Version 12.12.2012

Antibody-Oligonucleotide All-In-One Conjugation Kit

Technical Manual

Cat. No. A-9202-001

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Safety Information

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

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Chapter 1: Introduction

A. Purpose of Kit

Each Antibody-Oligonucleotide All-in-One Conjugation Kit provides all the necessary reagents to generate one antibody-oligonucleotide conjugate. The kit requires the user to supply the antibody (polyclonal or monoclonal mammalian IgG, 100 µg) and one HPLC-purified, 3' or 5' amino-modified oligonucleotide (15–25OD_{260} units). Typically, a 1 µmol synthesis provides sufficient amino oligo for modification. Kit instructions are specifically designed for researchers with limited or no conjugation experience. A specific**conjugation calculator** is directly integrated with the protocol and avoids the need to perform numerical calculations throughout the procedure. Each kit yields between 20–60 µg of highly purified, ready-to-use, antibody-oligonucleotide conjugate. Yield is dependent on both the specific antibody and oligo size. Final conjugate concentrations typically range from 0.1–0.3 mg/mL.

B. Important Parameters

The Antibody-Oligonucleotide All-in-One Conjugation Kit is designed to perform optimally with amino-modified oligonucleotides in the 20–60 nucleotide range. Oligos shorter than 20 nucleotides cannot be successfully conjugated with this kit; oligos longer than 60 bases *may* be used, albeit at the expense of conjugate yield. While successful use of oligonucleotides greater than 60 bases (and up to 120 bases) has been reported, we do not recommend exceeding the 60 base limit the kit was optimized to conjugate. The kit has been optimized for the modification of 25OD_{260}units of oligonucleotide; however, a minimum of 15OD_{260}units may be used if required.

Antibodies which are suitable for use with this conjugation procedureare mammalian antibodies of the IgGisotype**only**. Non-mammalian or non-IgGisotypeantibodies must not be used with this kit. Please ensure your antibody preparation does not contain carrier molecules such as gelatin or BSA, as these will interfere with the conjugation process. Likewise, high concentrations of glycerol (>25%) are not suitable due to the increased viscosity of these solutions. Small-molecule amines such as Tris or sodium azide are acceptable, as they are removed in the desalting process prior to antibody modification.

C. Customer Service and Technical Support

For assistance or additional technical information, please contact us:

Telephone **Email**

Chapter 2: Overview of Conjugation

A. All-in-OneConjugation Technology

1) Conjugation Chemistry

The Antibody-Oligonucleotide All-in-One Conjugation kit uses Solulink's patented conjugation chemistry to link an antibody to an oligonucleotide, as illustrated in **Figure 1**.The first stage of the process begins with the labeling of a 3' or 5'- amino-modified oligonucleotide with**S-4FB**. This amine-reactive NHS ester incorporates an aromatic aldehyde functional group, formylbenzamide (4FB), at the desired terminus of the oligonucleotide.

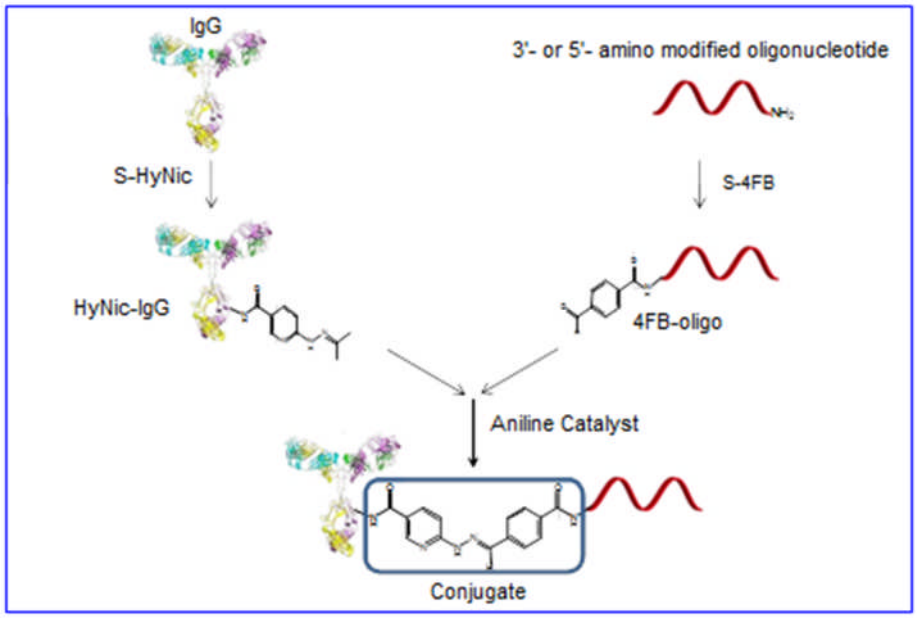


Figure 1.Reaction of a HyNic-modified IgG with a 4FB-modified oligo leads to the rapid formation of a stable antibody-oligonucleotide conjugate.

In stage two of the process, a polyclonal or monoclonal antibody (100 µg) is modified using the complementary linker**S-HyNic**. This NHS-ester reacts with lysine residues, incorporating HyNic functional groups (hydrazinonicotinamide) onto the antibody. In the **third and final stage**, the two modified biomolecules are mixed together in the presence of a reaction catalyst (aniline) to form the conjugate, after which purification is carried out using a magnetic affinity solid phase.

2) Conjugate Purification

Antibody-oligonucleotide conjugates produced with the All-in-One kit are ready to be used in the most demanding and sensitive of downstream applications. The kit delivers high-purity conjugate virtually free of residual antibody or oligonucleotide (>98%). Reaction conditions are optimized to convert nearly 100% of the antibody into conjugate, leaving only free, excess 4FB-oligo to be removed. Complete conversion of antibody to conjugate simplifies conjugate purification as illustrated in **Figure 2**. Antibody-oligonucleotide conjugate is purified to near homogeneity by selectively binding the conjugate

to a magnetic affinity matrix, allowing excess 4FB-oligonucleotide to be washed away. Affinity-bound conjugate is then gently eluted from the matrix and buffer exchanged into long-term storage buffer. **Antibody-oligonucleotide conjugates produced with this kit are stable for up to 1 year when kept at 4°C in storage buffer.**

