



S-HyNic Bioconjugation Technical Manual

(Cat. # 9002-2)

S-HyNic Bioconjugation Technical Manual

I.	Introduction	3
	A. SoluLink Bioconjugation Process	3
	B. Process Overview	3
	C. HydraLink Chemistry	4
	D. Linker and Conjugate Stability	6
	E. Linker and Conjugate Traceability	7
	F. Kit Components	8
	G. Additional Components Required	9
II.	Bioconjugation	9
	A. Getting Started	9
	B. Summary of Conjugation Steps	10
	C. General Guidelines for Modification of Proteins with S-HyNic and S-4FB	12
	D. Conjugation Protocol	13
	1.) Desalt	13
	2.) Protein Determination	15
	3.) Concentrating Proteins	17
	4.) Modification Protocol	19
	a. S-HyNic Modification	19
	b. S-4FB Modification	20
	5.) Desalting the Modification Reactions	20
	6.) Determining the Molar Substitution Ratio	21
	7.) Conjugation	23
	8.) Analysis	24
	9.) Purification	24
III.	TroubleShooting	26
	A. Common bioconjugation problems	26
IV.	Appendix	27
	A. Bioconjugation: Some Examples	27
	B. Bioconjugation Calculators	29
	C. Sample Calculators	30

I. INTRODUCTION

A. SoluLink 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.

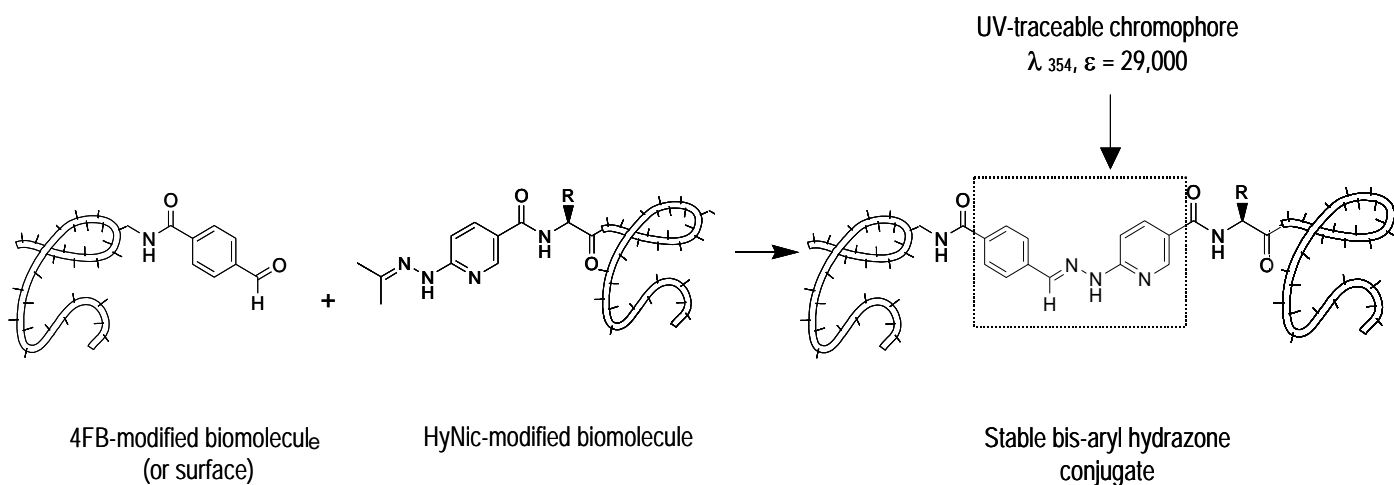


Figure 1: Linking chemistry behind HydraLink™ technology.

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).

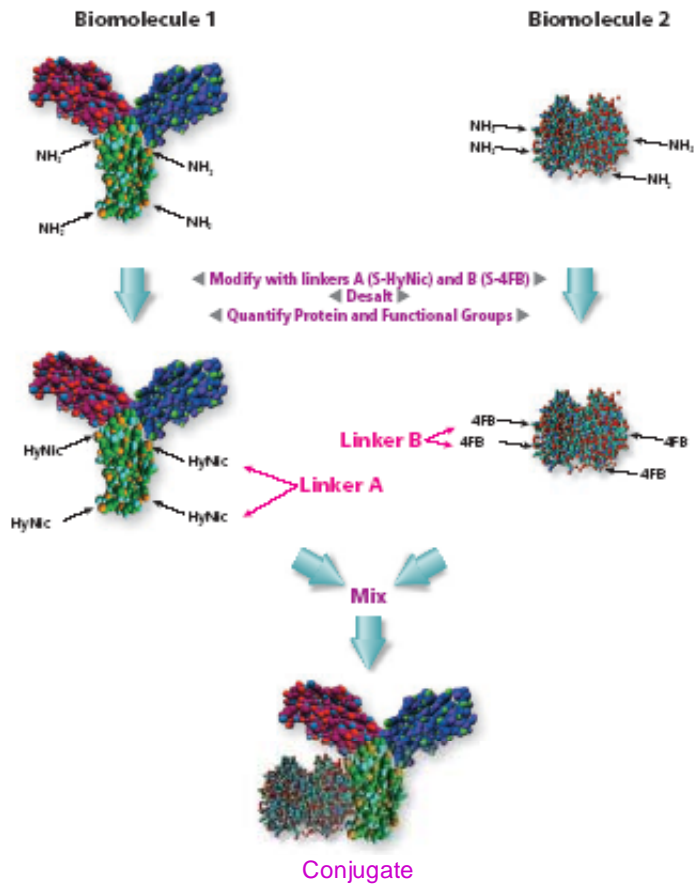


Figure 2. HydraLink bioconjugation process

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. HydraLinkTM Chemistry

HydraLinkTM 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.

