



HRP-Antibody All-In-One Conjugation Kit

Technical Manual

Catalog # A-9002-001

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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. User Manual

This manual provides instructions for using the HRP-Antibody All-in-One™ Conjugation Kit.

This chapter contains the following sections:

Purpose of Manual

Intended Users

Customer Service and Technical Support

B. Purpose of Manual

Each HRP-Antibody All-in-One™ Conjugation Kit provides all the necessary reagents and components to produce two (2) HRP-antibody conjugates.

Use of the kit (for each antibody) results in:

- The modification of a user-supplied antibody (100 µg) with S-HyNic.
- The conjugation of a HyNic-modified antibody with 4FB-HRP, resulting in the formation of an HRP-antibody conjugate.
- The isolation of highly purified HRP-antibody conjugate using a rapid spin filter (Q spin filter)

C. Intended Users

The HRP-Antibody All-in-One™ Kit is designed for users with minimal or no conjugation experience allowing them to prepare customized, high purity, ready-to-use HRP-antibody conjugates in a single day.

D. Customer Service and Technical Support

Additional technical information can be found at:

Telephone

1-888-625-0670 (Toll Free)

Fax

1-858-625-0770

Email

Solulink@Solulink.com

Address

Solulink-The Conjugation Company
9853 Pacific Heights Blvd, Ste H
San Diego, CA 92121

Chapter 2: Overview of Conjugation

A. Product Description

Each HRP-Antibody All-in-One™ Conjugation Kit provides all the necessary components to generate two (2) highly purified antibody-HRP conjugates. The kit requires the user to provide 100 µg of starting antibody for each conjugate. The components of this unique kit feature a pre-activated, high-activity horseradish peroxidase (>250U/mg) as well as a novel Q spin filter to purify the conjugate in high yield. Conjugates produced are free of both residual antibody and HRP, thus providing maximum signal to noise in your assay. Any suitably purified monoclonal or polyclonal mammalian antibody (regardless of IgG subclass) can be conjugated and purified within 5 hours (~1 h hands-on).

All-in-One™ conjugation kits are based on Solulink's proven HydraLink™ chemistry. This chemistry involves the reaction of an aromatic hydrazine with an aromatic aldehyde to form a stable hydrazone bond. HydraLink™ conjugation is so efficient that it converts 100% of the antibody to the conjugate form. This linking efficiency is made possible because of the recent discovery that small quantities of aniline catalyze hydrazone bond formation between the two functional groups (1, 2, 3). Aniline increases both the rate and efficiency of conjugate formation under mild reaction conditions; leading to quantitative conversion of free antibody to HRP conjugate.

Complete conversion of antibody to conjugate greatly simplifies downstream purification. Purification consists of selectively binding the conjugate to a novel Q spin filter membrane that allows excess HRP to flow through unbound. The spin filter provides high purity without sacrificing conjugate yield. Conjugates made with All-in-One™ kit are compatible with all downstream applications such as Westerns, ELISAs, or IHC. Each kit provides sufficient reagents to perform two (2) conjugation reactions; each yielding between 50-70 µg of high purity HRP-antibody conjugate.

B. All-in-One™ Technology

Conjugation Chemistry

HydraLink™ chemistry is based on the use of two complementary heterobifunctional linkers; **S-HyNic** and **Sulfo-S-4FB** (Figure 1). **S-HyNic** (Succinimidyl-6-hydrazino-nicotinamide) is first used to modify and incorporate protected aromatic hydrazines (HyNic groups) into the antibody via acylation of lysine residues. In a similar fashion a second linker, **Sulfo-S-4FB** (Sulfo-N-succinimidyl-4-formylbenzamide) is used by Solulink to provide a pre-activated high activity HRP called 4FB-HRP (included). Incubation of HyNic-modified antibody with pre-activated 4FB-HRP in the presence of aniline catalyst leads to rapid and efficient conversion of the antibody to conjugate through formation of stable bis-arylhydrazone bonds (Figure 2).

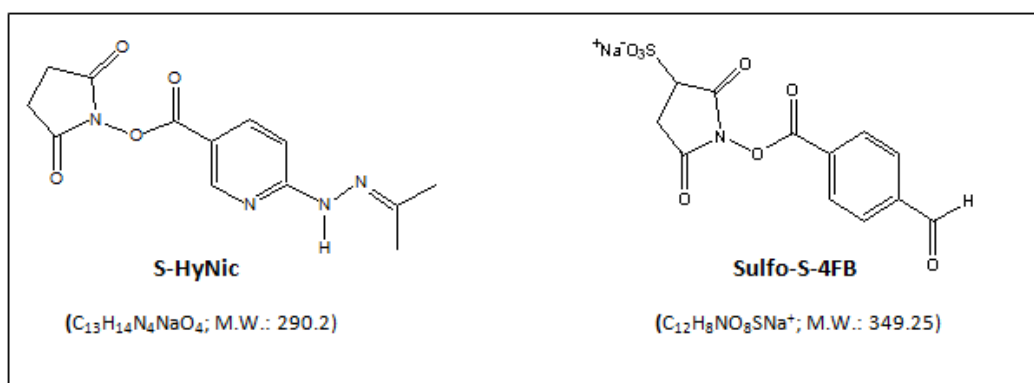


Figure 1. Structure of S-HyNic and Sulfo-S-4FB linkers used for conjugating HRP to antibody.

