchanged into a suitable conjugation buffer.

Step 6

A protein determination step is then used to quantify the two proteins, and the concentrations used to determine the proportions of each to mix together during the conjugation reaction.

Step 7

After step 6, small aliquots of each modified protein are used to determine the molar substitution ratio (MSR) using the colorimetric reagents, 2-SBA and 2-HP. Molar substitution ratios confirm that 4FB and HyNic groups are incorporated in sufficient quantity to permit conjugation.

Step 8

Conjugation involves combining the two modified proteins at known equivalents in a suitable conjugation buffer. Often an excess of one modified protein is used to drive the reaction. Conjugation reactions are generally incubated overnight at room temperature to obtain maximum efficiency.

Step 9

After conjugation, crude reaction mixtures routinely go through some type of analysis. Crude reactions are analyzed by SDS-PAGE or NovexTM (Bis-Tris) gradient gels and visualized by Coomassie blue or silver-stain and appropriate M.W. protein markers

Step 10

After confirming conjugate formation, crude conjugation reactions are purified using conventional gel filtration chromatography. Superdex 200 gel filtration media is preferred for purifying conjugates. Eluted column fractions can be spectrally traced and used to confirm conjugate formation through their absorbance signature at 354nm. Purification requires a suitable chromatographic workstation preferably equipped with a scanning diode-array detector. A final SDS-PAGE gel is often used to analyze the purified conjugate.

General Guidelines for Modifying Proteins with S-HyNic and S-4FB

The modification process is the 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-4FB or 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.), number of excess linker equivalents (e.g. 10X, 15X or 20X), reaction pH, the isoelectric point of the protein, the local hydrophilicity/hydrophobicity surrounding available amino groups, and the type of heterobifunctional NHS-ester employed (e.g. S-HyNic vs. S-4FB).

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. Proteins are so diverse in form and sequence that small-scale S-HyNic and S-4FB optimization reactions (e.g. different equivalents) are sometimes required. Modification reactions generally use a 10 to 20-fold excess of linker over protein.

Data has been compiled in Table 1 as an aid in determining the number of equivalents of S-HyNic or S-4FB required to achieve a given molar substitution ratio (MSR). This table is only a 'guide' for modifying proteins. Each protein will have its own variables that may need to be considered.

| [IgG] mg/mL | Equiv. added | MSR (pH 7.2) | MSR (pH 8.0) |
|----------------|-----------------|-----------------|-----------------|
| 1.0 | 5 | 2.38 | 2.07 |
| | 10 | 4.73 | 4.05 |
| | 15 | 6.20 | 6.14 |
| 2.5 | 5 | 3.08 | 2.91 |
| | 10 | 6.58 | 5.85 |
| | 15 | 8.26 | 7.59 |
| 5.0 | 5 | 3.74 | 3.34 |
| | 10 | 6.80 | 6.04 |
| | 15 | 9.76 | 8.51 |

Table 1. Guide for modification of proteins. Actual results may differ from protein to protein. Modification conditions used 1x Modification Buffer (100 mM phosphate, 150 mM NaCl (pH 7.2)) or (100 mM HEPES, 150 mM NaCl (pH 8.0)) in a volume of 50 ul for 2 hours at room temperature. Protein concentrations were determined using a BCA protein assay and hydrazine incorporation was monitored using a spectrophotometer.

Conjugation Protocol

Prior to starting the conjugation protocol, confirm that both proteins to be conjugated are in the appropriate concentration range of 2-5 mg/ml and in a volume range of 0.2 to 0.5 ml. Once confirmed, proceed to step 1 below.

1. Desalt

Solulink recommends the use of ZebaTM Desalt Spin Columns (Pierce Chemical, Cat. #89882 or 89889) to desalt proteins as required by our conjugation protocol. These rapid spin columns are recommended because they do not significantly dilute biomolecules during desalting.

Two sizes are available, a 0.5 ml and a 2 ml ZebaTM Desalt Spin Column. Figure 9 illustrates both the 0.5 ml and the 2 ml ZebaTM Desalt Spin Columns. Choose a spin column based on the available protein sample volume. For example, the 0.5 ml ZebaTM spin column desalts a volume that ranges from 30-130 ul. The 2 ml spin column desalts volumes that range between 200-700 ul. **Both 0.5 and 2 ml desalt spin column protocols are provided below. Use the appropriate protocol for your specific sample volume requirements.**

Note - The larger 2 ml ZebaTM spin column does not fit into a standard high-speed microcentrifuge that holds 1.5 ml tubes. The larger 2 ml ZebaTM spin column requires a tabletop centrifuge capable of spinning 15 ml tubes. Use the appropriate size column for processing your individual samples. Protocols for both the 0.5 ml and 2 ml ZebaTM columns are provided below.

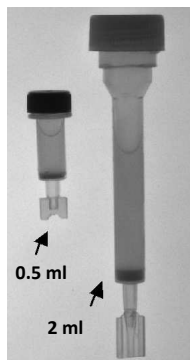


Figure 9. Zeba™ Desalt Spin Columns (0.5 and 2 ml) used to desalt S-HyNic proteins and modification reactions.

Protocol

0.5ml Zeba™ Spin Column Preparation (Sample volumes 30-130uL)

1. Remove spin column's bottom closure and loosen the top cap (do not remove cap).
2. Place spin column in a 1.5 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 ul of 1x Modification buffer (pH 7.4) to the top of the resin bed and centrifuge at 1,500 x g for 1 minute to remove buffer.
6. Repeat step 4 and 5 two additional times, discarding buffer from the collection tube.
7. Column is now ready for sample loading.