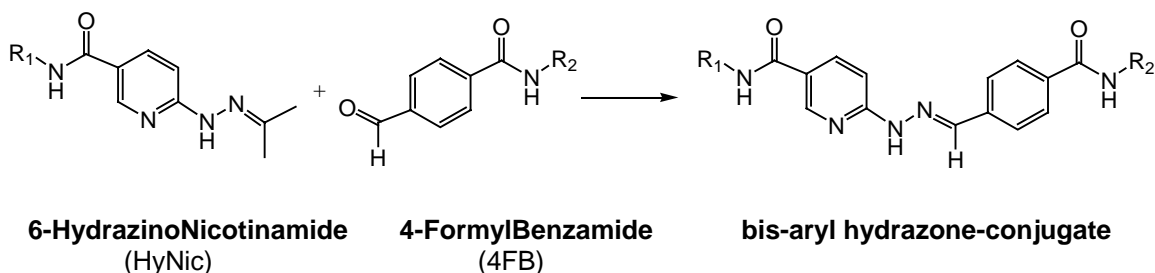


Figure 3. Specific chemical reaction between HyNic and 4-formylbenzamide (4FB) forms the bis-aryl hydrazone conjugate.

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 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. -COOH, -NH₂, -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.

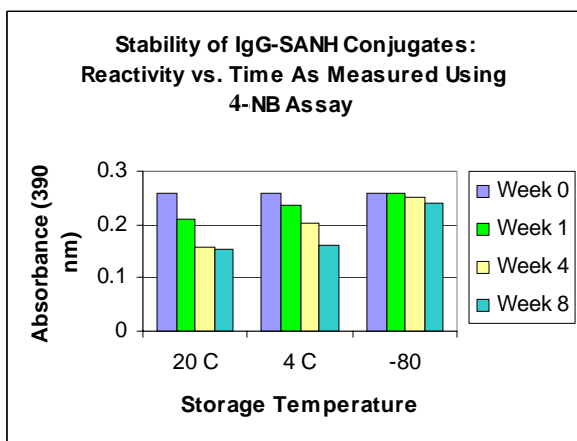


Figure 4. Reactive stability of HyNic functional groups conjugated to IgG. Stability was monitored by reaction of 4-nitrobenzaldehyde (4-NB, a colorimetric reagent) with HyNic-modified IgG to form a UV-traceable hydrazone. This absorbance signature was used to track the continued reactivity of HyNic-modified 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.

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.

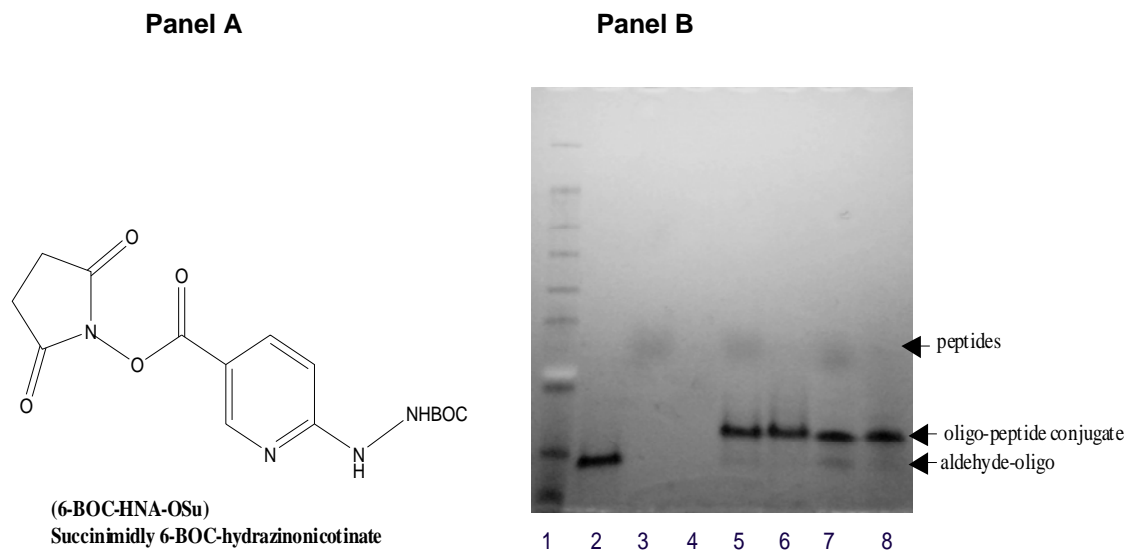


Figure 5. 6-Boc-HNA reagent (Panel A) was used to modify the NH₂-terminus of two 15-mer synthetic peptides with a HyNic moiety. SDS-PAGE was used to analyze conjugation products. Reaction of the HyNic-modified peptides with a 4FB-modified oligonucleotide (lane 2) formed conjugates (lanes 5, 6). Bond 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 4-NB) 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 p-nitrobenzaldehyde (4-NB, Cat. # S-2001-100) while 4FB groups are measured using 2-hydrazinopyridyl HCl (2-HP, Cat. # S-2002-100).