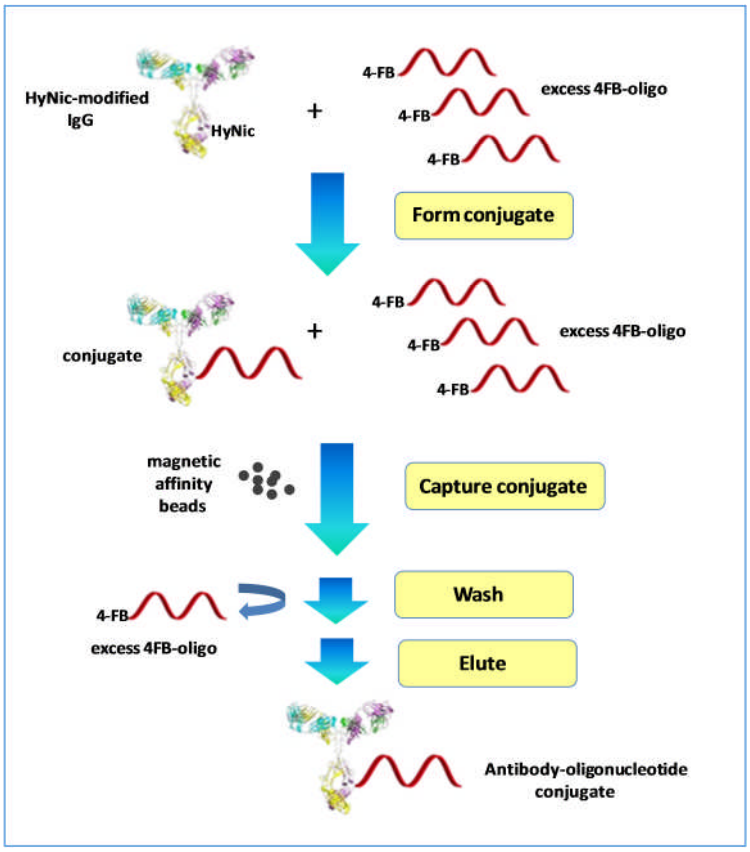
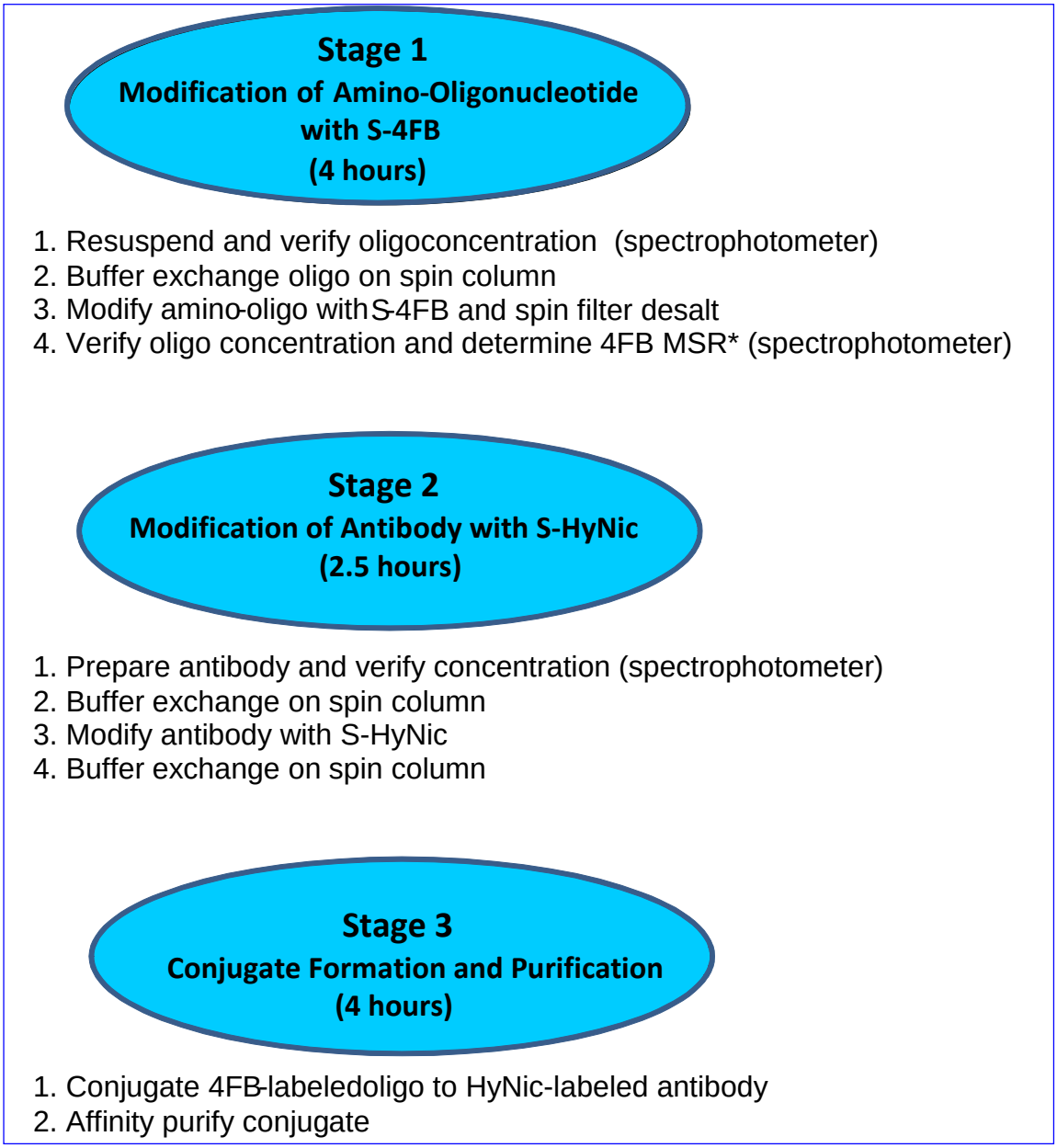


Figure 2.All-in-One conjugate purification strategy.

B. All-in-One Conjugation Overview



* 4FB MSR is an acronym for **4**-**f**orml**b**enzamide **m**olar **s**ubstitution **r**atio.

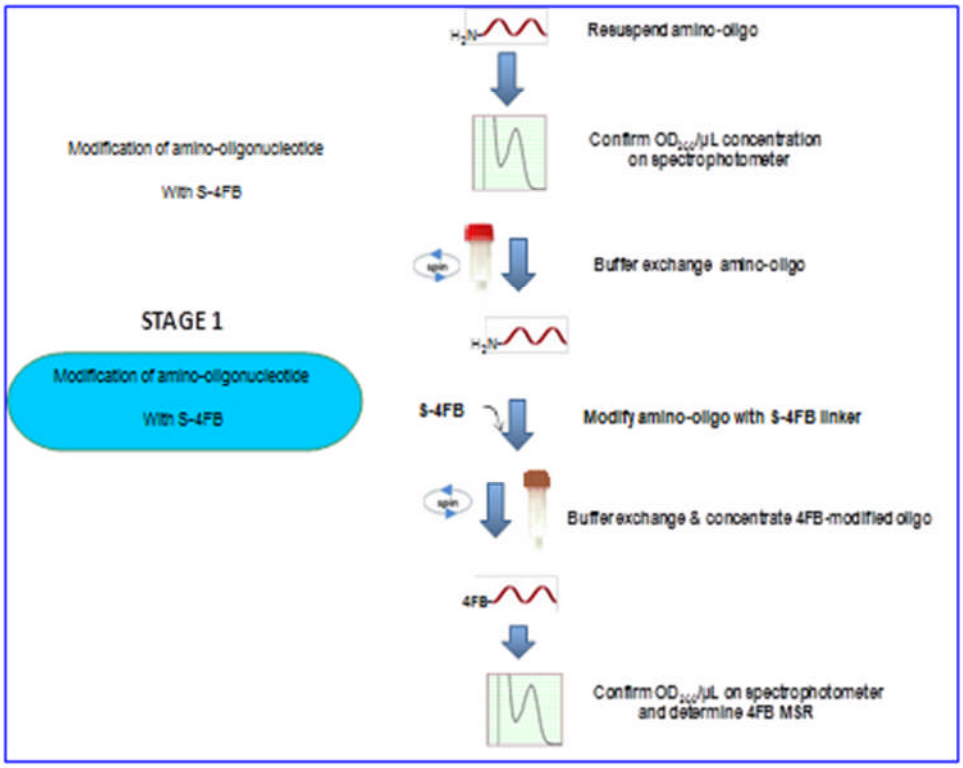


Figure 3. Stage 1 of the process illustrates the modification of an amino-oligonucleotidewith the S-4FB linker.