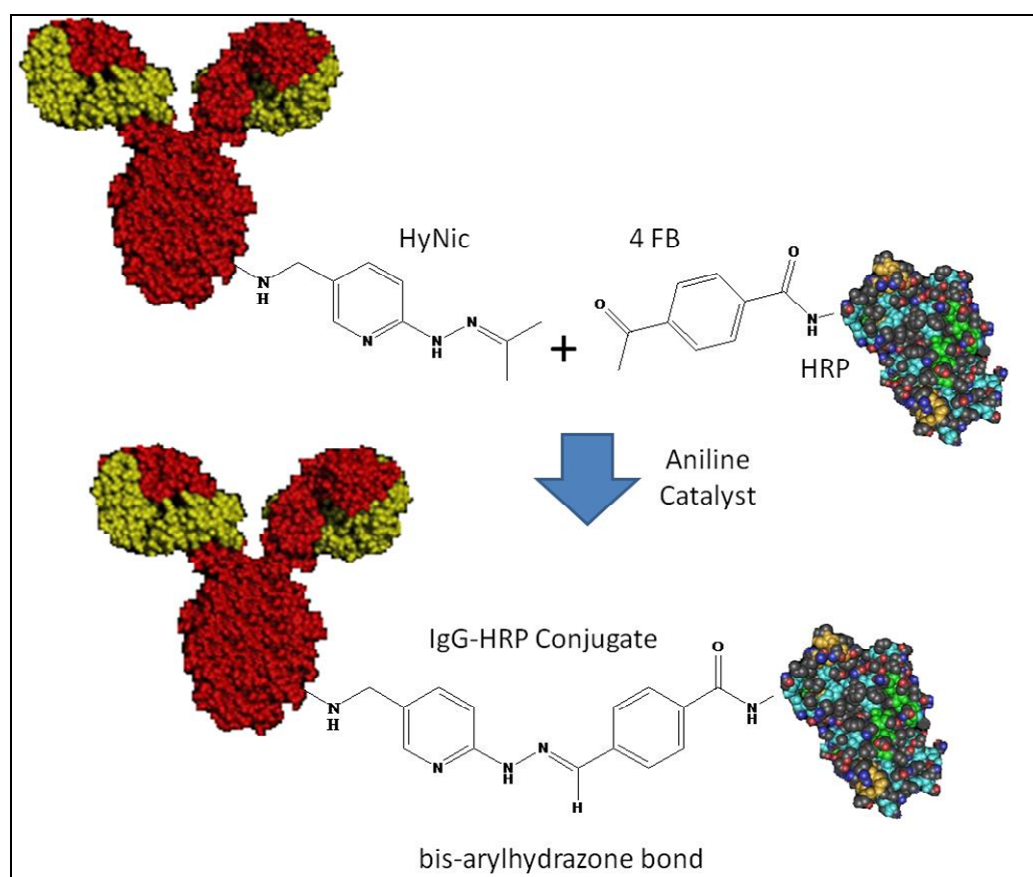


Figure 2. Aniline catalyzed conjugation of HyNic-modified antibody with pre-activated 4FB-HRP.

Conjugate Purification

The efficiency of aniline-catalyzed hydrazone bond formation greatly simplifies conjugate purification. Aniline's ability to increase both the rate and efficiency of conjugate formation under mild reaction conditions leads to quantitative conversion of free antibody to conjugate. The complete absence of free antibody at the end of the catalyzed reaction leaves only two components; excess HRP and conjugate. After conjugation, a novel Q spin filter is used to selectively bind the conjugate based on known biophysical properties of IgG (4, 5) while allowing free HRP to flow through. Purified conjugate can then be eluted from the filter membrane free of both residual antibody and HRP in high yield (50-70 µg).

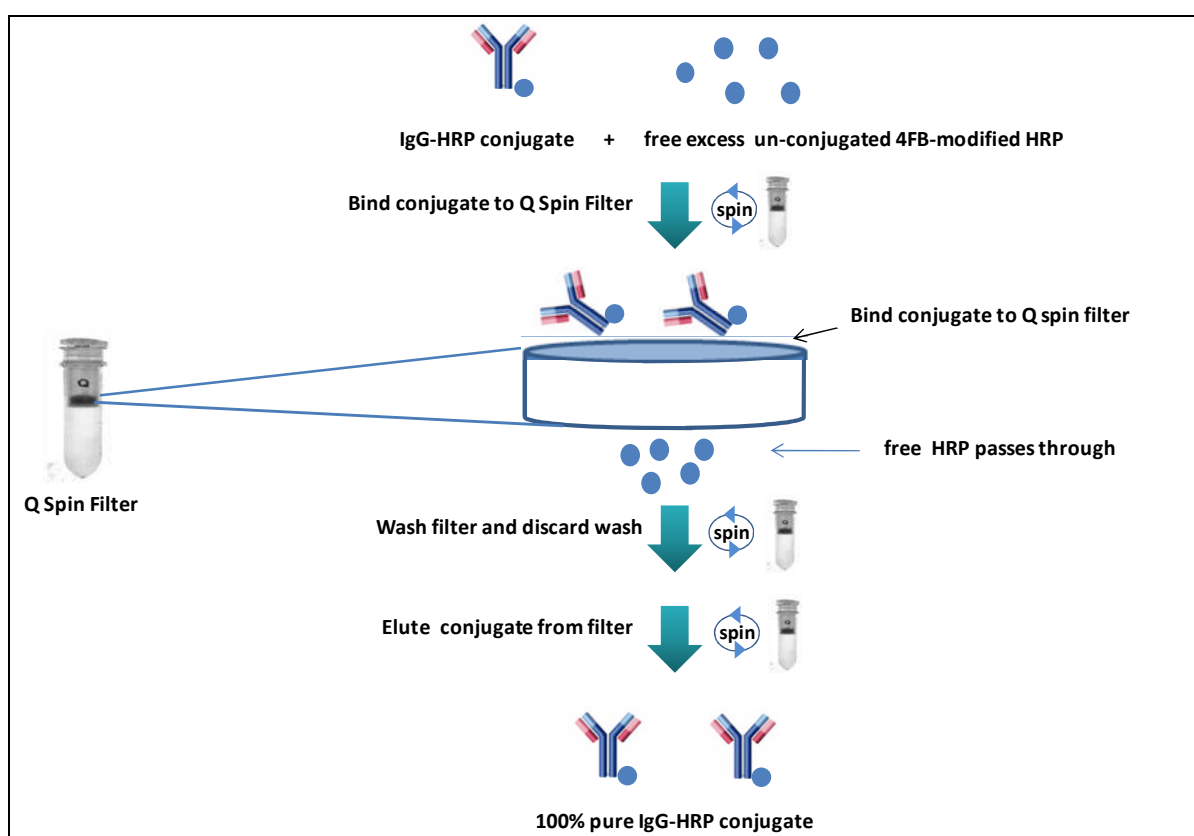


Figure 3. Q spin filter purification of HRP-IgG conjugate.