Reaction between 4-NB reagent and HyNic-modified proteins leads to the formation of a traceable absorbance signature @ 390 nm with a molar extinction coefficient at ϵ_{390} -24,000 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} - 18,000 l·mol⁻¹·cm⁻¹. These signatures are used in conjunction with Modification calculators provided by SoluLink at www.solulink.com/technology.htm. Together, they accurately quantify the degree of linker incorporation for each modified protein.

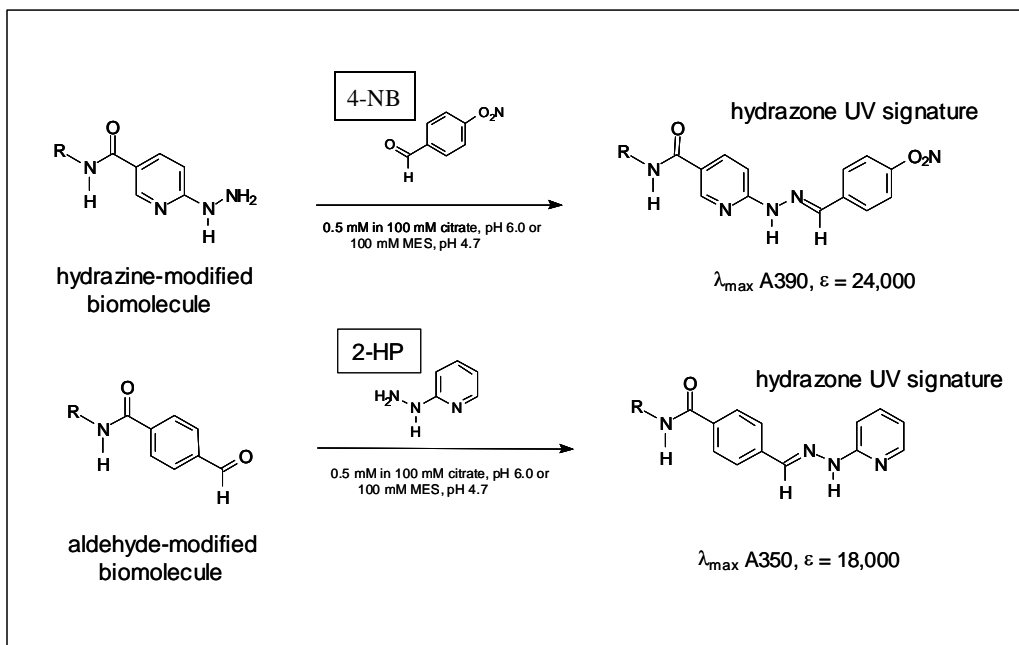


Figure 6. Colorimetric reactions used for quantifying HyNic and 4FB functional groups.

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

Product	Description	Catalog #	Size/Quantity
S-HyNic (SANH) Kit		S-9002-2	Kit
Kit Components	S-HyNic (SANH)		10 mg
	S-4FB (SFB)		10 mg
	4-nitrobenzaldehyde		100 mg
	2-hydrazinopyridine.2 HCl		100 mg
	2-sulfobenzaldehyde		100 mg
	DMF		1.0 mL
	10x Modification Buffer		1.5 mL
	10x Conjugation Buffer		1.5 mL
	Diafiltration Apparatus (5K MWCO)		5 per kit

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.