Protein Sample Loading

1. Place the equilibrated spin column in a new 1.5 ml collection tube, remove cap and slowly apply 30-130 µl sample volume to the center of the compact resin bed.

Note- for sample volumes less than 70 µl apply a 15 uL buffer (stacker) to the 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.

2. Centrifuge at 1,500 x g for 2 minutes to collect desalted sample.
3. Discard desalting column after use.
4. Protein sample is now desalted and ready for a protein determination.

2 ml Zeba™ Spin Column Preparation (Sample volumes that range from 240-700ul)

1. Twist off the column's bottom closure and loosen the top cap. Place column in a 15 ml conical collection tube.
2. Centrifuge column at 1,000 x g for 2 minute to remove storage solution.
3. 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.
4. Add 1ml of 1x Modification buffer (pH 7.3) to the top of the resin bed.
5. Centrifuge at 1,000 x g for 2 minute to remove buffer.
6. Repeat step 4 and 5 two or three additional times, discarding buffer from the collection tube.
7. Column is now ready for sample loading.

Protein Sample Loading

1. Place column in a new 15 ml conical collection tube, remove cap and slowly apply a sample volume (200-700 ul) to the center of the compact resin bed.

Note- for sample volumes less than 350 µl apply additional buffer (stacker) to the top of the resin bed (40 ul) after the sample has fully absorbed to ensure maximal protein recovery. Avoid contact with the sides of the column when loading.

2. Centrifuge at 1,000 x g for 2 minutes to collect desalted sample.
3. Discard column after use and retain the desalted protein in the 15 ml conical tube.
4. Desalted protein sample is now ready for a protein determination assay.

2. Protein Determination

Once protein samples are desalted and before modification, a protein determination assay is used to quantify

the amount of recovered protein. Although there are numerous assays for determining protein concentration, Solulink recommends using the BCA Protein Assay. A BCA Protein Assay (or similar) is required before proceeding with the next step in the conjugation

process. The BCA assay is based on the reduction of a copper/bicinchoninic acid solution by proteins. (Note-BCA is a trademark of Pierce Chemical, Rockford, Illinois).

This protein assay is simple, rapid, and highly reproducible. Solulink routinely employs a 96-well plate format to expedite absorbance measurements. Most common plate readers come equipped with automatic standard curve analysis software for determining protein concentrations using this reagent. A simplified 96-well BCA Protein Assay protocol for determining protein concentration is given below. Follow this protocol (or similar) to determine the protein concentration of your protein samples before proceeding to the modification step below.

96-Well Plate BCA Protein Assay Protocol

BCA Reagents (sufficient for 25 assays)

| | | |
|-----------------------|--------|---------------------------|
| BCA Reagent A | 5 ml | 96-well polystyrene plate |
| BCA Reagent B | 100 ul | 40° C water bath |
| BSA standard: 2 mg/ml | | 1X PBS (10 ml) |

Preparation of BCA Working Reagent

1. Prepare a working solution of BCA reagent just prior to use by adding 5 ml BCA reagent A to a clean 15 ml conical tube followed by 100 ul of BCA reagent B. Mix the two solutions until a clear green solution forms.

Note- Prepare this working reagent fresh daily.

2. Prepare a 2-fold BSA standard curve with a serial dilution using an Albumin Standard (Pierce Chemical, Product Number 23209) as follows:

| | | |
|----------|--|---------------|
| Well #1- | Add 50ul 1X PBS and 50ul 2mg/ml BSA standard to a well | (1mg/ml) |
| Well #2- | Add 50ul 1X PBS and 50ul from the 1 st well to a 2 nd well | (0.5mg/ml) |
| Well #3- | Add 50ul 1X PBS and 50ul from the 2 nd well to a 3 rd well | (0.25mg/ml) |
| Well #4- | Add 50ul 1X PBS and 50ul from the 3 rd well to a 4 th well | (0.125mg/ml) |
| Well #5- | Add 50ul 1X PBS and 50ul from the 4 th well to a 5 th well | (0.0625mg/ml) |
| Well #6- | Add 50ul 1X PBS to the 6 th well (Buffer blank). | |

Note- If sufficient quantities are available, the BCA standard curve can be made using the actual protein being assayed rather than an albumin standard.

3. After completing standard curve dilutions, transfer 20 ul of each of the above dilutions into wells containing 150 ul fresh BCA working reagent.

4. Immediately prepare the protein samples to be assayed. Dilute each sample into PBS at a final volume of 50 ul. Dilute the samples so that the final protein concentration falls into the middle range of the BSA standards. Add two 20 ul aliquots of the diluted protein into 2 wells containing 150 uL of BCA working reagent (duplicates). Record the dilution factor.

Example 1: Transfer 5 ul of a ~2.5 mg/ml protein sample to 45 ul 1x PBS (1:10 dilution factor), mix, make duplicate wells by transferring 20 ul into wells containing 150 ul BCA working reagent.

Example 2: Transfer 10 ul of a 1mg/ml protein solution into 40 ul 1x PBS, mix, make duplicate wells by transferring 20 ul into wells containing 150 ul BCA working reagent.

Note-protein samples may need to be diluted (more or less) depending on their initial concentration such that the final dilution falls within the range of the BSA standards.

5. Seal the samples in the 96-well plate using clear adhesive film and incubate in a 40°C water bath for 20 minutes.
6. Remove the plate from the bath, dry the bottom of the plate to remove excess water and proceed to read the plate using a suitable plate reader (e.g. Molecular Devices) @ 562nm. A typical 96-well BCA protein assay result is illustrated in Figure 10.