C. Il-in-One™ Conjugation Process Summary

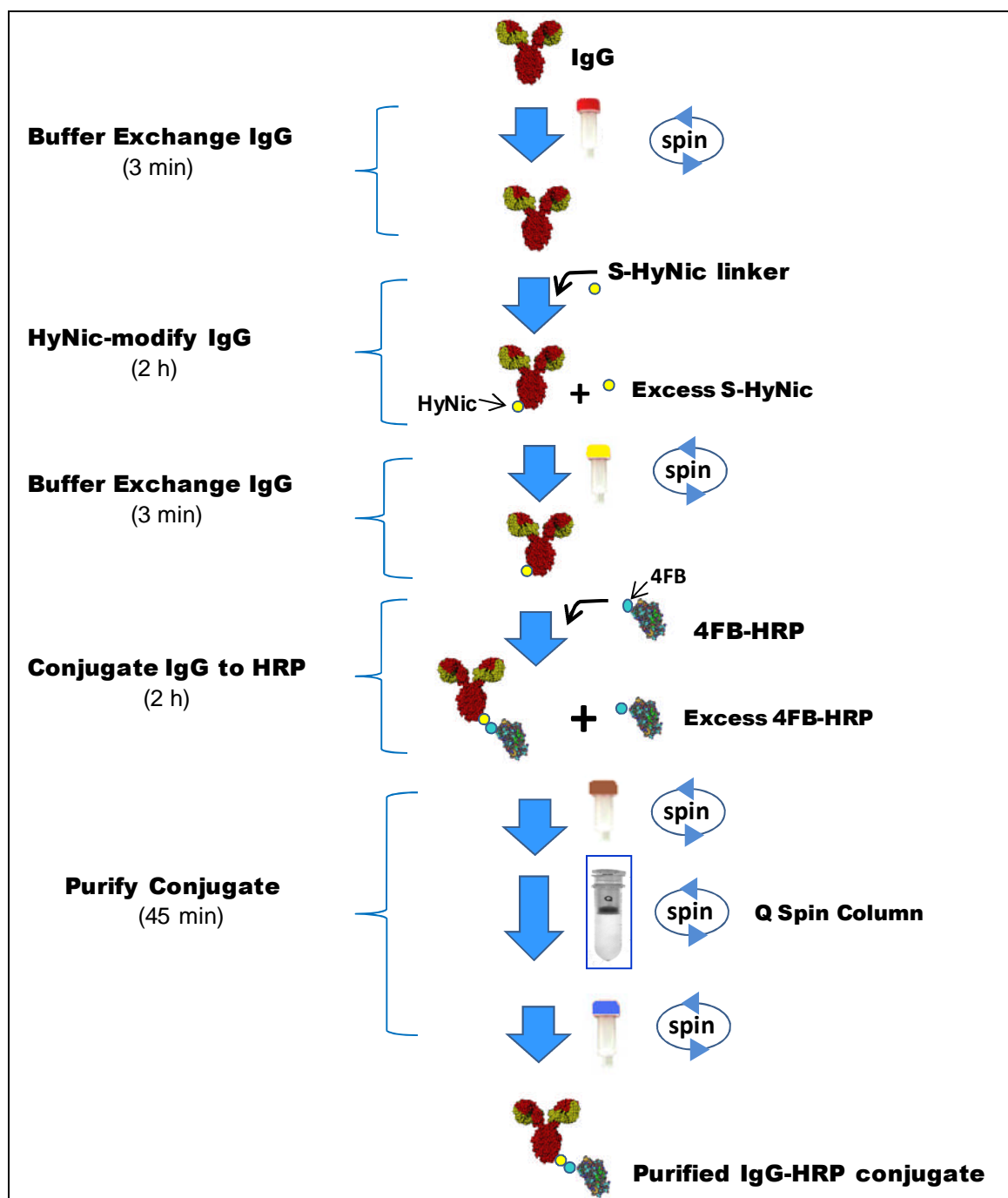


Figure 4. HRP-Antibody All-in-One™ conjugation process.

D. Materials Provided and Storage Conditions

Components	Amount	Storage conditions
S-HyNic	2 x 100 µg	Keep refrigerated within desiccated sealed aluminum pouch
4FB-Modified HRP	2 x 50 µl	Keep refrigerated (2-8°C)
Buffer A	5 mL	Keep refrigerated (2-8°C)
Buffer B	5 mL	Keep refrigerated (2-8°C)
Buffer C	0.5 mL	Keep refrigerated (2-8°C)
Spin Column (Red cap)	2	Keep refrigerated (2-8°C)
Spin Column (Yellow cap)	2	Keep refrigerated (2-8°C)
Spin Column (Brown cap)	2	Keep refrigerated (2-8°C)
Spin Column (Blue cap)	2	Keep refrigerated (2-8°C)
Q Spin Filters	2	Room temperature or refrigerated (2-8°C)
Q Collection tubes	4	Room temperature or refrigerated (2-8°C)
DMF	0.5 mL	Room temperature or refrigerated (2-8°C)
Diafiltration Spin Filters (30k)	2	Room temperature or refrigerated (2-8°C)
Collection Tubes	16	Room temperature or refrigerated (2-8°C)

E. Additional Materials Required But Not Provided

Bradford Protein Assay Reagents (verification of initial IgG concentration)
Conventional UV-VIS or NanoDrop™ Spectrophotometer (optional)
Semi-micro quartz cuvette (50-100 µL, 1-cm path length) (For conventional UV-VIS Spectrophotometer)
Calibrated pipettes (P-2 or P-10, P-100, P-1000) and tips
Variable speed centrifuge (e.g. Eppendorf or MicroMax)
1.5 ml microfuge tubes

Chapter 3: All-in-One™ Conjugation Protocol

Note - Before using the kit, remove from refrigerated storage and allow components to warm up to ambient or room temperature for at least 30 minutes.

A. IgG Sample Preparation (10 minutes)

Antibodies come in two physical forms, solids or liquids. Individual samples can vary significantly in the amount of packaged IgG (protein mass) and/or concentration (mg/ml). We highly recommend that IgG concentrations be confirmed either by Bradford protein assay or A280 whenever possible. The All-in-One™ conjugation protocol requires antibody samples to be free of protein carriers such as BSA or gelatin before proceeding. A 100 µg mass of antibody is required at the start of the procedure. Depending on the initial form of your sample (solid or liquid), proceed as follows:

Antibody is in Solid Form (e.g. lyophilized powder)

Resuspend lyophilized antibody (100 µg free of protein additives gelatin or BSA) in 25 µl **Buffer A** to obtain a 4 mg/mL solution. If the antibody sample contains less than 100 µg per vial (e.g. 50 µg), resuspend the requisite number of vials equivalent to a 100 µg in 25 µl **Buffer A** to obtain a 4 mg/mL solution.

Proceed to step B below.

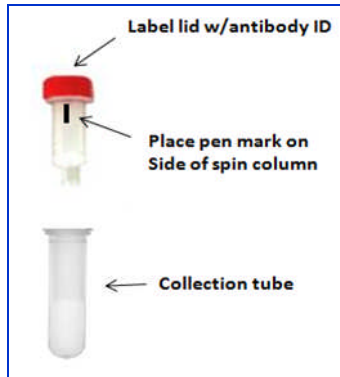
Antibody is in Liquid Form (e.g. PBS or TBS Buffer)

If the antibody sample is in liquid form at 4 mg/ml, simply transfer 25 µl to a labeled microfuge tube. If the sample is in liquid form at a concentration greater than 4 mg/ml, transfer a volume equivalent to 100 µg antibody to a labeled microfuge tube and add **Buffer A** to obtain 4 mg/ml solution. If a sample is at a concentration less than 4 mg/ml, concentrate the sample to 25 µL and 4 mg/mL using a diafiltration spin filter (e.g. Amicon or VivaSpin 500) as described in the Appendix. A concentration filter is provided in the kit for this purpose (if necessary).

Proceed to step B below.

B. Buffer Exchange IgG (3 minutes)

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 sample and place a vertical mark on the side of each spin column as shown on the next page.



3. Place the assembly into the centrifuge and orient the vertical mark on the spin column aiming outward and away from the center of the rotor.
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. Remove the red cap; load the antibody sample (25 μ L at 4 mg/mL) to the top of the dry resin bed; loosely recap 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**. Use an appropriate balance tube opposite the assembly.