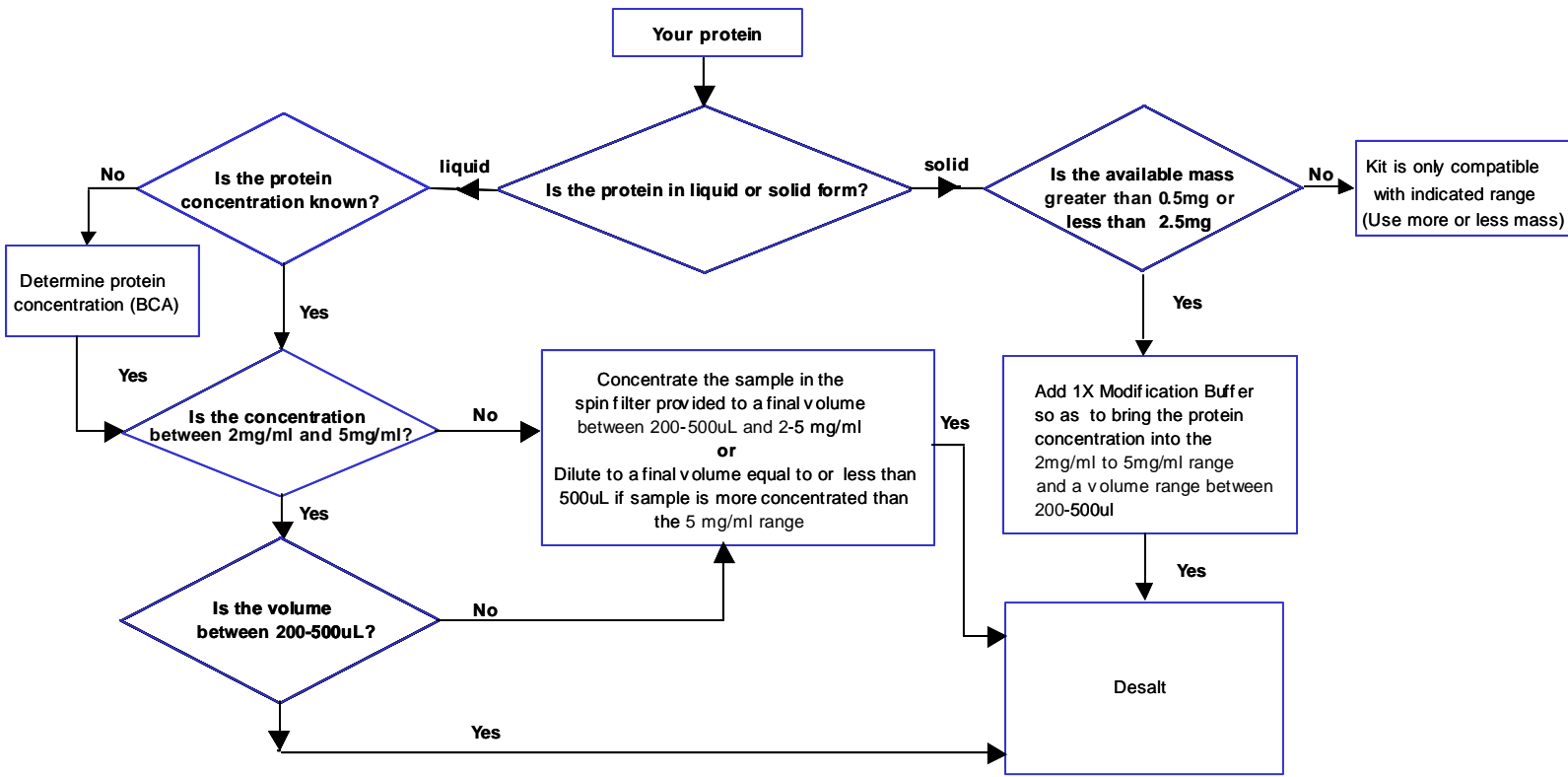


Figure 7. Flow-chart used for guiding a user to the start of the conjugation process.

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, 4-NB 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 Novex™ (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 hydrazone 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 Zeba™ 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 Zeba™ Desalt Spin Column. Figure 9 illustrates both the 0.5 ml and the 2 ml Zeba™ Desalt Spin Columns. Choose a spin column based on the available protein sample volume. For example, the 0.5 ml Zeba™ spin column desalts a volume that ranges from 30-130 μ l. The 2 ml spin column desalts volumes that range between 200-700 μ l. **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 Zeba™ Spin Column does not fit into a standard high-speed microcentrifuge that holds 1.5 ml tubes. The larger 2 ml Zeba™ 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 Zeba™ columns are provided below.

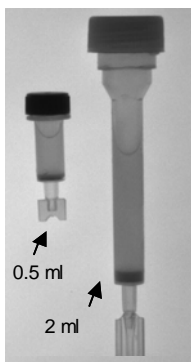



Figure 9. Zeba™ Desalt Spin Columns (0.5 and 2 ml) used to desalt S-HyNic proteins and modification reactions.

Protocol

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

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 μ l 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 μ l sample volume to the center of the compact resin bed.

Note- for sample volumes less than 70 μ l apply a 15 μ l 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 240-700 μ l)

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 μ l) to the center of the compact resin bed.

Note- for sample volumes less than 350 μ l apply additional buffer (stacker) to the top of the resin bed (40 μ l) 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 μ l	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 μ l 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 1st well to a 2nd well (0.5mg/ml)
 Well #3- Add 50ul 1X PBS and 50ul from the 2nd well to a 3rd well (0.25mg/ml)
 Well #4- Add 50ul 1X PBS and 50ul from the 3rd well to a 4th well (0.125mg/ml)
 Well #5- Add 50ul 1X PBS and 50ul from the 4th well to a 5nd well (0.0625mg/ml)
 Well #6 -Add 50ul 1X PBS to the 6th 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.

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

- Seal the samples in the 96-well plate using clear adhesive film and incubate in a 40°C water bath for 20 minutes.
- 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.

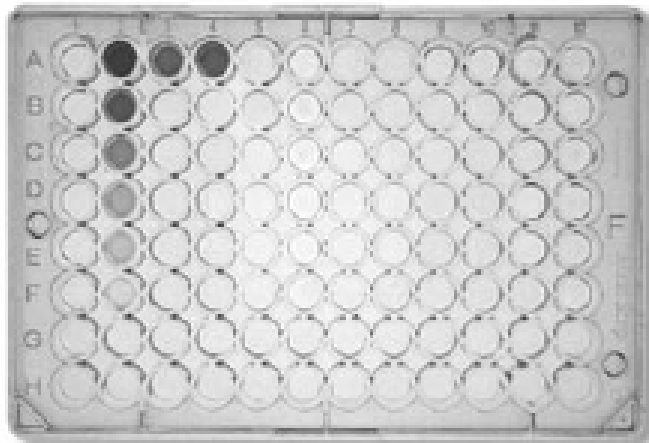


Figure 10. 96-well BCA Protein Assay plate containing a dilution series

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.