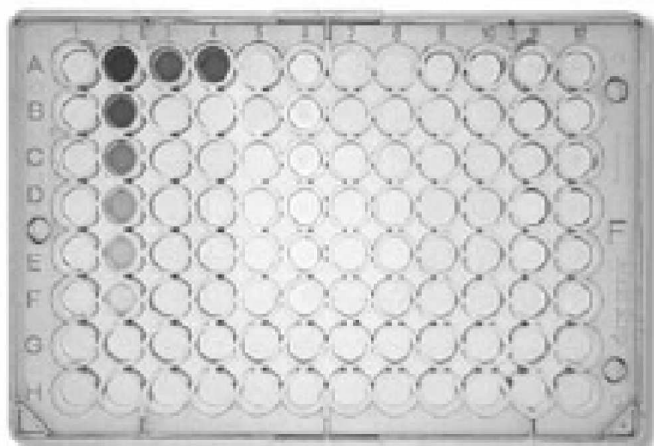


Figure 10. 96-well BCA Protein Assay plate containing a dilution series of a BSA standard (A2-F2) and duplicates of a protein sample (A3, A4). A Molecular Devices 96-well plate BCA Assay printout is illustrated in Figure 11. Similar print outs are available from other plate readers manufacturers.

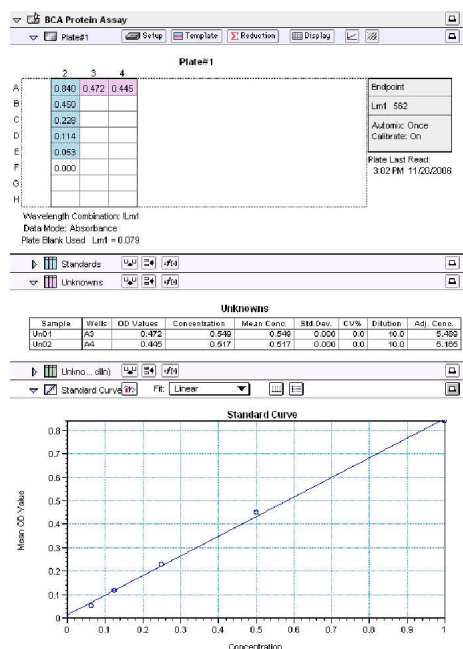


Figure 11. BCA protein assay printout from a Molecular Devices plate reader.

3. Concentrating Proteins

At times, initial protein concentrations are too dilute for desalting and modification to proceed. Such dilute protein samples must first be concentrated. In some instances, one or both proteins may need to be concentrated before they are desalted and modified.

For best results and as previously outlined in the flow chart, proteins to be modified must be within a defined concentration range (2mg/ml to 5mg/ml). If your protein sample is at a lower concentration, then use the VIVASPIN 500 concentration filter unit (Figure 12) provided with the kit to concentrate your sample. When concentrating proteins, two separate filter units are often used in tandem to balance the centrifuge and to expedite the concentration process. In general, we do not recommend attempting to concentrate proteins that are too dilute (i.e. more than 10-fold).

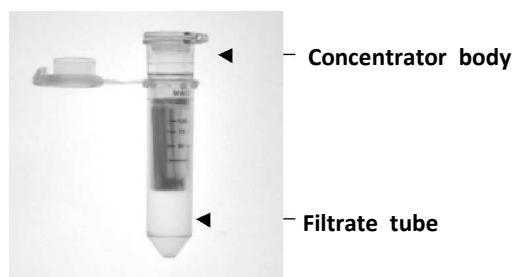


Figure 12. VIVASPIN 500 spin filter used to concentrate dilute protein samples prior to modification.

Protocol

Note- VIVASPIN 500 spin filters are made to contain and process volumes of 500 ul or less. If volumes greater 1ml are to be concentrated and processed, then multiple filters or loadings may be required.

- 1). Open the lid of two VIVASPIN 500 filter devices.
- 2). Transfer equal volumes of dilute protein solution to each of two VIVASPIN 500 filters.

For example, if the original sample is at 0.2 mg/ml in a volume of 1ml, then transfer 500 ul of protein solution into each VIVASPIN 500 filter device and concentrate the samples to a final volume of 100 ul each.

Note- When transferring or mixing solutions in the concentrator body, make sure never to contact or puncture the membrane in the process. Never bring a pipette tip in contact with the membrane. Spin filters only process 500 ul at a time, multiple time-consuming concentration and loading steps may be required when attempting to concentrate larger volumes. For this reason we do not recommend concentration of volumes much greater than 1 ml with these spin filters.

- 3). After loading the two filter units, orient the VIVASPIN 500 spin filters in the centrifuge so that the volume markers face toward the center of the centrifuge rotor each time.
- 4). Centrifuge for exactly 3 minutes @ 7,500 x g.
- 5). Open the filters and using a pipette, gently and slowly pipette the protein solutions up and down several times to mix them within the concentrator body.

Note- Re-suspending the protein solutions during each centrifugation step prevents irreversible aggregation of the protein to the membrane surface. After the solution is properly mixed, replace the cap on the concentrator body tube. Avoid foaming the solutions.

- 6). Re-orient the spin filter back into the centrifuge as before.
- 7). Centrifuge for exactly 3 minutes @ 7,500x g
- 8). Repeat steps 4 through 7 as many times as required until the final volume in each unit reaches the desired concentration (i.e. 2-5 mg/ml).