Note - Rotor speed must be set to 1500 x g (RCF) and not 1500 x rpm (RPM). The volume recovered should always be approximately the same volume loaded onto the spin column (e.g. 25 \pm 5 μ L). If the recovered volume is low, the centrifuge may require recalibration. If recovered volume is low; re-centrifuge at the appropriate speed in an attempt to recover the full volume (i.e. 25 μ L).

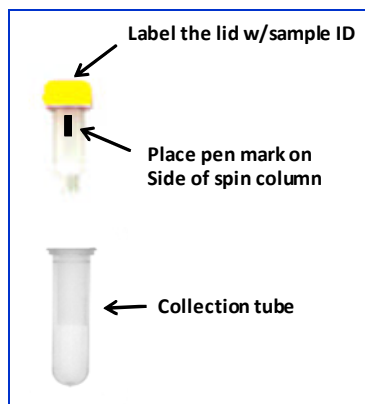
7. Transfer the buffer exchanged IgG solution (25-30 μ L) from the bottom of the collection tube to a new 1.5 mL tube and label appropriately.

C. HyNic Modify IgG (2 hours)

1. Add 20 μ L DMF to the vial of S-HyNic reagent. Pipette the solution up and down to resuspend the reagent pellet. **Note**- a small but visible pellet can be seen at the bottom of the vial.
2. Add 1.5 μ L dissolved S-HyNic reagent to the antibody solution (25 μ L @ \sim 4 mg/mL). Pipette the solution up and down to mix.
3. Incubate the reaction for 2 h at room temperature.

D. Buffer Exchange IgG (3 minutes)

1. Five minutes before the end of the HyNic modification reaction (section c step 3), prepare a spin column (yellow cap) by twisting off the bottom closure and loosening the yellow cap (do not remove). Place the spin column into a collection tube (provided).
2. Mark the top of the yellow cap using an indelible pen to identify the sample. Also place a vertical mark on the side of each spin column as shown on the next page.



3. Place the assembly 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 assembly.

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 yellow cap; load the now completed antibody/HyNic modification reaction (~ 25-35 μ L) 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 and use an appropriate balance tube opposite the spin filter. Centrifuge at **1,500 x g for 2 minutes**.
7. After centrifugation, transfer the solution (~ 25-35 μ L) from the bottom of the collection tube to a 1.5 mL tube. Label the sample appropriately (e.g. HyNic-IgG).

E. Conjugate Formation (2 hours)

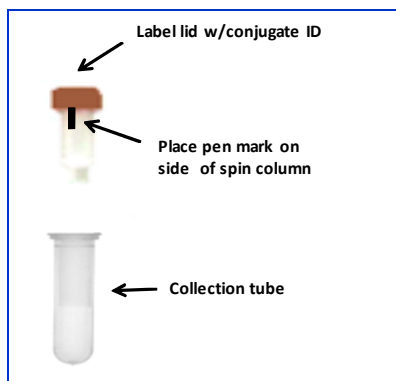
1. Briefly spin the dark brown vial containing 4FB-modified HRP (**5 seconds @ 1000 x g**) to collect the contents at the bottom of the tube.
2. Transfer 50 μ L 4FB-modified HRP to the tube containing HyNic-modified antibody (25-35 μ L); pipette up and down to mix.
3. Incubate the reaction for 2 h at room temperature to form the conjugate.

F. Buffer Exchange Conjugate (3 minutes)

1. After completion of the conjugation reaction, prepare a spin column (brown cap) by twisting off the bottom closure and loosening the brown cap (do not remove). Place the spin column into a collection tube (provided).

Remember- equilibrate all kit components to ambient room temperature before use.

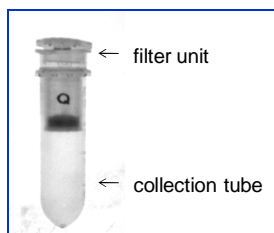
2. Mark the top of the brown cap using an indelible pen to identify the sample. Also place a vertical mark on the side of each spin column as shown below.



3. Place the assembly 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 filter
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 brown cap; load ~75-90 μL conjugate solution (section e, step 3) 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 and centrifuge at **1,500 x g for 2 minutes**.
7. After centrifugation, add 350 μL **Buffer B** to the conjugate solution located at the bottom of the collection tube and pipette up and down to mix. Set aside on the bench for the time being.

G. Q Spin Column Purification (40 min)

1. Pre-wet a Q Spin Filter (refer to next page) by adding 200 μL **Buffer B** to the top of the filter unit and incubate for 2 minutes.



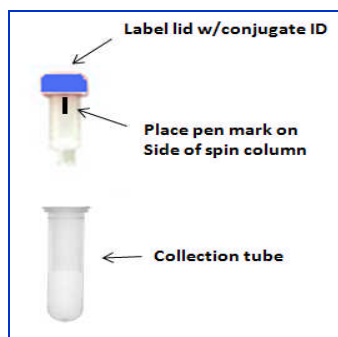
2. Place the filter assembly into the centrifuge and orient the letter Q towards the center of the rotor; spin at **2,000 x g for 4 minutes** using an appropriate balance tube; discard the flow-through from collection tube and place the filter back into the empty collection tube.
3. Load the antibody-HRP conjugate (~430-450 µL) from the previous section (section f, step 7) to the top of the filter unit and allow it to incubate inside the filter for 2 minutes on the bench top.
4. Place the oriented assembly in the centrifuge and spin at **2,000 x g for 4 minutes** using an appropriate balance tube opposite the filter unit; discard the flow-through from the bottom collection tube and place the filter back into the empty collection tube.

Note- a light brown coloring will appear on the top
of the Q filter membrane (bound conjugate).

5. Add 400 µL **Buffer B** to the filter unit; orient and balance in the centrifuge and spin at **2,000 x g for 4 minutes**; discard the flow-through from the bottom collection tube and place the filter back into the empty collection tube.
6. Repeat step 5 **two (2) additional** times to complete the washing procedure.
7. Remove the top filter unit from the collection tube and place it into a new collection tube (provided)
8. Add 100 µL **Buffer C** to the top of the brown-colored filter membrane and incubate for 5 minutes on the bench top.
9. Place the oriented assembly in the centrifuge and balance, spin at **2,000 x g for 4 minutes**; add an additional 50 µL **Buffer C** to the top of the filter membrane and spin the oriented assembly **for another 4 minutes at 2,000 x g**. A slightly brown-colored conjugate solution (150 µL) will now be visible at the bottom of the collection tube. Set the collection tube aside on the bench.

H. Buffer Exchange Conjugate (3 minutes)

1. Prepare a spin column (blue cap) by twisting off the bottom closure and loosening the blue cap (do not remove). Place the spin column into a collection tube (provided).
2. Mark the top of the blue cap using an indelible pen to identify the sample. Place a vertical mark on the side of each spin column as shown on the next page.