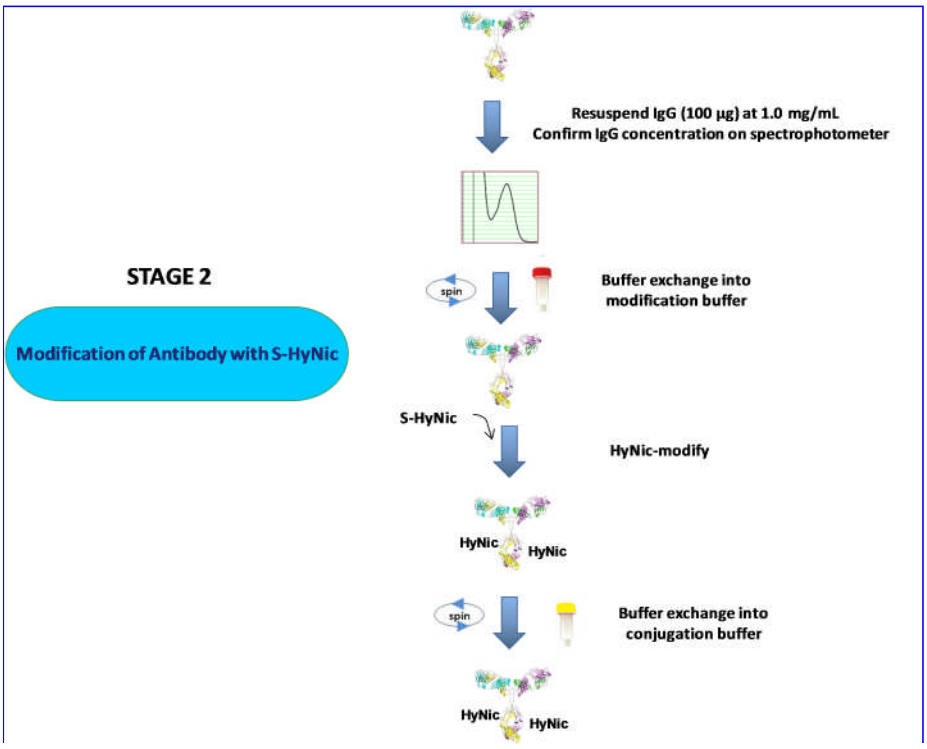


Figure 4. Stage 2 of the process illustrates the modification of IgG using the S-HyNic linker.

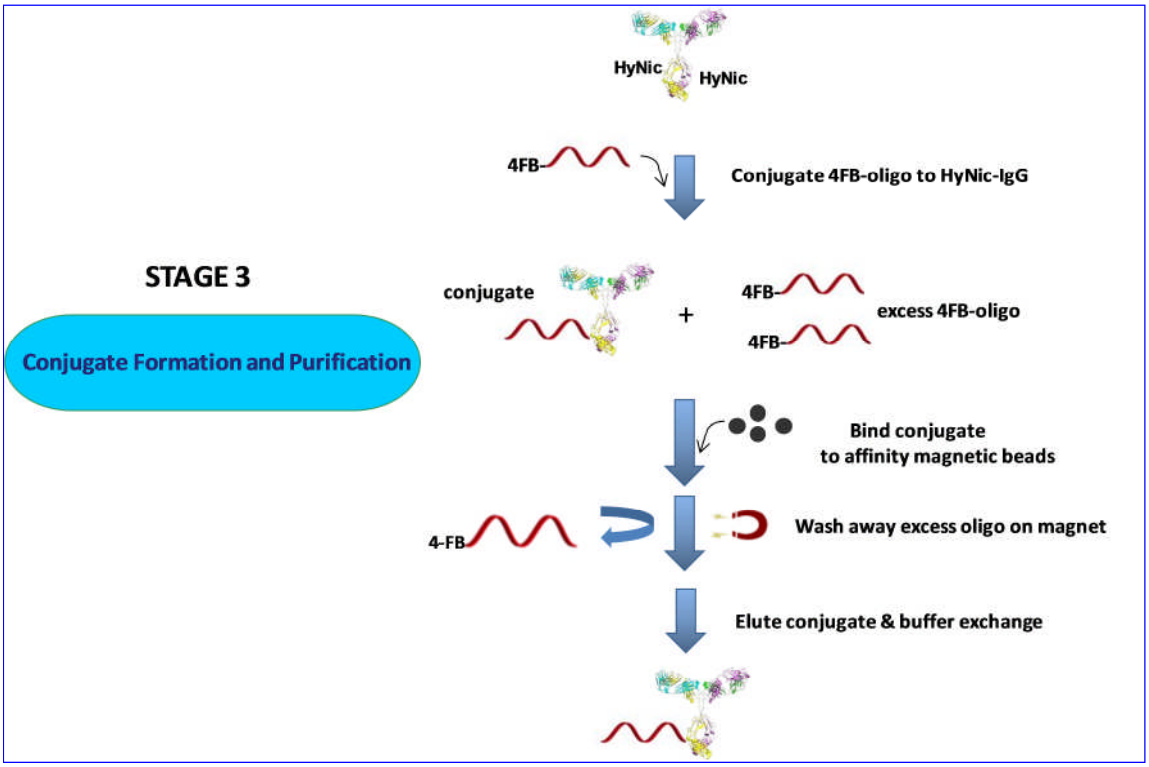


Figure 5. Stage 3 of the process illustrates both the formation and purification of the conjugate.

C. Kit Components

S-HyNic	A-9202-001-01	100 µg
OligoResuspensionSolution	A-9202-001-03	250µL
1X Modification Buffer	A-9202-001-04	500 µL
Bead Wash Buffer	A-9202-001-06	5 mL
Bead Elution Buffer	A-9202-001-07	0.25 mL
Red Cap Spin Column	A-9202-001-08	2 columns
Yellow Cap Spin Column	A-9202-001-09	1 column
Blue Cap Spin Column	A-9202-001-10	2 columns
Anhydrous DMF	A-9202-001-11	1.0 mL
2-HP	A-9202-001-13	500 µL
Affinity Magnetic Beads	A-9202-001-14	75 µL
2 mL Collection Tubes	A-9202-001-16	14 tubes
S-4FB	A-9202-001-17	1.0 mg
Brown Cap Spin Column	A-9202-001-18	2 columns

D. Materials to be Provided by the User

Variable high-speed microcentrifuge (*e.g.,*Eppendorf5415D or equivalent)
Magnetic single 1.5 mL tube stand
UV-VIS scanning spectrophotometer or NanoDrop™ spectrophotometer
UV-VIS scanning plate reader (Bradford Assay) (optional)
Micro-volume quartz cuvette (50–100 µL) if a NanoDrop™ is not available
1.5 mL microcentrifuge tubes
Bradford protein assay reagents (Bio-Rad, Cat. No. 500-0006)
Bovine IgG concentration standards (Pierce, Cat. No. 23212)
Calibrated pipettes (P-2, P-20, P-100, P-200, and P-1000) and barrier tips
Table-top centrifuge (holds 50 mL conical tubes)(optional)

NOTE: PLEASE DO NOT USE A PLATE READER FOR ANY SPECTROPHOTOMETRIC MEASUREMENTS FOR THIS PROTOCOL.