Note- If the target concentration is exceeded (>5 mg/ml) then simply add a sufficient volume of 1x Modification Buffer to bring the final concentration into range (2-5 mg/ml).

- 9). Carefully transfer and pool the contents of the two units into a single clean, 1.5 ml microfuge tube. The final protein volume should be in the range of 200-500 ul.
- 10). Remove an aliquot of the concentrated sample (~5 ug) and determine the protein concentration using the BCA protein assay protocol.
- 11). After concentrating, proceed to desalt and exchange the concentrated protein sample into 1x

Modification Buffer using either a 0.5 ml or 2 ml Zeba™ Desalt Spin Column as described in the Desalt section of this manual.

4. Modification

After desalting proteins into 1x modification buffer (pH 7.4) and determining their concentrations (e.g. 2 to 5 mg/ml range), you are ready to proceed with the modification step. In principle, either linker can be used on either protein. We recommend using the modification protocol below in concert with our [Modification Calculators](#) to modify your specific protein. Typically, modification reactions are conducted in a volume that ranges from 100-700 µl so that the reactions can be desalted (after modification) with either a 0.5 ml or 2 ml Zeba Desalt Spin Column. These spin columns do not significantly alter protein concentrations after use.

S-HyNic Modification Protocol

(Requires the use of the [Protein-Modification with an NHS-Ester Calculator](#)).

1. Using an analytical balance, weigh approximately 5 mg of S-HyNic solid (M.W. 290.2) into a clean, 1.5 ml polypropylene tube. Record the exact weight and input the value into the calculator.

Note- although smaller quantities can be used, in our experience most analytical balances are not as precise (or accurate) below 5 milligrams.

2. Dissolve S-HyNic (SANH) in a suitable volume of anhydrous DMF, usually in the range of 500-1000 µl. Record the exact volume and input the value into the calculator.
3. Add the required volume of S-HyNic to the protein, a volume that typically represents 10 to 20 mole equivalents over the amount of protein. To calculate the exact volume of S-HyNic/DMF required, input the number of equivalents into the calculator.
4. Mix the protein/S-HyNic reaction well.

Note- Solulink highly recommends the use of our [Protein Modification with an NHS-ester Calculator](#) to determine the exact volume of S-HyNic/DMF to add to your specific protein. The calculator functions by the input of 7 user-defined variables. These 7 variables include the exact mass of S-HyNic weighed, the exact volume of DMF used to dissolve the S-HyNic, the desalted protein concentration, the protein mass (mg) being modified, the name and molecular weight of the protein being modified, and finally the number of S-HyNic equivalents used in the reaction. The two outputs include the volume of S-HyNic/DMF to mix with the requisite volume of protein solution.

Note- Always maintain the percentage of DMF (vol/vol) in the final S-HyNic modification reaction at or below 5% of the total reaction volume.

Note- PBS (10mM phosphate, 150mM sodium chloride, pH 7.2) is not recommended as a modification buffer due to its poor buffering capacity.

Note- It is important to have a final protein concentration @ 2-5 mg/ml for efficient HyNic modification.

5. Incubate the reaction at room temperature for 2 hours.

6. Proceed to desalt the S-HyNic modification reaction (Step 1).

S-4FB Modification Protocol

(Requires the use of the [Protein-Modification with an NHS-Ester Calculator](#)).

1. Using an analytical balance, weigh between 5 mg of S-4FB solid (M.W. 247.1) into a clean, 1.5 ml polypropylene tube. Record the exact weight and input the value into Solulink's S-4FB Modification Calculator.

Note- although smaller quantities can be used, in our experience most analytical balances are not as precise (or accurate) below 5 milligrams.

2. Dissolve S-4FB (SFB) in a suitable volume of anhydrous DMF, usually in the range of 500-1000 ul. Record the exact volume and input the value into Solulink's S-4FB Modification Calculator.

3. Add the required volume of S-4FB to the protein, a volume that typically represents 10 to 20 mole equivalents over the amount of protein. To calculate the exact volume of S-4FB/DMF required, input the number of equivalents using Solulink's S-4FB Modification Calculator.

4. Mix the protein/S-4FB reaction well.

Note- Solulink highly recommends the use of our [Protein Modification with an NHS-ester Calculator](#) to determine the exact volume of S-4FB/DMF to add to your specific protein. The calculator functions by the input of 7 user-defined

variables. These 7 variables include the exact mass of S-4FB weighed, the exact volume of DMF used to dissolve the S-4FB, the desalted protein concentration, the protein mass (mg) being modified, the name and molecular weight of the protein being modified, and finally the number of S-4FB equivalents used in the reaction. The two outputs include the volume of S-4FB/DMF to mix with the requisite volume of protein solution.

Note- Always maintain the percentage of DMF (vol/vol) in the final S-4FB modification reaction at or below 5 % of the total reaction volume.

Note- PBS (10 mM phosphate, 150 mM sodium chloride, pH 7.2) is not recommended as a modification buffer due to its poor buffering capacity.

Note- It is important to have a final protein concentration @ 2-5 mg/ml for efficient 4FB modification.

5. Incubate the reaction at room temperature for 2 hours.

6. Proceed to desalt the S-4FB modification reaction

5. Desalting Modification Reactions

Desalt modification reactions (refer to Desalt Protocol, Step 1) using Zeba desalt spin columns equilibrated in 1x conjugation buffer (pH 6.0). Since the modified proteins will be conjugated at pH 6.0, remember to equilibrate the Zeba Desalt Spin Columns in 1x conjugation buffer (pH 6.0) rather than 1x modification buffer (pH 7.4). **Note- Remember to buffer exchange modification reactions into 1x conjugation buffer (6.0).** Use the

appropriate Zeba Desalt Spin Column (0.5 ml or 2 ml) equilibrated in 1x conjugation buffer (pH 6.0) to desalt your modified proteins.