3. Place the assembly 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 filter
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. Remove the blue cap; load 150 μ L conjugate from the bottom of the Q spin filter collection tube (section g, step 9) to the top of the dry resin bed; loosely recap 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. After centrifugation, transfer the buffer exchanged conjugate (150 μ L) from the bottom of the collection tube to a new 1.5 mL tube and label appropriately.

8. Measure the final protein concentration of the purified conjugate using either a Bradford or BCA protein assay (see Appendix).

Chapter 4: Appendix

A. Polyclonal & Monoclonal IgG-HRP Conjugates: Some Examples

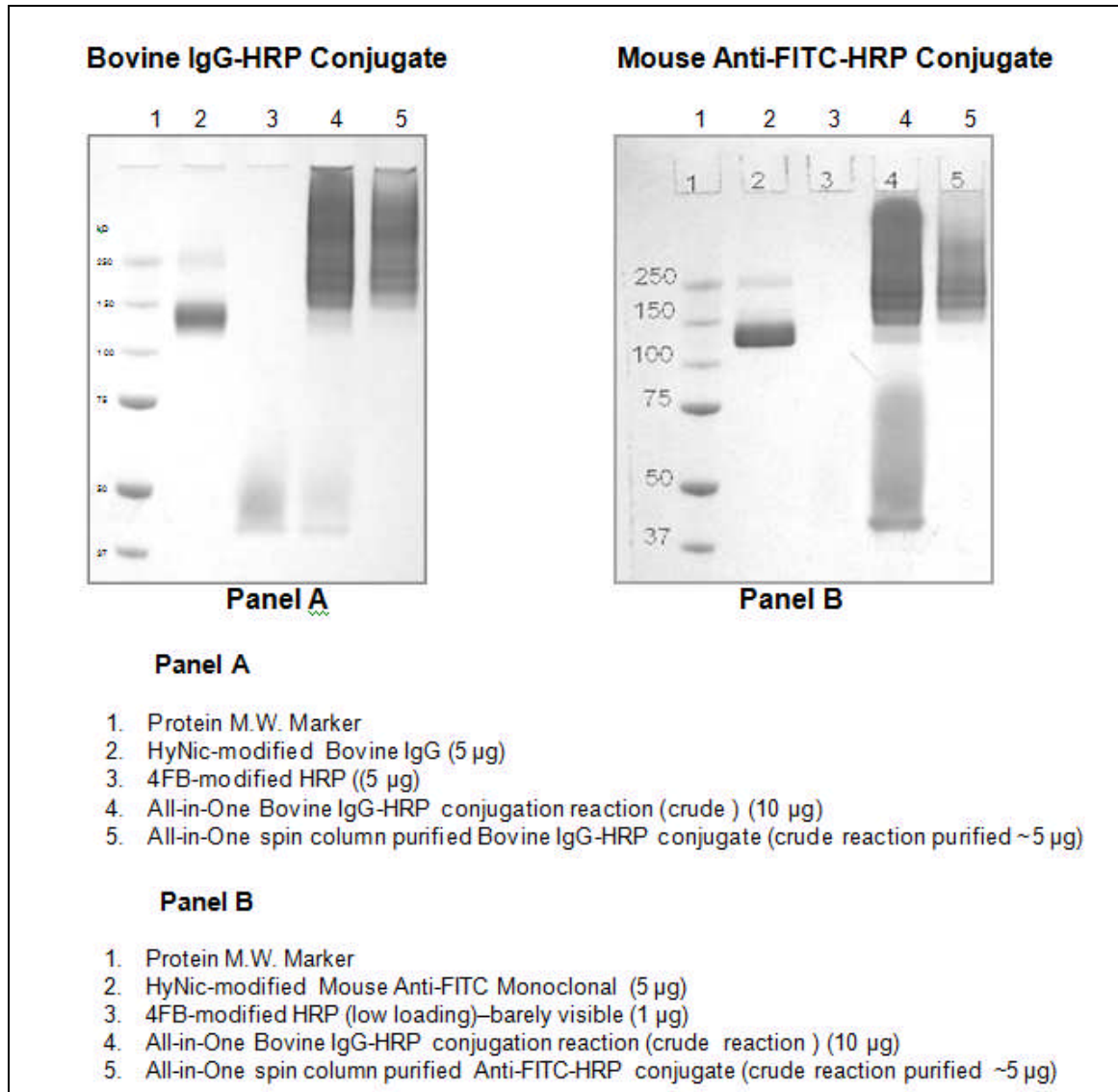


Figure 5. Coomassie-stained (4-12% SDS-PAGE) gels illustrating typical conjugation results.

Note- horseradish peroxidase is a 44 kD highly glycosylated protein that migrates as a broad band when the protein sample is not heated (70°C) before loading on an SDS-PAGE gel.