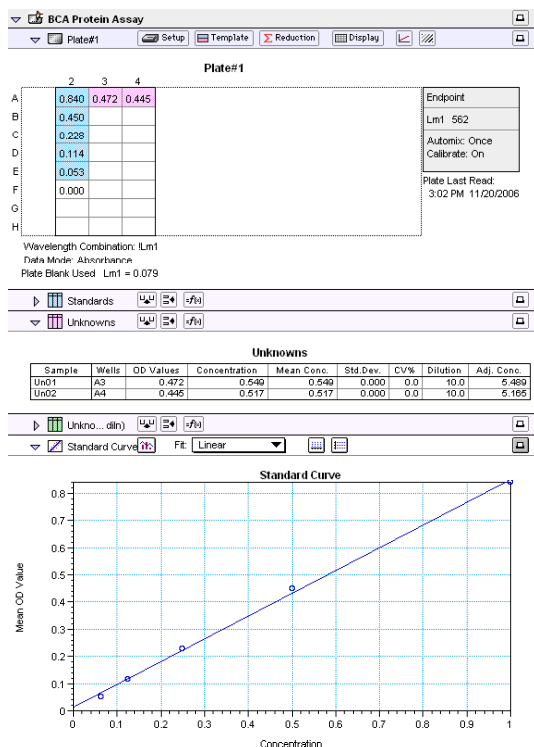


Figure 11. BCA protein assay printout from a Molecular Devices plate reader.

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).

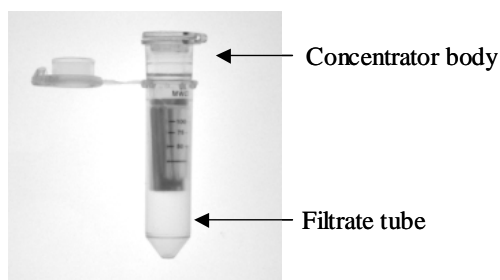


Figure 12. VIVASPIN 500 spin filter used to concentrate dilute protein samples prior to modification.

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 SoluLink's on-line modification calculators (S-HyNic and S-4FB Modification Calculators) located at www.solulink.com/technology.htm to modify your specific protein. Typically, modification reactions are conducted in a volume that ranges from 100-700 μ l 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

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 SoluLink's S-HyNic Modification Calculator (www.solulink.com/technology.htm).
- 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 μ l. Record the exact volume and input the value into SoluLink's S-HyNic Modification 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 using SoluLink's S-HyNic Modification Calculator.
4. Mix the protein/S-HyNic reaction well.

Note- SoluLink highly recommends the use of our on-line S-HyNic Modification Calculator at www.solulink.com/technology.htm 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

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 μ l. 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 on-line S-HyNic Modification Calculator at www.solulink.com/technology.htm 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 p-nitrobenzaldehyde solution in 0.1 M MES buffer, pH 5.0 as follows:
 - a) Dissolve 5 mg p-nitrobenzaldehyde in 100ul DMF.
 - b) Add 76ul of this solution to a 50ml conical tube containing 50ml 100mM MES Buffer (pH 5.0). Label this solution 0.5mM 4-NB 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 4-NB 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 10ul of HyNic-modified (desalted) protein solution (~2-5mg/ml in 1x conjugation buffer) to a new 1.5 ml microfuge tube containing 490ul 4-NB reagent. Prepare another reaction tube (negative control) containing 490ul 4-NB reagent and 10uL 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 A_{390} of both reactions using a quartz cuvette as follows:
 - a) blank the spectrophotometer @ 390nm using 500ul 0.5mM 4-NB solution in MES (pH 5.0) in a 1 ml quartz cuvette.
 - b) record the A_{390} 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 HyNic-modification, it may require a volume greater or lesser than 10ul of protein to get a detectable A_{390} reading on the spectrophotometer.
5. Using the values obtained, calculate the HyNic/protein MSR with the aid of our on-line Modification Calculator found at (<http://www.solulink.com/support.htm>) or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e. 24,000 @ 390 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 A_{350} 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 A_{350} 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 A_{350} reading on the spectrophotometer.

5. Using the values obtained, calculate the 4FB/protein MSR with the aid of our on-line S-4FB Modification Calculator found at (<http://www.solulink.com/support.htm>) or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e. 18,000 @ 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

- 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 SoluLink's protein-protein calculator at (<http://www.solulink.com/support.htm>) 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), it's 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.

	Reaction 1	Reaction 2	Reaction 3	
Protein 1	IgG-HyNic	IgG-HyNic	IgG-HyNic	
Source	Biosource	Biosource	Biosource	Insert source modified protein 1
M.W.	150000	150000	150000	Insert M.W. protein 1
mg req'd	1	1	1	Insert mg protein 1 to be conjugated
vol req'd	333.33	333.33	333.33	volume of protein solution to be reacted (uL)
mmol	6.67E-03	6.67E-03	6.67E-03	mmol of protein to be reacted
equivalents	1.0	1.0	1.0	# equiv of protein 1
	Reaction 1	Reaction 2	Reaction 3	
Protein 2	Alk-Phos-4FB	Alk-Phos-4FB	Alk-Phos-4FB	
Source	Biozyme	Biozyme	Biozyme	Insert source modified protein 2
M.W.	140000	140000	140000	Insert M.W. protein 2
mg/mL	2.5	2.5	2.5	Insert protein 2 conc. (mg/mL)
mg req'd	0.93	1.87	2.80	mg protein 2 to be conjugated
vol req'd	373.33	746.67	1120.00	volume (uL) of protein required
mmol	6.67E-03	1.33E-02	2.00E-02	mmol of protein2 used
equivalents	1.0	2.0	3.0	Insert # equiv. of protein 2 req'd

Figure 13. Sample protein-protein calculator available on-line at www.solulink.com/support.htm

- Incubate the two proteins at room temperature for 16 hr.
- 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 μ l.