E. Component Storage Conditions

Component	Storage
Kit	4°C (do not freeze)
S-HyNic (desiccated)	4°C (do not freeze)
S-4FB (desiccated)	4°C (do not freeze)
All other components	4°C (do not freeze)
HyNic-modified antibody	4°C (do not freeze)
4FB-modified oligo	4°C (do not freeze)
Antibody-oligo conjugate	4°C (do not freeze)

Chapter 3: Conjugation Protocol

Prior to Starting

The conjugation protocol is a three stage process **(~11 hours in duration)** where each step takes several hours to complete.**If desirable, the end-user can complete Stage 1 on the first day (~4 hours), then proceed with Stages 2 and 3 on day two (6.5 hours).Keep in mind that we do not recommend stopping the procedure after Stage 2**.

Stage 1: Modification of Amino-Oligonucleotide with S-4FB

A. Enter Amino-Oligo Information into Conjugation Calculator

1. Enter the following amino-oligo parameters directlyfrom the Oligo vendor's Certificate of Analysisinto the**Antibody-Oligonucleotide Conjugation Calculator (Section A)**.

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

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

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

B. Resuspend Amino-Oligo

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

2. Place the vial containing lyophilized oligonucleotide in a microcentrifuge and centrifuge at 15,000 x g for 15 seconds to pellet the lyophilizate at the bottom of the tube. If the tube contains between 15 and 25 OD_{260} units of oligo, add 50 µL of **OligoResuspension Solution**to the tube. If the tube contains more than 25 OD_{260} units of oligo, add a sufficient volume of OligoResuspension Solutionto create a 0.5 OD_{260}/µL solution (example: if there are 31 OD_{260} units of oligo, add 62 µL of OligoResuspension Solutionto create a 0.5 OD_{260}/µL solution).

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

C. Measure Amino-Oligo Concentration on a Spectrophotometer

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

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

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

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

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

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

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

    e) **Divide this number by 5** to calculate the OD_{260}/µL concentration of the stock oligo solution.

2. Multiply the OD_{260}/µL value calculated in (e) above by 50 to determine the OD_{260} units available after dissolution (do not enter this information into the calculator at this point).

3. **Important:If less than 15 OD_{260} units are recovered after resuspension (from 2 above), obtain additional amino-oligo.**

Conventional UV-VIS Spectrophotometer

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

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

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

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

    d) **Divide this number by 2** to calculate the OD_{260}/µL concentration of the stock oligo solution.

2. Multiply the OD_{260}/µL value calculated in (d) above by 50 to determine the OD_{260} units available after dissolution (do not enter this information into the calculator at this point).

3. **Important: If less than 15 OD_{260} units are recovered after resuspension, obtain additional amino-oligo.**

D. Buffer Exchange Amino-Oligo

1. Prepare the oligo desalting spin column (red cap) by loosening the cap one-quarter turn, twisting off the bottom closure, and placing it in an empty collection tube. Centrifuge the column at **1,500 x *g* for 1 minute** to remove excess interstitial buffer. **Note:**Using a Sharpie marker, place a vertical line on the outside of the desalting column. **Ensure that this line faces *away* from the center of the rotor in this and all subsequent steps.**

2. Remove the spin column from the collection tube (discard the collection tube containing excess buffer) and place the column in a new collection tube. *Slowly* and carefully pipet exactly 50 µL of oligo solution into the center of the resin bed. Be careful not to let the oligo solution contact the tube wall; it must channel down through the resin itself. Replace the cap and loosen one-quarter turn.

3. Centrifuge the column again at **1,500 x *g* for 2 minutes** to recover the desalted oligo into the collection tube. Transfer this solution into a new microcentrifuge tube(not provided) and measure the volume with a P-100 pipet. Enter this volume (µL) of desalted amino-oligo recovered into the conjugation calculator (Section B).

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

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

E. Dissolve S-4FB Reagent

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

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

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

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

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

G. Removal of excess S-4FB

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

1. Remove both of thebrown-capped spin columns from the kit. Prepare the desalting columns by loosening the capsone-quarter turn, twisting off the bottom closures, and placing them in empty collection tubes. Centrifuge the columns at **1,500 x *g* for 1 minute** to remove excess interstitial buffer. **Note:** Using a Sharpie marker, place a vertical line on the outside of the desalting columns. **Ensure that this line faces *away* from the center of the rotor in this and all subsequent steps.**

2. Remove the spin columns from the collection tubes (discard the collection tubes containing excess buffer) and place the columns in new collection tubes.

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

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

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

6. *Immediately* transfer the entire eluatefrom step 3 to the other brown-capped spin column and repeat the desalting process by centrifuging at **1,500 x *g* for 2 minutes.** This “double-desalting” will ensure that all traces of un-incorporated 4FB are removed from the oligo.

7. Transfer the desalted oligo solution to a microcentrifuge tube, measuring the volume with a P-200 pipette. Vortex the solution to mix thoroughly before proceeding to Part H.

H. Measure4FB-Oligo Concentration

Measure the 4FB-Oligo concentration as follows:

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

1. In a microcentrifuge tube prepare a 1:50 dilution of the 4FB-oligo by transferring 2 µL oligo with a calibrated P-2 pipette into 98 µL molecular grade H_2O. Vortex well to mix.
2. Select the "Nucleic Acid" menu option on the NanoDrop and initialize the instrument using molecular grade water (ND-1000 only).
3. Clean the sample pedestal and blank the instrument with 2µL molecular grade H_2O.
4. Clean the sample pedestal and measure the A_{260} of the 1:50 4FB-oligo solution as displayed in the **10 mm path length window.** Record the A_{260} value.
5. **Divide this number by 20** to calculate the OD_{260}/µL concentration of the stock 4FB-oligo solution.
6. Enter the calculated OD_{260}/µL concentration from part "5" above into the Conjugation Calculator (Section D).

Using a conventional 1cm pathlength spectrophotometer

1. In a microcentrifuge tube prepare a 1:500 dilution of the 4FB-oligo by transferring 2 µL oligowith a calibrated P-2 pipette into 998µL molecular grade H_2O.
2. Blank the spectrophotometer at 260 nm using 1mL molecular grade H_2O.
3. Measure the A_{260} of the 1:500 4FB-oligo. Record the A_{260} value.
4. **Divide this number by 2** to calculate the OD_{260}/µL concentration of the stock 4FB-oligo solution.
5. Enter the calculated OD_{260}/µL concentration from part "4" above into the Conjugation Calculator (Section D).