B. Direct ELISA Assay Using an IgG-HRP All-in-One Conjugate

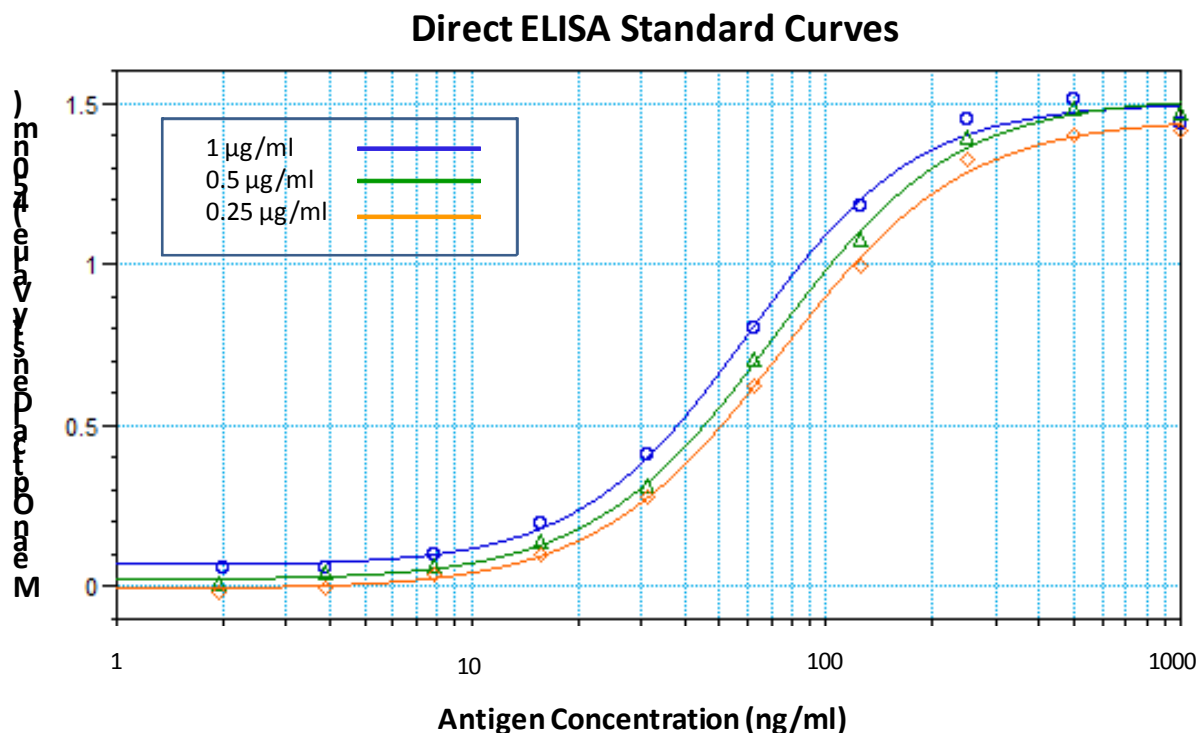


Figure 6. Direct *ELISA* curves generated using an HRP conjugate made with the All-in-One kit. A mouse anti-FITC monoclonal antibody was conjugated to HRP as described in the manual. Antigen consisting of FITC-labeled BSA (FITC MSR = 2) was coated on plates in a 2-fold dilution series (100 µl @ 500, 250, 125, 62.5, 31.25, 15.625, 7.8, 3.90, and 1.95 ng/ml) using standard methods. Immobilized antigen was then detected at 3 different conjugate concentrations (1 µg/ml, 0.5 µg/ml, 0.25 µg/ml) using TMB substrate (20 minutes @ 450 nm) on a Molecular Devices plate reader.

C. Bradford Protein Assay

Solulink highly recommends that whenever IgG is not limiting or its concentration, source, or quality are unknown that the sample be assayed for initial protein concentration using a Bradford protein assay prior to conjugation. The starting quality and quantity of antibody is critical to the success of the procedure. A reference assay protocol is provided for measuring antibody or conjugate protein concentrations using Bradford protein reagents (not provided in the kit).

Bradford Microtiter Plate Procedure

Required Materials

Bradford Reagent (Bio-Rad, Hercules, CA, Cat. #500-0006)

96-well microtiter plate (standard flat bottom)

PBS (phosphate buffered saline)

P-200 and P-1000 pipettes

Bovine IgG Antibody Standard: 2 mg/ml (Pierce/ThermoFisher, Cat. # #23212)

Molecular grade water

Assay Protocol

- 1) Prepare 2 ml of a Bradford working solution by adding 400 μ l dye reagent to 1600 μ l molecular grade water (1:4 ratio).

- 2) Prepare the following protein dilution standards and blank as follows:

Add 160 μ l 2 mg/ml bovine IgG standard to 240 μ l PBS (0.8 mg/ml standard)
Add 150 μ l 0.8 mg/ml standard to 50 μ l PBS (0.6 mg/ml standard)
Add 75 μ l 0.6 mg/ml standard to 25 μ l PBS (0.4 mg/ml standard)
Add 50 μ l 0.4 mg/ml standard to 50 μ l PBS (0.2 mg/ml standard)
Add 50 μ l 0.2 mg/ml standard to 50 μ l PBS (0.1 mg/ml standard)
Add 50 μ l PBS (buffer blank)

- 3) Pipette 5 μ l of each standard (and blank) along with duplicates of antibody sample into separate microtiter wells.

- 4) Add 100 μ l of previously diluted dye reagent (1:4) to each well and mix thoroughly.
Always replace pipette tips between additions.
- 5) Incubate at room temperature for 5-10 minutes (but no more than 60 minutes).
- 6) Measure absorbance at 595 nm on a suitable microtiter plate reader.
- 7) A typical Bradford microtiter assay result from a commercial plate reader is illustrated below in Figure 7.

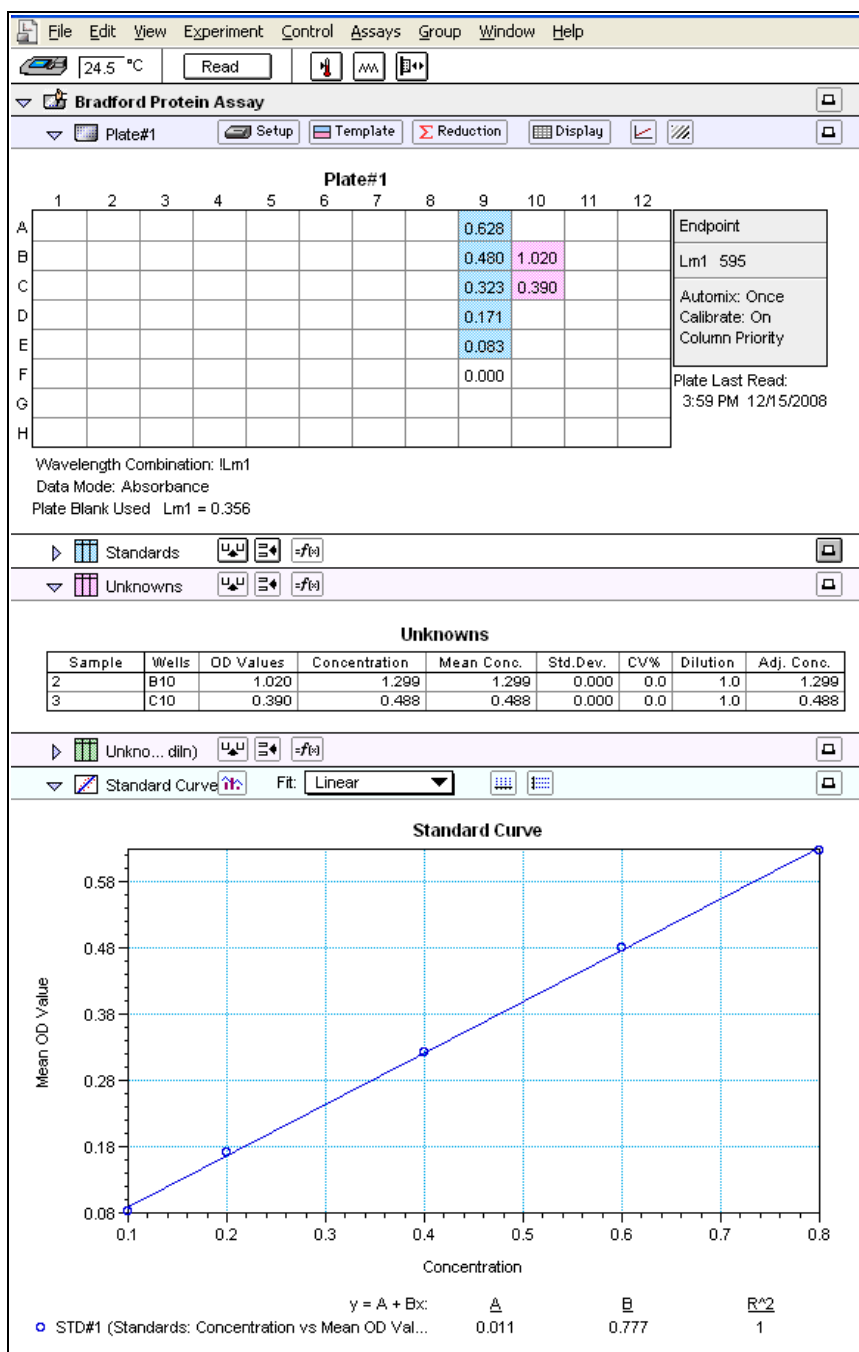


Figure 7. Print out from a Bradford microplate-based protein assay.