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 bioconjugates

Protein-protein conjugate purification

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.

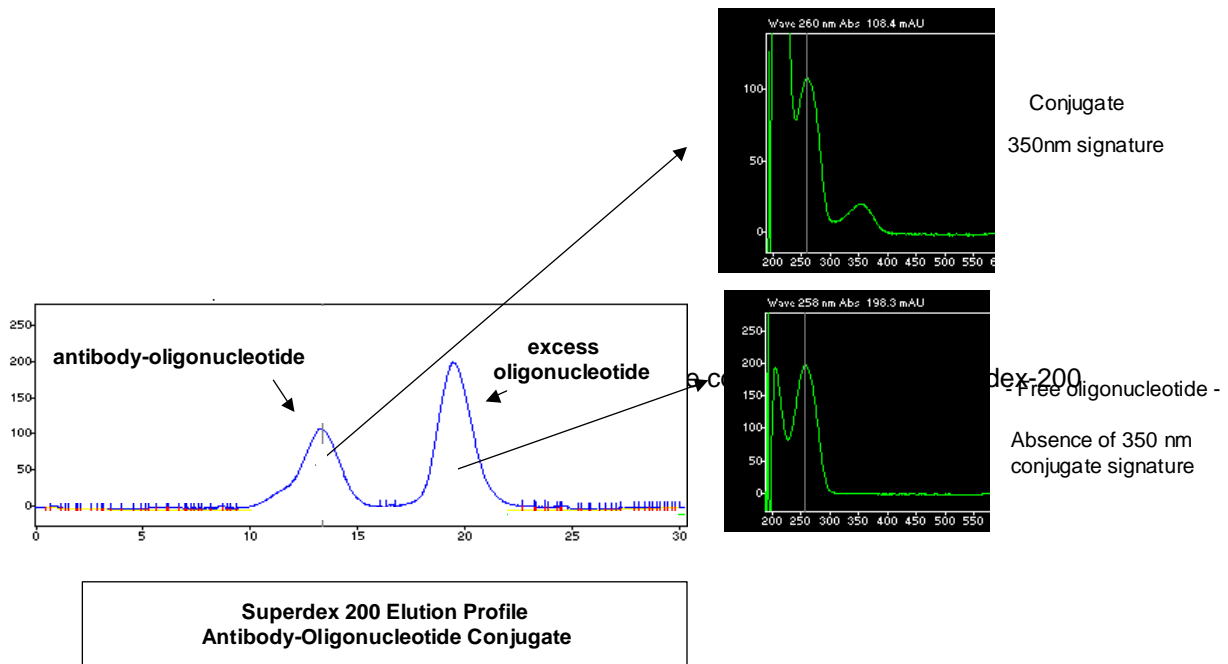


Figure 15. Antibody-oligonucleotide chromatographic elution profile.

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 (p-NB or 2-HP) can lead to spurious readings	-for HyNic modified proteins use 2-sulfobenz-4FB for quantification
Precipitation of protein on modification	- over-modification of the protein	-exchange the protein buffer 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.

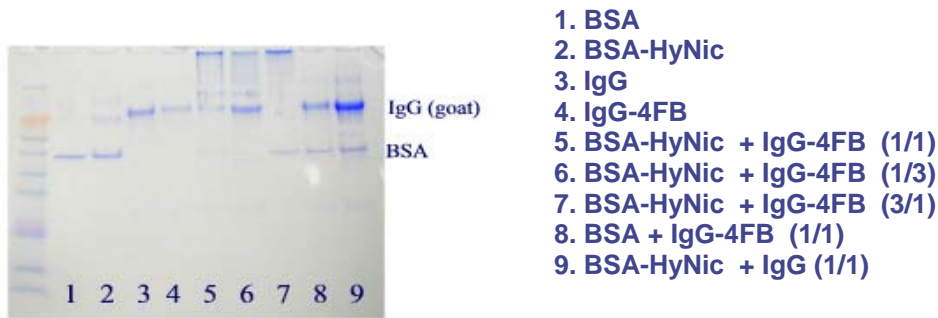


Figure 16. HyNic-modified BSA (lane 2), 4FB-modified IgG (lane 4). Reactions of 1/1, 1/3, 3/1 molar equivalents of BSA-HyNic over IgG-4FB (Lanes 5, 6, and 7). The subscripts denote the molar equivalents. Lanes 8 and 9 are negative control reactions.

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%).