I. Quantify 4FB Molar Substitution Ratio

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

NanoDrop4FB Molar Substitution Ratio Assay

1. Prepare a 2-HP blank solution by adding exactly 2 µL water to exactly 18 µL 2-HP Reagent in a clean reaction tube (not provided); label '2-HP Blank'.
2. Prepare a 4FB-oligo MSR sample by adding exactly 2 µL 4FB-modified oligo to exactly 18 µL 2-HP reagent in a clean reaction tube (not provided); label '4FB-Oligo'. Vortex both

solutions well to mix, and then briefly spin the tubes to pool the entire contents to the bottom of each tube.

3. Incubate the 2-HP blank and 4FB-Oligo reactions at 37°C for **60 minutes**. After the incubation period, centrifuge both tubes at 15,000 x *g* for 15 seconds to collect condensation at the bottom of the tube. Gently vortex to thoroughly mix.

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

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

6. Place a 2 µL aliquotof the 4FB-Oligo MSR reaction on the pedestal and click the 'Measure' icon. The 1.0 mm A_{360} absorbance will appear. Enter this value directly into the MSR calculator (Section E).

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

1. Prepare a 2-HP blank solution by adding exactly 2 µL molecular-grade water to exactly 18 µL 2-HP Reagent; label '**2-HP Blank'**.

2. Prepare a 4FB-oligo MSR sample by adding exactly 2 µL 4FB-modified oligo to exactly 18 µL 2-HP reagent; label '**4FB-Oligo**'. Vortex both solutions well to mix and then briefly spin the tubes to pool the entire contents to the bottom of each tube.

3. Incubate the 2-HP blank and 4FB-Oligo reactions at 37°C for **60 minutes**. After the incubation period, centrifuge both tubes at 15,000 x *g* for 15 seconds to collect condensation at the bottom of the tube. Gently vortex to thoroughly mix and then briefly spin the tubes to pool the entire contents to the bottom of each tube.

4. Prepare a 1:10 dilution of the **2-HP blank** by transferring 10 µL from the completed reaction mixture into 90 µL molecular grade H_2O.

5. Prepare a 1:10 dilution of the **4FB-oligo**MSR reaction by transferring 10 µL from the completed reaction mixture into 90 µL molecular grade H_2O. Vortex both solutions well to mix.

6. In a 1 cm, 100 µL quartz micro-cuvette, blank the spectrophotometer at λ = 360 nm with 100 µLof 1:10 diluted 2-HP blank from step 4 above.

7. Measure the A_{360} of the1:10 4FB-OligoMSR sample from step 5 above in the cuvette. Record the A_{360} and enter this value directly into the MSR calculator (Section E).

Conventional Spectrophotometer 4FB Molar Substitution Ratio Assay (1mL cuvette method)

1. Prepare a 2-HP blank solution by adding exactly 10 µL molecular-grade water to exactly 90 µL 2-HP Reagent; label '**2-HP Blank'**.

2. Prepare a 4FB-oligo MSR sample by adding exactly 10 µL 4FB-modified oligo to exactly 90 µL 2-HP reagent; label '**4FB-Oligo'**. Vortex both solutions well to mix and then briefly spin the tubes to pool the entire contents to the bottom of each tube.

3. Incubate the 2-HP blank and 4FB-Oligo reactions at 37°C for **60 minutes**. After the incubation period, centrifuge both tubes at 15,000 x *g* for 15 seconds to collect condensation to the bottom of the tube.

4. Next, add exactly 900 µL moleculargrade water to both reactions to bring the volumes to 1mL each. Vortex to thoroughly mixand then briefly spin the tubes to pool the entire contents to the bottom of each tube.

5. In a 1 cm, 1 mL quartz cuvette, blank the spectrophotometer at λ = 360 nm with 1 mL of 1:10 diluted 2-HP blank from step 4 above.

6. Measure the A_{360} of the1:10 4FB-Oligo MSR sample from step 4 above in the cuvette. Record the A_{360} and enter this value directly into the MSR calculator (Section E).

Stage 2: Modification of Antibody with S-HyNic

Antibodies are packaged in two different physical forms, solids and liquids. Individual samples can vary greatly from vendor to vendor and are often sold in a variety of different sizes and/or concentrations. In all cases, Solulink highly recommends starting with the highest-quality/purity antibody available.Depending on the initial form (solid or liquid), follow the instructions that apply to your particular sample.

A. Antibody Preparation

If the IgG is in a solid lyophilized form (100 µg)

1. Add 100 µL 1X Modification Buffer to lyophilized antibody (100 µg solid). Cap the sample vial and vortex for 1 minute and then briefly spin the tube to pool the entire contents to the bottom.

2. Open the lid and using a P-200 gently pipette the solution up and down while rinsing the wall of the container from top to bottom. Lyophilized antibody can often adhere to the upper walls of a product vial. Visually inspect the vial and lid for any residual lyophilized antibody residue that may have become trapped during the vendor packaging process in order to maximize sample recovery.

Important: Although careful resuspension of the antibody is tedious,it is a critical step in the conjugation process. Antibody vendors rarely overfill product vials, so to achieve efficient recovery of expensive antibodies, great care and diligence is recommended.

3. Briefly centrifuge the resuspended antibody at **1,000 x g for 10 seconds** to collect the entire liquid contents at the bottom of the vial and proceed to confirm antibody concentration.

   Note: If the original IgG product is packaged in a product vial that is too large to fit inside a standard microcentrifuge, such larger vials (e.g., glass vials) can first be placed inside a 50 mL disposable conical tube and briefly spun at **1,000 x g for 10 seconds** using a larger tabletop centrifuge. If a larger tabletop centrifuge is not available, use a rapid and brisk downward flick of the sample vial in an attempt to collect as much of any adhering liquid at the bottom of the vial.

If the IgG is already in liquid form

1. If the initial antibody sample is already in liquid form at 1 mg/mL, transfer 100 µL to another labeled tube (100 µg). If the initial antibody sample is in liquid form at a concentration greater than 1 mg/mL, transfer a volume equivalent to 100 µg to another tube and add the necessary volume (µL) of 1X Modification Buffertoobtain 100 µL at 1 mg/mL. And finally, if the initial antibody sample is less than 1 mg/mL, the sample must first be concentrated to 1 mg/mL and 100 µL using a suitable ultra-filtration spin filter. Spin filters are available from Solulink(http://store.solulink.com/collections/accessories). An ultra-filtration spin filter is not provided with this kit.

2. Briefly centrifuge the resuspended antibody at **1,000 x g for 10 seconds** to collect the entire liquid contents at the bottom of the original vial and proceed to confirm antibody concentration.

   Note: If the original IgG product is packaged in a product vial that is too large to fit inside a standard microcentrifuge, such larger vials (e.g., glass vials) can first be placed inside a 50 mL disposable conical tube and briefly spun at **1,000 x g for 10 seconds** using a larger tabletop centrifuge. If a larger tabletop centrifuge is not available, use a rapid and briskdownward flick of the sample vial in an attempt to collect as much of any adhering liquid at the bottom of the vial.