D. Using a NanoDrop™ to Measure Antibody Concentration

If an antibody sample is free of protein-based carriers (e.g. BSA, gelatin) or certain interfering preservatives such as thimerosal, then a simple non-destructive scan of the IgG sample on a NanoDrop™ spectrophotometer can be used to estimate antibody concentration saving the trouble of conducting a Bradford protein assay to confirm concentration. To estimate antibody concentration using a NanoDrop™ spectro-photometer, proceed as follows.

1. Turn on the NanoDrop™ spectrophotometer and click on the NanoDrop™ icon to launch the software.
2. Place a 2 µl drop of molecular grade water on the clean pedestal, click OK.
3. When the main menu appears, select the A₂₈₀ menu option. **Note-** do not use the UV-VIS menu option on the NanoDrop™ to read an antibody sample.
4. After the A₂₈₀ menu appears, **click-off the 340 nm normalization option** using the mouse. **Note-**some instruments do not have this normalization feature in which case this step can be ignored.
5. In the window labeled Sample Type, select 'Other protein E1%' option from the pull-down menu. Enter the appropriate E1% value (**Table 1 on the next page**) corresponding to your particular antibody sample type. For example, 14.00 for mouse IgG.
6. Blank the NanoDrop™ spectrophotometer by placing a 2 µl drop of the appropriate sample buffer (e.g. PBS) and click on the 'Blank' icon.
7. Immediately re-click the 'Measure' icon to validate the baseline (i.e. flat across the bandwidth). Clean the pedestal and repeat (if necessary) until a flat baseline is obtained. **Note-**sometimes air bubbles can become trapped on the pedestal during sample loading and cause baseline offsets. If necessary, remove air bubbles and rescan to insure a proper baseline.

8. Place a 2 μ l volume of antibody solution on the clean pedestal and click the 'Measure' icon. Wait until the spectrum (220-350 nm) appears in the window. **Note**-for precious or limited samples the majority of the 2 μ l aliquot can be recovered from the pedestal.
9. Record the antibody concentration directly from the NanoDrop™ display window [mg/ml]. Alternately, calculate the antibody concentration (manually) as illustrated on the following page.

Example: A mouse IgG sample at 1 mg/ml in PBS (100 μ l) was scanned as described and its concentration confirmed using equation #1 below.

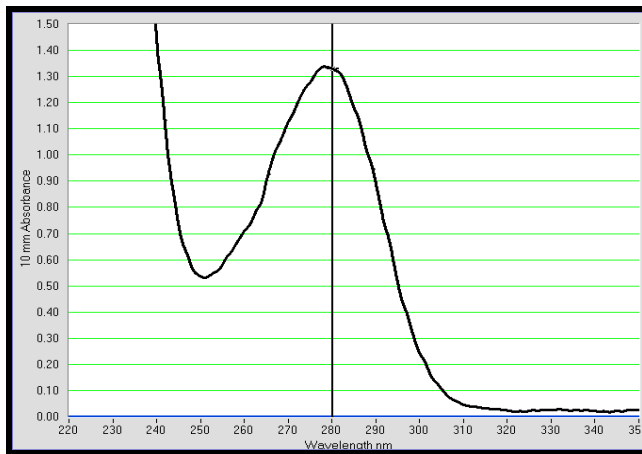


Figure 8. A mouse IgG sample 100 μ l @ 1 mg/ml in PBS pH 7.2, scanned on the NanoDrop™ as described in the text.

Equation #1: $[A_{280} / E1\% \text{ value}] \times 10 \text{ mg/ml} = \text{protein concentration (mg/ml)}$

E1% (mass extinction coefficient, from Table 1)

Example: Mouse IgG @ 1 mg/ml (Fig. 8)

A_{280} reading (from scan in Figure 8) = 1.34

Antibody E1% value (Table 1) = 14.00

$[A_{280} / E1\% \text{ bovine IgG}] \times 10 \text{ mg/ml} = \text{protein concentration (mg/ml)}$

$$[1.34 / 14.00] \times 10 \text{ mg/ml} = 0.96 \text{ mg/ml}$$

Antibody Source	Antibody E1% (1-cm path)
Human IgG	13.60
Human IgE	15.30
Rabbit IgG	13.50
Donkey IgG	15.00
Horse IgG	15.00
Mouse IgG	14.00
Rat IgG	14.00
Bovine IgG	12.40
Goat IgG	13.60

Table 1. Mass extinction coefficients (E1%) used for calculating antibody concentrations. The E1% is the A_{280} of a 10 mg/ml solution in a 1-cm path.

E. HRP Absorption Spectrum (Unmodified Horseradish peroxidase)

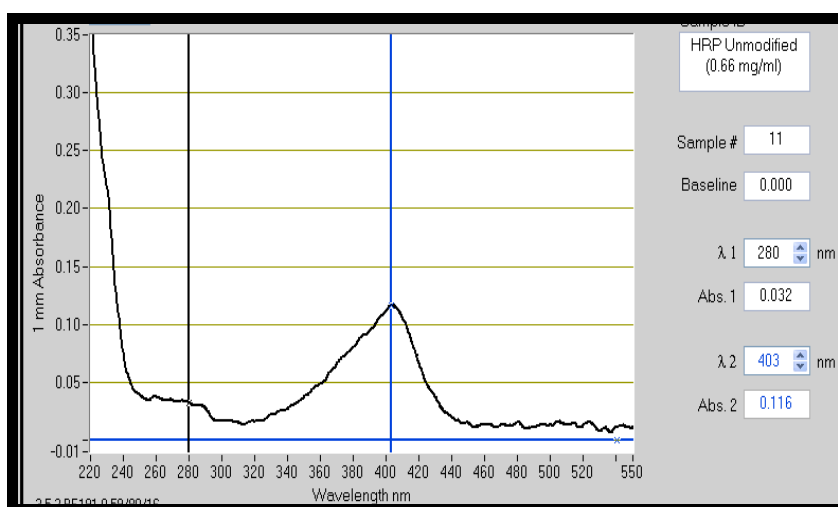


Figure 9. NanoDrop™ absorption spectrum of unmodified horseradish peroxidase (220-550 nm) @ 0.66 mg/ml (sodium phosphate buffer, pH 6.0, 1 mm path length)