6. Determining the Molar Substitution Ratio (MSR)

After desalting on Zeba™ Spin columns to remove excess linker from the modification reaction, protein concentrations are determined using the BCA assay. The modified protein samples are then ready for their respective MSR assay.

HyNic MSR

1. Prepare a 0.5mM working solution of 2-sulfobenzaldehyde solution in 0.1 M MES buffer, pH 5.0 as follows:

- a) Make a 100 mg/mL solution of 2-sulfobenzaldehyde in nuclease-free H₂O.
- b) Add 52 μ L of this solution to a 50ml conical tube containing 50 mL 100mM MES Buffer (pH 5.0). Label this solution 0.5mM 2-SBA solution.
- c) Protect the solution from light and keep refrigerated. This solution remains stable for up to 30 days at 4°C.

Note- The pH of the buffer may need to be changed if the protein precipitates during the MSR reaction. This occurs because exhaustive reaction of HyNic groups with 2-SBA alters the isoelectric point of the protein. Changing the pH of the 100 mM MES Buffer to 6.0 or 7.0 can sometimes prevent unwanted precipitation of the protein although the reaction is slower at a higher pH.

2. Fill three microcentrifuge tubes with 18 μ L of 0.5 mM 2-SBA solution.

- a) Add 2 μ L of 1X Conjugation Buffer, pH 6.0 to the first tube, which will be the blank.
- b) Add 2 μ L of desalted HyNic-modified protein/antibody solution (~2-5 mg/mL in 1X Conjugation Buffer, pH 6.0) to the second and third tube to create duplicates.
- c) Label all tubes as desired.

3. Incubate all reaction tubes at 37°C for 30 minutes or at room temperature for 2 hours.

4. Remove the reaction tubes from the 37°C incubator and measure the A₃₄₅ of both reactions using a quartz cuvette as follows:

- a) Blank the spectrophotometer @ 345 nm using the blank prepared above (18 μ L 0.5mM 2-SBA with 2 μ L 1X Conjugation Buffer).
- b) Record the A₃₄₅ of each sample.

Using the values obtained, calculate the HyNic/protein MSR with the aid of the calculator that is appropriate for your application: [HyNic-Protein Colorimetric MSR Calculator](#), [HyNic-Oligonucleotide Colorimetric MSR Calculator](#); alternatively, you can calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e. 28,500 @ 345 nm) and dividing by the known molar protein concentration.

4FB MSR

After modification, the proteins are desalted using Zeba™ Spin columns to remove excess linker and protein concentrations are determined (BCA assay) before proceeding with the MSR assay.

1. Prepare a 0.5mM working solution of 2-hydrazinopyridine-2HCl solution in 0.1 M MES buffer, pH 5.0 as follows:
 - a) Dissolve 5 mg 2-hydrazinopyridine-2HCl solid in 100 ul DMF.
 - b) Add 91 ul of this solution to a 50 ml conical tube containing 50 ml 100 mM MES Buffer (pH 5.0). Label this solution 0.5 mM 2-HP solution.
 - c) Protect the solution from light and keep refrigerated. This solution remains stable for up to 30 days at 4°C. Label the solution 0.5 mM 2-HP solution.

Note- The pH of the buffer may need to be changed if the protein precipitates during the MSR reaction. This occurs because exhaustive reaction of 4FB groups with 2-HP alters the isoelectric point of the protein. Changing the pH of the 100 mM MES Buffer to 6.0 or 7.0 can sometimes prevent unwanted precipitation of the protein although the reaction is slower at a higher pH.

2. Transfer 10 ul of 4FB-modified (desalted) protein solution (~2-5 mg/ml in 1x conjugation buffer) to a new 1.5 ml microfuge tube containing 490 ul 2-HP reagent. Prepare another reaction tube (negative control) containing 490 ul 2-HP reagent and 10 uL of 1x conjugation buffer.
3. Incubate all reaction tubes at 37°C for 30 minutes or at room temperature for 2 h.
4. Remove the reaction tubes from the 37°C incubator and measure the A₃₅₀ of both reactions using a quartz cuvette as follows:
 - a) blank the spectrophotometer @ 350 nm using 500 ul 0.5 mM 2-HP solution in MES (pH 5.0) in a 1 ml quartz cuvette.
 - b) record the A₃₅₀ of each sample and no protein controls.

Note- In rare instances that depend on the protein concentration of the desalted protein being measured and the final degree of 4FB modification, it may require a volume greater or lesser than 10 ul of protein to get a detectable A₃₅₀ reading on the spectrophotometer.

5. Using the values obtained, calculate the 4FB/protein MSR with the aid of our [4-FB-Protein Colorimetric MSR Calculator](#) or the [4-FB-Oligonucleotide Colorimetric MSR Calculator](#), or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e. 24,000 @ 350 nm) and dividing by the known molar protein concentration.

7. Conjugation

Conjugate formation is initiated by mixing the desired equivalents of each modified protein together. Often one protein is added in excess (2-3 fold) over the other in order to more efficiently drive the conjugation reaction to completion. Refer to Solulink's protein-protein conjugation calculators in concert with the following protocol.

Protocol

1. Mix the desired volume of 4FB-modified (desalted and quantified) protein #1 with the desired volume of HyNic-modified (desalted and quantified) protein #2. We recommend the use of our [Protein-Protein Conjugation Calculator](#) or [Protein-Oligonucleotide Conjugation Calculator](#) to calculate desired volumes.