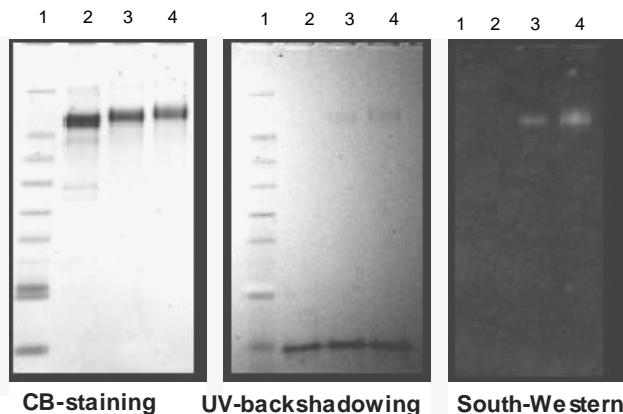


Figure 17. Lane 1, protein molecular weight marker. Lane 2 is HyNic-IgG. Lane 3 is IgG-HyNic (7.5 equiv.) reacted with a 10-fold excess of a 4FB-oligo (24-mer). Lane 4 is IgG-HyNic (15 equiv.) reacted with a 10-fold excess of the same oligonucleotide. The gel was stained with Coomassie blue (CB). Same 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.

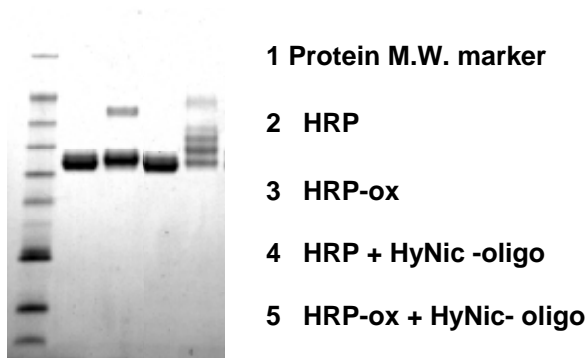


Figure 18. Commassie-stained PAGE gel of a 5'-HyNic-modified oligonucleotide (22-mer) conjugated to periodate oxidized horseradish Peroxidase (Lane 5). Lanes 2 and 3 are HRP and ox-HRP, respectively. Lane 4 is a negative control reaction using non-oxidized HRP.

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.

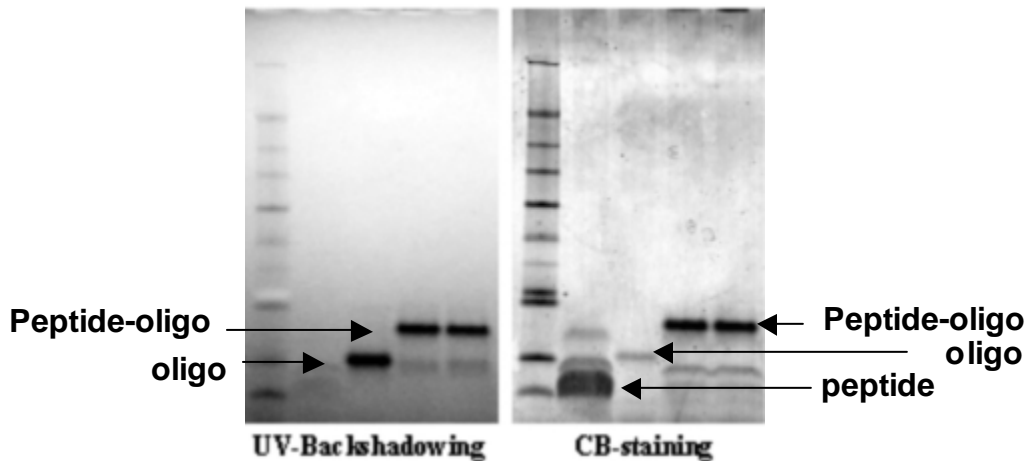


Figure 19. HyNic-peptide (15-mer) was conjugated to a 5'-4FB oligonucleotide. PAGE analysis clearly demonstrates the formation of an oligo-peptide conjugate.

B. Bioconjugation Calculators

SoluLink provides a comprehensive group of on-line bioconjugation calculators at www.solulink.com/support.htm. These calculators aid the experimenter in three distinct ways. They allow for easy set-up of the modification reaction, they aid the user in calculating and determining the MSR (molar substitution ratio) after modification, and finally they calculate conjugation volumes based on equivalents and concentrations.

Each calculator is specific to the linker being used (e.g. S-HyNic or S-4FB) and which biomolecule (e.g. protein or oligonucleotide) is being modified or conjugated. They make estimating the amount (mass) and volume (ul) of each component in these reactions easy to calculate. These calculators require only simple user-defined inputs such as the exact mass of S-HyNic or S-4FB weighed by the user, the volume of DMF used to dissolve linkers, and the number of equivalents needed. Two additional calculators are provided on-line, the S-HyNic Protein Modification Calculator and the S-4FB Protein Modification Calculator. We specifically recommend the use of these simplified calculators when using the modification protocol outlined in this technical manual that is specifically focused on protein-protein conjugations.

These calculators offer flexibility for custom conjugation experiments because each protein (or biomolecule) has its own intrinsic molecular weight, concentration, and batch size. In addition, they provide enhanced flexibility for weighing out (different) user-defined masses of linker to obtain the correct 'equivalents' needed for each specific batch size.