B. Confirm Antibody Concentration on a Spectrophotometer

Confirm the resuspended antibody concentration by measuring the sample's A_{280} on a spectrophotometer. As before, either a micro-volume UV-VIS scanning spectrophotometer (e.g.,NanoDrop™ ND-1000) or conventional spectrophotometer can be used. When using a conventional spectrophotometer a quartz micro-cuvette (50–100

µL, 1-cm path length) is required. Use the appropriate instructions that follow depending on the specific type of spectrophotometer available to you (NanoDrop™ orconventional).

Antibody Concentration on a NanoDrop™ Spectrophotometer(ALL MODELS)

1. Launch the NanoDrop™ software by clicking the desktop icon.

2. Select the **ProteinA_{280} menu** option.

3. Initialize the instrument with 2 µL molecular grade water on a clean pedestal.

4. When the scan window appears, turn off the 340 nm normalization feature by clicking the appropriate box. **Note:**Some NanoDrop™ instruments do not have a 340 nm normalization feature.

5. Blank the spectrophotometer using 2 µL of the appropriate buffer blank solution (e.g., the solution used to resuspend the antibody). Click the "Reblank" icon to verify a flat baseline (i.e., no offsets).

6. Clean the pedestal and measure the A_{280} of the antibody sample with a 2 µL aliquot of antibody sample. Record the A_{280}.

7. Enter the name of the antibody, the measured A_{280} (10 mm path length) and the total volume of antibody solution into the **Antibody-Oligonucleotide Conjugation Calculator(Section F).** The calculator displays the protein concentration (mg/mL) and the total mass of antibody to be conjugated into the **Antibody-Oligonucleotide Conjugation Calculator(Section F)**. A concentration of 1 ± 0.2 mg/mL is required to proceed; otherwise, obtain additional IgG or adjust the concentration to 1 mg/mL.

   Note: The calculator uses the "average" known mass extinction coefficient (E1%) of IgG to calculate protein concentration (e.g., E1% = 14)

Antibody Concentration on a Conventional Spectrophotometer

1. Blank the spectrophotometer at 280 nm using an appropriate blank solution (e.g., the solution used to resuspend the antibody) with a quartz micro-cuvette (50–100 µL, 1-cm path length). Empty the cuvette.

2. Measure the A_{280} of the antibody sample. Record the A_{280} and recover the antibody sample from the cuvette back to its sample tube.

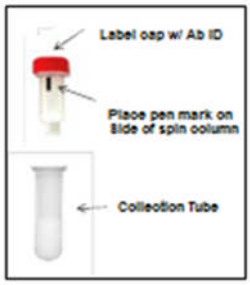
3. Enter the name of the antibody, the A_{280} (1-cm path length) and the volume of antibody solution (e.g., 100 µL) into the **Antibody-Oligonucleotide Conjugation Calculator(Section F).** The calculator then displays the protein concentration (mg/mL) and total mass of antibody available to be conjugated into the **Antibody-Oligonucleotide Conjugation Calculator(Section F**). A concentration of 1 ± 0.2 mg/mL is required to proceed; otherwise, obtain additional IgG or adjust the concentration to 1 mg/mL.

Note: The calculator uses the "average" known mass extinction coefficient (E1%) of IgG to calculate protein concentration (E1% = 14).

C. Buffer Exchange Antibody

1. Prepare a spin column **(red cap)** by twisting off the bottom closure and loosening the red cap (do not remove). Place the spin column into a collection tube (provided).

2. Mark the top of the red cap using an indelible pen to identify the antibody sample. Also place a vertical mark on the side of the spin column as shown below.



3. Place the entireassembly into the centrifuge and orient the vertical mark on the spin column aiming outward and away from the center of the rotor. Use an appropriate balance tube opposite the spin column.

4. Centrifuge at **1,500 x g for 1 minute**. Discard the flow through from the collection tube. The column matrix will appear white in color. Place the column back into a new empty collection tube (provided).

5. Open the red cap; load the antibody sample (~100 µL at 1 mg/mL) to the top of the dry resin bed; loosely cap and place the column back into the collection tube.

6. Orient the spin column mark outward as before and centrifuge at **1,500 x g for 2 minutes**.

7. Transfer the eluate from the bottom of the collection tube to a new labeled 1.5 mL tube (not provided); measure the volume (µL) recovered from the collection tube with a P-200 pipette. Label the tube with the appropriate volume (µL) recovered.

8. Confirm the antibody concentration on a spectrophotometer (same procedure as stage 2, step B – be sure to use 1X Modification Buffer to blank the instrument).

9. Enter the antibody A280 into Section F of the conjugation calculator.

D. Dissolve S-HyNicReagent

1. Add 35 µL DMF to a vial of S-HyNic reagent. Pipette the solution up and down for 60 seconds to dissolve the pellet and then briefly spin the tube to pool the entire contents to the bottom.

E. Modify IgG with S-HyNic Reagent and Buffer Exchange

1. Add 2.0 µL of dissolved S-HyNic modification reagent to the antibody sample. Gently pipette the solution to mix.
2. Incubate the antibody/HyNic modification reaction at **room temperature** for **2 hours**.
3. Exactly five minutes prior to the end of the HyNic modification reaction, prepare a spin column **(yellow cap)** by twisting off the bottom closure and loosening the cap (do not remove). Place the spin column into a collection tube and mark the top of the yellow cap with an indelible pen to identify the antibody sample. Also place a vertical mark on the side of the spin column as shown below.

4. Place the assembly into the centrifuge and balance appropriately. Orient the mark on the side of the spin column aiming outward and away from the center of the rotor. Use an appropriate balance tube opposite the spin column.
5. Centrifuge at **1,500 x g for 1 minute**. Discard the flow through from the bottom of the collection tube. The column matrix will appear white in color. Place the column back into a new empty collection tube (provided).
6. After the HyNic modification reaction is complete, apply the HyNic/IgG reaction mixture (~100 µL) to the top of the dry resin bed. Loosely recap and orient the spin column in the centrifuge. Centrifuge at **1,500 x g for 2 minutes**.
7. Transfer the recovered volume (µL) of HyNic-modified IgG using a P-200 pipette from the bottom of the collection tube to a new 1.5 mL tube. Measure and record the volume recovered and immediately proceed to conjugate formation.