An example

A protein-protein conjugation calculator is illustrated in Figure 13. User inputs are inserted into the yellow-shaded cells.

In this example, two proteins are being conjugated. Protein 1 is IgG-HyNic and Protein 2 is Alk-Phos-4FB. The data input into the calculator included: the source of Protein 1 (HyNic-IgG), its molecular weight, the concentration before mixing, and the milligrams of HyNic-IgG a user wishes to react.

The same information was input for Alk-Phos-4FB (Protein 2) into the corresponding yellow-shaded areas. The desired number of mole-equivalents are input in the last cell (Protein 2). The volume of Protein 2 to be added to Protein 1 is automatically calculated and displayed. Note that the calculator has inputs for 3 different reactions, namely 1 mole-equivalent, 2 mole-equivalents, and 3 mole-equivalents for Protein 2.

2. Incubate the two proteins at room temperature for 16 hr.
3. After incubation, the crude conjugate mixture is ready for analysis and purification.

8. Analysis

After completion of the conjugation reaction and before attempting to purify the conjugate, a small aliquot of the crude reaction mixture is often analyzed using a 4-12% Bis-Tris gel (Invitrogen) in a MOPS or MES denaturing buffer system. (Refer to the manufacturer's detailed protocols for the use of these systems).

The amount of crude sample loaded on these gels depends on the type and sensitivity of the staining method being used to detect the reaction products. For example, silver stains can detect between 150-1000 ng of conjugate whereas Coomassie blue stains are best visualized with at least 5 micrograms of

conjugate per lane. To analyze the crude samples, a suitable aliquot of the crude sample is mixed with loading buffer (e.g. 4X LDS Invitrogen) and water to a final loading volume of ~10 ul.

Appropriate protein molecular weight standards are loaded side by side on these gels to confirm conjugate size. Often, HyNic and 4FB modified proteins are also loaded on the gels as controls. Gels are electrophoresed in 1x NuPAGE SDS Running Buffer (MOPS or MES) at 200V (constant) for about 35 minutes. They are stained using manufacturer's suggested protocol (GE Healthcare PlusOne™ Silver Stain) or using standard Coomassie staining protocols followed by a de-stain step. Gels are visualized and documented using digital CCD imaging systems and a white light. These systems are available from numerous manufacturers (e.g. Kodak, Alpha Innotech, etc.) Conjugates migrate and are confirmed as higher molecular weight species on gels (see examples provided in Appendix IV). Once conjugate formation is confirmed the remainder of the crude reaction mixture is purified.

9. Purification

All conjugation reactions will be a mixture of crude reaction products consisting of the desired conjugate along with un-conjugated HyNic and 4FB-modified proteins. For this reason, most conjugation reactions are purified using chromatographic methods. Various FPLC or HPLC chromatography workstations are available for this purpose.

Solulink routinely purifies conjugates using gel filtration or ion exchange chromatography media on a Beckman Gold Chromatography Workstation (Figure 14) equipped with a scanning diode array detector. This detector is capable of continuous, real-time spectral monitoring of the elution profile (200-600 nm). This particular system allows the purification process to be 'traced' at various wavelengths such as 260 nm (DNA signature), 280 nm (protein signature), or more often at 350 nm (conjugate signature).

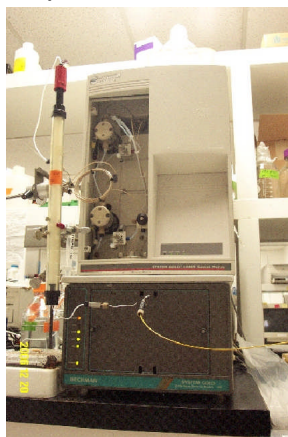


Figure 14. HPLC chromatography workstation used to purify bioconjugated protein-protein conjugate.

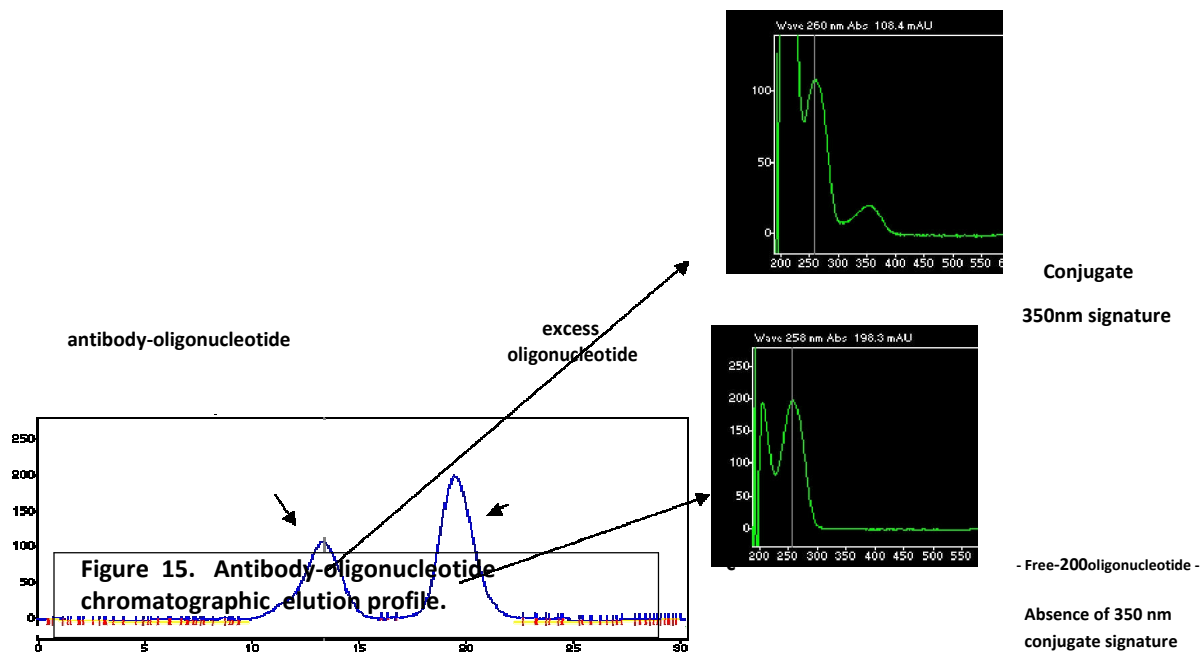
Protein-protein and protein-peptide conjugates are routinely isolated and purified according to their molecular weight using a size exclusion column (Superdex 200) (10 cm x 40 cm). Standard run conditions are 1.25 ml/min for 0.5 hr in 1x PBS buffer (pH 7.2). Injection volumes range from 0.1 to 1 ml at a protein concentration of 1 to 2.5 mg/ml (for this size column). Conjugate peaks are monitored using the scanning