Modification Calculators available on-line:

Worksheet 01: MES Buffer Calculator
Worksheet 02: S-HyNic/Oligonucleotide Modification Calculator
Worksheet 03: S-4FB/Oligonucleotide Modification Calculator
Worksheet 04: S-HyNic/Protein Modification Calculator
Worksheet 05: SHNH/Protein Modification Calculator
Worksheet 06: S-4FB/Protein Modification Calculator
Worksheet 07: 2-HP and 4-NB MSR Buffer Calculator
Worksheet 08: Protein/Protein Conjugation Calculator
Worksheet 09: Protein/Oligonucleotide Conjugation Calculator
Worksheet 10: Oligo/Oligo Conjugation Calculator
Worksheet 11: Oligo/Small Molecule Conjugation Calculator
Worksheet 12: S-HyNic Protein Modification Calculator
Worksheet 13: S-4FB Protein Modification Calculator

Sample Calculator

SoluLink provides interactive calculators on our website.

These calculators make the modifications with our linkers and conjugates simpler by estimating the amounts of the materials needed to perform conjugations. The following example illustrates the steps in one such calculator.

Step	Protein Information	Input/Result	Explanation
1	Protein to be modified	BSA	Insert protein name
2	Reference #	5550102	Insert protein source/lot#
3	Modification type	HyNic	
4	M.W. Protein	50,000	Insert protein M.W.
5	Protein conc. (mg/mL)	2.30	Insert protein concentration (mg/mL)
Protein Information			
6	M.W. protein (from above)	50,000	M.W. protein (as inserted above)
7	mg/mL (from above)	2.30	Protein conc. (as inserted above)
8	mg required	3.1	Insert mg of protein to be conjugated
9	Volume (µL)	1347	Volume (µL) protein to be used
10	mMol	6.20E-05	mMol protein reacted
11	mM	4.60E-02	mMol protein concentration
S-HyNic (SANH) Information			
12	Lot#	S-1002	Insert S-HyNic (SANH) lot #
13	Molecular weight of S-HyNic (SANH)	290.2	M.W. S-HyNic (SANH)
14	Weight of S-HyNic (SANH) (mg)	6	Insert # mg S-HyNic (SANH) weighed
15	mMol	2.07E-02	mMol S-HyNic (SANH) weighed
16	Volume of DMF (µL)	600	Insert volume (µL) DMF added to S-HyNic (SANH)
17	Equivalentents	25	Insert # equiv to be added to protein
18	mMol S-HyNic (SANH) required	1.55E-03	mMol S-HyNic (SANH) to be added to protein
19	Volume S-HyNic (SANH)/DMF required (µL)	44.98	Volume (µL) S-HyNic (SANH)/DMF added to protein solution
Purification/Desalting			
20	Modified protein	BSA	Protein name
21	Volume of recovered Protein (mL)	1.4	Insert volume of recovered protein
22	Concentration (mg/mL)	1.9	Insert protein concentration (mg/mL)
23	Total weight recovered (mg)	2.66	Total mg recovered
24	Total mMol recovered	5.32E-05	Total mMol recovered
25	Concentration of protein (mM)	3.80E-02	Concentration (mM) recovered protein

The following instructions detail the use of the above calculator in more detail

Step **User input steps are highlighted; remaining steps are results and require no user input.**

1	Input the name of the protein to be modified with S-HyNic (SANH) reagent, e.g., BSA (Bovine serum albumin)
2	Input the commercial source or lot number of the protein to be modified
3	Refers to the type of functional group (name) to be incorporated into the protein, e.g., hydrazine.
4	Input the known molecular weight of the protein (Daltons) to be modified with S-HyNic (SANH)
5	Input the concentration ¹ of the (desalted) protein, e.g., BSA, to be modified with S-HyNic (SANH) (mg/mL).
6	Displays the molecular weight of the protein to be modified with S-HyNic (SANH) from step 4
7	Calculates the concentration (mg/mL) of the protein about to be modified, e.g., BSA, from step 5
8	Input the number of milligrams of protein (BSA) one wishes to conjugate
9	Calculates the volume in microliters of the protein (BSA) of step 8
10	Calculates the number of mMol of BSA to be modified
11	Calculates the concentration of the protein to be S-HyNic (SANH)-modified, e.g., BSA, in millimoles
12	Input the lot number of S-HyNic (SANH) to be used in the reaction
13	Displays the molecular weight of S-HyNic (SANH) reagent in Daltons
14	Input the mass of S-HyNic (SANH) weighed on the analytical balance
15	Calculates the millimoles of S-HyNic (SANH) from step 14
16	Input the volume in μL of anhydrous DMF used to dissolve S-HyNic (SANH) (solid) prior to its use in the reaction
17	Input the number of mole equivalents (excess) S-HyNic (SANH) to be added relative to the protein to be modified
18	Calculates the millimoles of S-HyNic (SANH) corresponding to the number of equivalents from step 17
19	Calculates the volume in μL of S-HyNic (SANH)/DMF reagent to be added to the protein labeling reaction required to give the millimoles shown in step 18.
20	Automatically shows the name of the S-HyNic (SANH)-modified protein from previous input
21	Input the final volume of S-HyNic (SANH)-modified protein recovered after desalting the protein/S-HyNic (SANH) reaction ²
22	Input the final desalted, S-HyNic (SANH)-modified protein concentration recovered ³
23	Calculates the total mass of S-HyNic (SANH)-modified protein recovered after desalting
24	Calculates the total number of millimoles of S-HyNic (SANH)-modified, desalted protein recovered

1 This value is usually determined using BCA or Bradford protein assay or by its known molar extinction coefficient.

2 This value is usually determined by BCA or Bradford protein assay.