Stage 3: Formation of Conjugate and Purification

A. Conjugate Formation

1. Enter the name of the antibody, the name of the 4FB-oligonucleotide, and the volume of HyNic-IgG to be conjugated into the **Antibody-Oligonucleotide Conjugation Calculator(Section G).Also, be sure that the correct oligoinformation is added to the calculator.**
2. Add the indicated volume (µL) of 4FB-modified oligonucleotide displayed in the **Antibody-Oligonucleotide Conjugation Calculator(Section G)**to the HyNic-modified antibody. Pipette the solution up and down to mix.
3. Incubate the antibody-oligo conjugation reaction for **2 hours at room temperature.**

B. Conjugate Purification

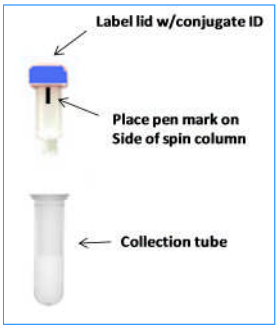
Ten minutes prior to the end of the *Conjugate Formation* reaction, prepared the affinity magnetic beads as follows:

1. Centrifuge the vial containing affinity magnetic beads (black slurry) at **1,000 x g for 5 seconds** to collect the bead contents at the bottom of the tube.
2. Place the tube containing the magnetic beads on a magnetic stand for 10 seconds; carefully remove and discard the bead storage solution.
3. Add 500 µL Bead Wash Buffer to the bead slurry; using a P-1000 pipette, pipette the solution up and down several times to mix the slurry. Quickly, before the beads resettle, place the tube on the magnet for 10 seconds; carefully remove and discard the supernatant using a P-200 pipette without disturbing the pellet.
4. Repeat step 2 **three (3) additional times** to fully wash the beads; removing the supernatant after each wash.
5. Immediately add the conjugation reaction (~115 µL) directly to the washed bead pellet.
6. Gently pipette the slurry/conjugate mix up and down 3–4 times with a P-1000 (barrier tips). Set a timer and allow the settled slurry to incubate for 40 minutes away from a magnet. Use a P-1000 to gently mix the beads every 10 minutes during the 40-minute incubation period. **Never vortex beads after conjugate addition**.
7. Gently pipette the settled slurry up and down one last time and immediately place the slurry on the magnet for 10 seconds before the beads have a chance to resettle.
8. The conjugate is now bound to the affinity matrix. With a P-200 pipette, carefully remove and discard the supernatant without disturbing the magnetized bead pellet.
9. Immediately add 500 µL Bead Wash Buffer to the bead pellet, remove the tube from the magnet, and pipette the slurry up and down with a P-1000 several times to wash. **Never vortex the beads**. Before the beads resettle, place them back on the magnet for 10 seconds. Remove and discard the supernatant without disturbing the pellet.

10. Repeat step 9 **three (3) additional times**; discarding the wash supernatant between washes.
11. Remove the tube from the magnet and add 100 µL Bead Elution Buffer directly to the bead pellet.
12. Using a P-1000, pipette the slurry up and down until the bead pellet adhered to the wall is rinsed to the bottom of the vial**. Never vortex the beads. Set P-1000 to 90 µL when mixing slurry.**
13. Incubate the settled slurry for 15 minutes away from the magnet. Use a P-1000 to gently mix the beads every 5 minutes during the 15-minutes incubation period.
14. Pipette the settled slurry up and down with the P-1000 one last time and immediately place the slurry on the magnet for 10 seconds before the beads have a chance to resettle
15. Without disturbing the pellet, carefully transfer the clarified supernatant (~100 µL) containing the eluted conjugate to a new, labeled 1.5 mL tube (not provided).
16. Repeat steps 11–17 **one (1) additional time**; pooling the two 100 µL conjugate fractions together in the same tube (200 µL final volume). **Proceed to Step C -Buffer exchange.**

C. Buffer Exchange into Storage Buffer

Prepare two spin columns (blue cap) by twisting off the bottom closure and loosening the cap (do not remove the cap). Place each spin column into a collection tube (provided) and mark the top of the blue caps with an indelible pen to identify the conjugate. Also place a vertical mark on the side of the spin column as shown on below.



1. Place the two spin columns in the centrifuge and orient the vertical mark on the spin column aiming outward and away from the center of the rotor.
2. Centrifuge at **1,500 x g for 1 minute**. Discard the flow through from each collection tube. Each column matrix will appear white in color. Place the columns back into new empty collection tubes (provided).
3. Open each blue cap; load 100 µL conjugate to the top of each dry resin bed; loosely cap and place them back into their empty collection tube.
4. Orient the spin column mark outward as before and centrifuge at **1,500 x g for 2 minutes.**

5. **Eluted conjugate is now in storage buffer at the bottom of the two collection tubes.** Pool the two 100 µL fractions containing antibody-oligo conjugate into a single 1.5 mL tube. **Label and store the tube at 4°C for up to 1 year.**

6. **Measure the protein concentration of the conjugate using a Bradford protein assayas described in the Appendix.**

Chapter 4: Appendix

A. Bradford Protein Assay

A Bradford or BCA Protein Assay is used to determine the final antibody-oligonucleotide conjugate concentration. A reference protocol is provided for each procedure.

Bradford 96-Well Procedure

Required Materials

Bradford Reagent (Bio-Rad, Cat.No. 500-0006)
96-well plate (polystyrene flat bottom)
PBS (phosphate buffered saline)
P-200 and P-1000 pipettes and sterile tips
Bovine IgG Antibody Standard: 2 mg/mL (Pierce/ThermoFisher, Cat. No. 23212)
Molecular grade water

Assay Protocol

1. Prepare 2 mL of Bradford working solution by adding 400 µL Bradford dye reagent to 1,600 µL molecular grade water (1:4 ratio).

Prepare IgG standards and a blank in 1.5 mL tubes as follows:

Add 100 µL 2 mg/mL bovine IgG standard to 300 µL PBS (0.5 mg/mL standard)
Add 200 µL 0.5 mg/ml standard to 200 µL PBS (0.25 mg/mL standard)
Add 200 µL 0.25 mg/mL standard to 200 µL PBS (0.125 mg/mL standard)
Add 200 µL 0.125 mg/mL standard to 200 µL PBS (0.0.0625 mg/mL standard)
100 µL PBS (buffer blank)

2. In a flat-bottom 96-well plate, prepare standards by pipetting 10 µL of each standard (and a blank) into 100 µLBradford working solution; mix. Replace pipette tips between additions.
3. In an adjacent well containing 100 µLBradford working solution, add 10 µL of the conjugate.
4. Incubate at **room temperature** (18–25°C) for 15 min (do not exceed 60 min).
5. Measure absorbance at 595 nm using pre-programmed Bradford assay software.
6. Data from a typical Bradford assay is provided as an illustration only in **Figure 6**.