diode array detector and specific conjugate peaks are detected by their UV-signature at 350 nm.

Protein-oligonucleotide conjugate purification

Protein-oligonucleotide conjugates can also be isolated and purified using an ion exchange column (Sephacrose Q, 1 cm x 10 cm). Standard run conditions use a buffered gradient from 300 mM NaCl, 20 mM Tris-HCl pH 8.0 to 700 mM NaCl, Tris-HCl pH 8.0 @ 1.25 ml/min for 1.5 hr. Conjugate samples can be diluted prior to injection to reduce the sodium ion content to below 300 mM. Injection volumes range from 0.1 to 1 ml at a protein concentration of 1 to 2.5 mg/ml (for this size column).

A typical antibody-oligonucleotide elution profile is illustrated in Figure 15. The profile was generated using a scanning diode array detector. The DNA-protein conjugate peak was detected @ 350 nm.



III. Troubleshooting

A. Common Bioconjugation Problems

| Problem | Possible Cause | Recommended Action |
|--|--|---|
| Poor modification of biomolecule | -initial biomolecule concentration is too low | -concentrate biomolecule using a diafiltration filter, -use an initial 2-5 mg/ml for efficient labeling of proteins |
| | -insufficient equivalents of modification reagent added | -Add more modification reagent, up to 50 equivalents can sometimes be added |
| | -amine contaminant, e.g. Tris or glycine buffer present in starting biomolecule solution | -exchange the protein buffer by diafiltration, dialysis or desalting column before modification -verify the protein or biomolecule being labeled has sufficient amino-groups using the NCBI protein database |
| Molar substitution assay readings are out of range | -precipitation of the modified protein on treatment with quantification reagents (2-SBA or 2-HP) can lead to spurious readings | -for HyNic modified proteins use 2-sulfobenz-4FB for quantification |
| Precipitation of protein on | - over-modification of the | -exchange the protein buffer |

| | | |
|---|--|---|
| modification | protein | by diafiltration, dialysis, or a desalting column |
| Protein-protein conjugate has a molecular weight that is much larger than predicted | -due to high modification levels on each protein a large M.W product may be formed | -lower modification levels by using lower equivalents of S-HyNic or S-4FB , lower the protein concentration during the modification reaction |
| 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, 6, 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, test the protein being conjugated for nuclease-activity by incubating with intact DNA |

IV. Appendix

Bioconjugation: Some examples

Protein-protein conjugation: S-HyNic (succinimidyl 6-hydrazinonicotinate acetone) and S-4FB (succinimidyl 4-formylbenzoate) were used to incorporate HyNic and 4FB moieties on BSA and IgG, respectively. SDS-PAGE (Figure 16) clearly demonstrates that mixing HyNic-modified BSA with 4FB-modified IgG forms a higher molecular weight conjugate.

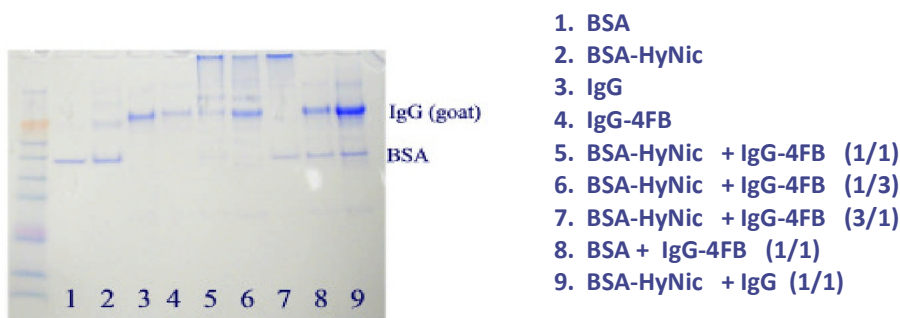


Figure 16. HyNic-modified BSA (lane 2), 4FB-modified IgG (lane 4). Reactions of 1/1, 1/3, 3/1 molar equivalents of BSA-HyNic over IgG-4FB (Lanes 5, 6, and 7). The subscripts denote the molar equivalents. Lanes 8 and 9 are negative control reactions.