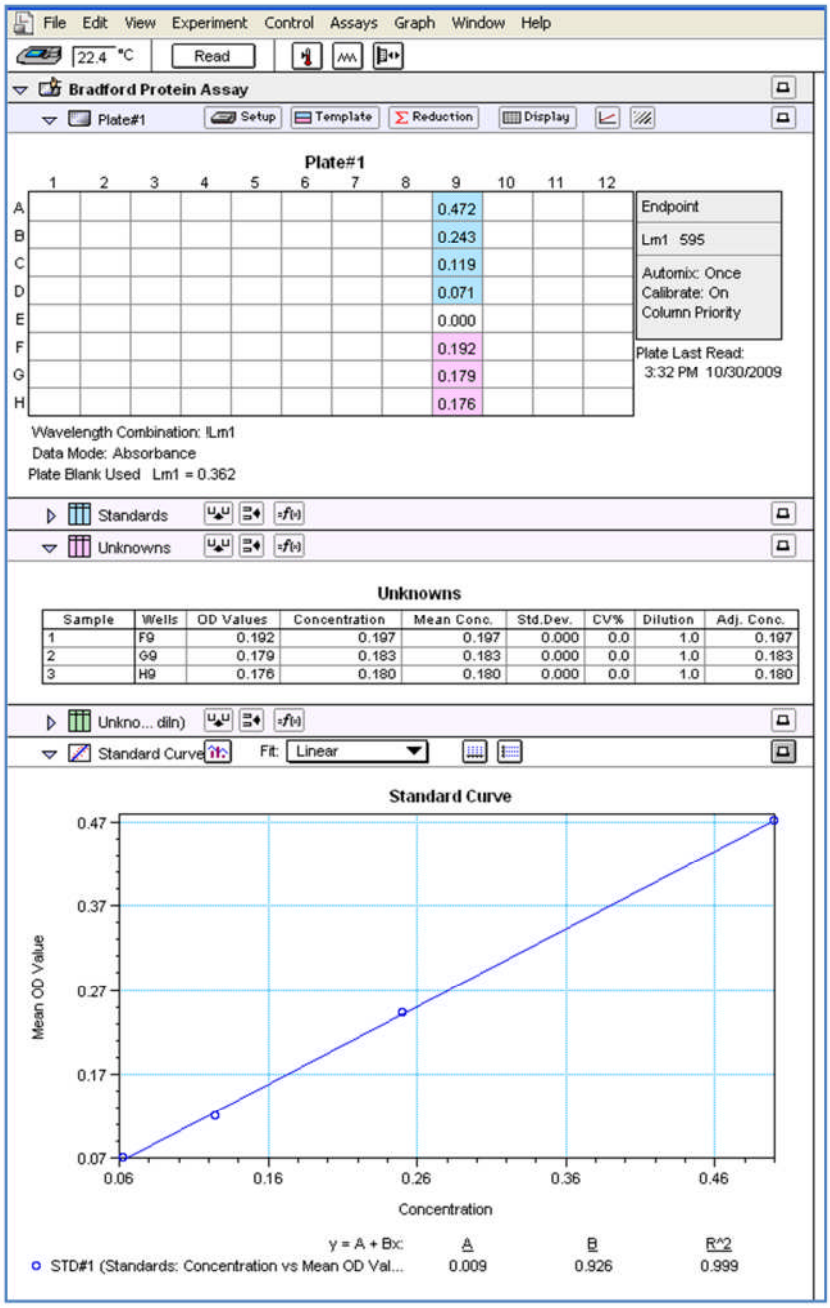
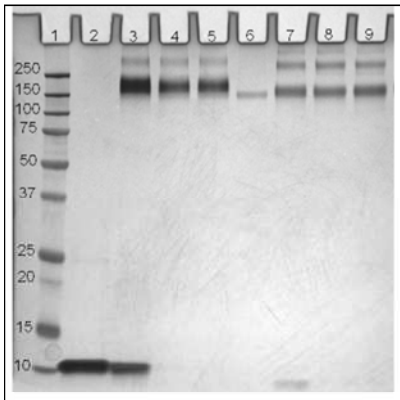


Figure 6. Bradford output from a commercial plate reader.

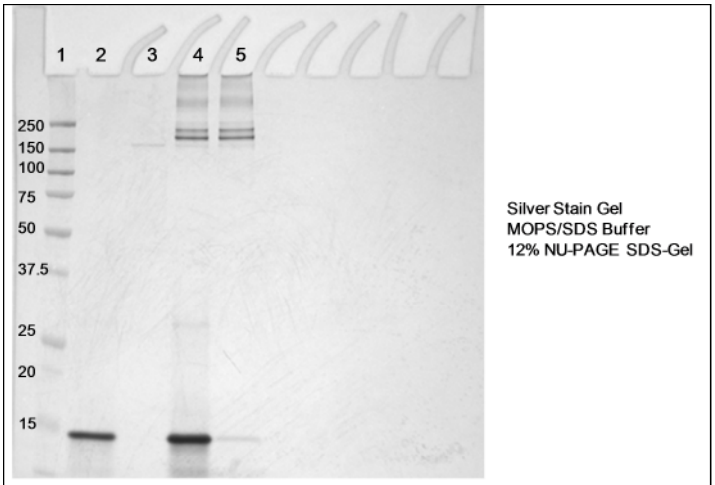
B. Antibody-Oligonucleotide Conjugates: Examples

Mouse mAb/oligonucleotide conjugates (44-mer and 22-mer)



1. Protein Molecular Weight Marker
2. 4FB-Oligonucleotide 44-mer
3. Crude mouse anti-FITC mAb/44-mer conjugation reaction 800 ng
4. Affinity purified mouse anti-FITC mAb/44-mer reaction 800 ng
5. Duplicate of lane 4
6. Mouse anti-FITC mAb 200 ng
7. Crude mouse anti-FITC mAb/22-mer conjugation reaction 800 ng
8. Affinity purified mouse anti-FITC mAb/22-mer reaction 800 ng
9. Duplicate of lane 8

Hamster mAb/oligonucleotide conjugate (60-mer)



1. Protein molecular weight marker
2. 60-mer 4FB-oligonucleotide standard
3. Hamster mAb anti-CD3 145-C211 standard (250 ng)
4. All-in-One™ crude conjugation reaction (60-mer/anti-CD3 mAb) 900 ng protein
5. Affinity purified anti-CD3-60-mer antibody-oligonucleotide conjugate 900 ng protein

C. Troubleshooting Guide

Problem	Possible Cause	Recommended Action
Poor or undetectable conjugate yield	•Amino-oligonucleotide may not be sufficiently 4FB-modified	•Verify 4FB MSR to ensure proper conjugation.
	•Quality and/or purity of starting oligois poor	•Concentrate 4FB-oligo into the required range (0.3–0.5OD_{260}/µL).
	•Low buffer exchange spin column recovery volume	•If antibody quality or source are undetermined, perform suitable test such as SDS-gel page analysis and/or a Bradford protein assay to confirm the purity and quantity of the starting material.
	•Low yield during affinity purification of conjugate	•Use a properly calibrated variable-speed centrifuge and follow recommended spin speed/time. Altered spin speeds will adversely compromise recovery. • Make sure to follow all the incubation times for binding and elution of conjugate.

Problem	Possible Cause	Recommended Action
Poor HyNic modification of antibody	•Presence of protein carriers such as BSA or gelatin may be contaminating antibody sample.	•Remove and purify the antibody sample of all protein carriers such as BSA or gelatin using affinity chromatography or other method before proceeding.
	•Concentration of S-HyNic modification reagent	•Make sure to thoroughly dissolve S-HyNic reagent before adding it to the antibody. •Use a calibrated pipette to ensure accuracy in small volume additions.
	•Presence of non-protein amine contaminants	•Remove all non-protein amine contaminants such as glycine or Tris before modifying the antibody with S-HyNic reagent.
	•Improper storage of S-HyNic reagent can lead to hydrolysis of the NHS ester	•Keep and store the S-HyNic reagent sealed in the pouch provided below 4°C.
	•Initial antibody concentration is low	•Confirm initial antibody concentration prior to S-HyNic modification on the spectrophotometer. If in doubt, perform a Bradford. • Dissolve the antibody sample carefully in the original product vial
Poor 4FB-modification of oligo	•Low MSR	•If using a NanoDrop spectrophotometer, follow the specific instructions