Protein-oligonucleotide conjugation: IgG that was reacted with 7.5 and 15 equivalents of S-HyNic (Figure 17). After desalting, HyNic-modified IgG (IgG-HyNic) was reacted with 10 equivalents of 5'-4FB modified oligonucleotide in 0.1 M MES, 0.9% NaCl, pH 6.0 for 2 h. The crude reaction was then loaded on the gel. Subsequently, proteins were transferred to PVDF membrane and hybridized with a 5'-fluorescein-labelled oligonucleotide complementary to the conjugated oligonucleotide. Solulink has successfully conjugated 5'-4FB modified oligonucleotides as large as 90-mers to HyNic-modified antibodies in excellent yield (60-75%).

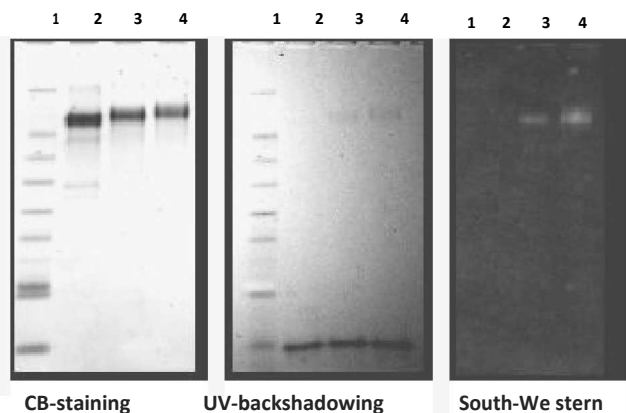


Figure 17. Lane 1, protein molecular weight marker. Lane 2 is HyNic-IgG. Lane 3 is IgG-HyNic (7.5 equiv.) reacted with a 10-fold excess of a 4FB-oligo (24-mer). Lane 4 is IgG-HyNic (15 equiv.) reacted with a 10-fold excess of the same oligonucleotide. The gel was stained with Coomassie blue (CB). Same gel visualized by UV-backshadowing (center) to detect antibody-oligo conjugate. A southwestern blot using a 5'-fluorescein-labelled oligonucleotide hybridized to the oligo-IgG conjugate.

Glycoprotein-oligonucleotide conjugation:

HyNic-modified oligonucleotides efficiently react with periodate-oxidized glycoproteins to yield glycoprotein-oligonucleotide conjugates. HyNic-groups were first incorporated on the amino group of an oligonucleotide using S-HyNic. Subsequently, the oligo was conjugated to an oxidized glycoprotein (HRP-ox). Figure 18 is a gel illustrating the reaction of the 5'-HyNic-modified oligonucleotide with periodate-oxidized horseradish peroxidase. Simple incubation of the HyNic-modified oligonucleotide with the protein produced the enzyme-oligonucleotide conjugate. No reduction was required.

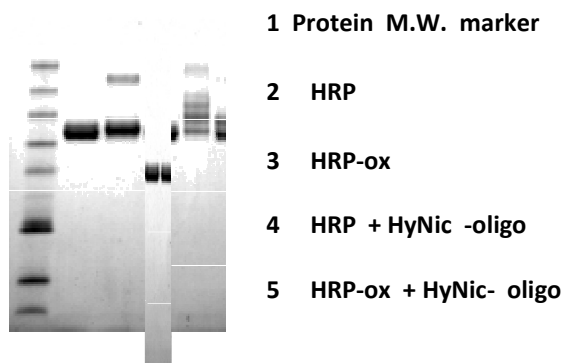


Figure 18. Coomassie-stained PAGE gel of a 5'-HyNic-modified oligonucleotide (22-mer) conjugated to periodate oxidized horseradish Peroxidase (Lane 5). Lanes 2 and 3 are HRP and ox-HRP, respectively. Lane 4 is a negative control reaction using non-oxidized HRP.

Oligonucleotide-peptide conjugates: Oligonucleotides and peptides are readily conjugated using HydraLink chemistry. Figure 19 illustrates the conjugation of a 5'-4FB modified oligonucleotide with a 15-mer HyNic-peptide that was prepared by incorporation of succinimidyl hydrazine reagent C₆-HNA during solid phase synthesis. Simple addition of the HyNic-peptide to a 4FB-modified oligonucleotide directly yields the peptide-oligonucleotide conjugate without the need of a reducing agent.

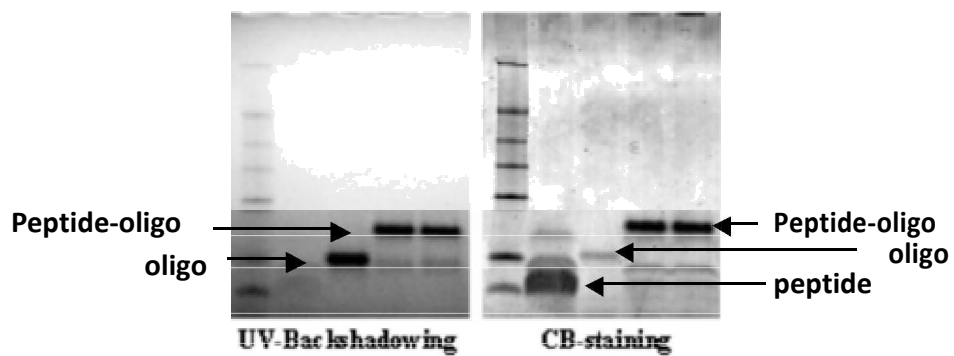


Figure 19. HyNic-peptide (15-mer) was conjugated to a 5'-4FB oligonucleotide. PAGE analysis clearly demonstrates the formation of an oligo-peptide conjugate.