F. 4FB-modified HRP Absorption Spectrum

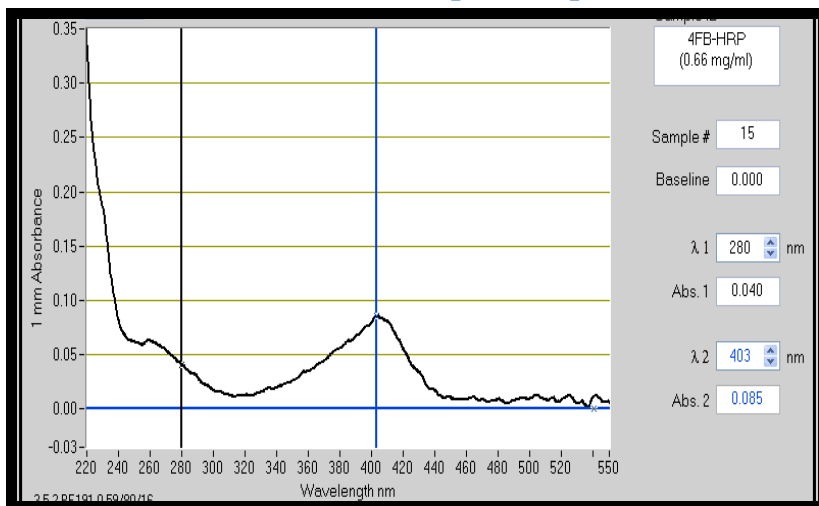


Figure 10. NanoDrop™ absorption spectrum of 4FB-modified horseradish peroxidase (220-550 nm) @ 0.66 mg/ml (sodium phosphate buffer, pH 6.0, 1 mm path length)

G. Bovine IgG-HRP Conjugate Absorption Spectrum (All-in-One Purified)

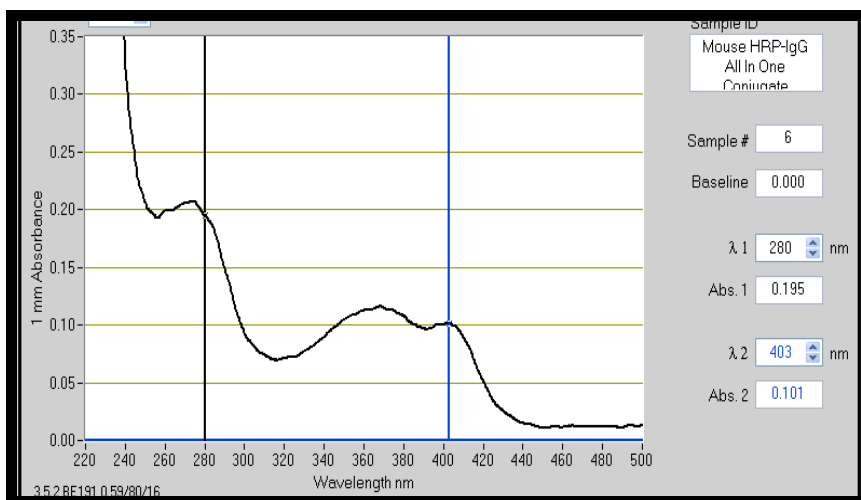


Figure 11. NanoDrop™ absorption spectrum of All-in-One IgG-HRP conjugate (220-550 nm) @ 0.96 mg/ml (sodium phosphate buffer, pH 6.0, 1 mm path length).

H. Concentration of Dilute Antibody Solutions

The HRP-Antibody All-in-One Conjugation protocol requires that initial antibody protein concentration be at 4-5 mg/ml and 25 μ l. Many antibody vendors package their products at significantly more dilute concentrations (e.g. 0.25 to 1.5 mg/ml). In these

instances, IgG samples will need to be concentrated to 4-5 mg/ml and 25 μ l before proceeding. The All-in-One kit provides two (2) diafiltration filters (M.W.C.O. 30 kD) for this purpose (**Figure 12**). Carefully follow these instructions to avoid antibody loss or aggregation on the filter's surface.

Note-Dilute antibody solutions require at least 125 μ g of starting antibody (e.g. 500 μ l @ 0.25 mg/ml) since diafiltration filters recover ~80% of input antibody. If antibody samples are not in limiting supply, we recommend antibody concentrations be confirmed using a Bradford protein assay before proceeding.

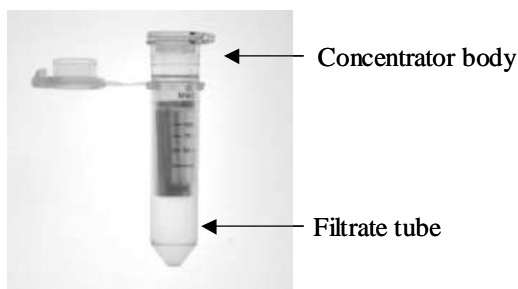


Figure 12. Diafiltration spin filter used for concentrating dilute antibody samples prior to the start of All-in-One conjugation protocol.

Antibody Concentration Protocol

Note - the diafiltration spin filters illustrated is made to contain and process a maximum volume of 500 μ l or less. If a volume greater than 500 μ l is to be concentrated, multiple loadings will be required.

- 1) Open the lid of a diafiltration spin filter device.
- 2) Transfer 500 μ l (or less) of dilute protein solution (equivalent to 125 μ g antibody) to the center of the filter cup.
- 3) Close the lid and orient the spin filter in the centrifuge so that the volume markers face toward the center of the centrifuge rotor. Use an appropriate balance tube opposite the spin filter.
- 4) Centrifuge for 2 minutes @ 5,000 x g.

- 5) Open the filter unit and visually inspect the remaining volume. If the volume remaining in the concentrator body is greater than 25 μ l, gently pipette the solution up and down to mix; taking care not to touch or puncture the filter surface during this step.
- 6) Repeat steps 4 and 5 until the volume in the filter cup reaches the 25 μ l mark. Once the final volume reaches 25 μ l, do not pipette up and down to avoid sample loss.
- 7) Carefully transfer the concentrated IgG solution (25 μ l) to a new 1.5 ml microfuge tube and proceed with the rest of the procedure in Chapter III, section B.

I. Troubleshooting Guide

Problem	Possible Cause	Recommended Action
Poor conjugate yield	-initial antibody concentration and volume were incorrect or unknown.	-whenever possible verify the original starting antibody concentration using a Bradford protein assay or NanoDrop™ to assure efficient conjugation. -concentrate or dilute the antibody sample to be conjugated into the required range (4-5 mg/ml and 25 µl)
Poor conjugate yield	Starting antibody concentration and volume are incorrect or unknown.	-preservatives can interfere with the accuracy of a Bradford protein assay. Remove all interfering preservatives such as thimerosal or proclin before performing a Bradford protein assay.
Poor HyNic modification	-presence of protein carrier (e.g. BSA or gelatin) is contaminating the antibody sample.	-remove and purify away all protein carriers such as BSA or gelatin using affinity chromatography or other methods
Poor HyNic modification	-improper mixing of HyNic reaction components -presences of amine contaminants -improper storage of S-HyNic reagent can lead to hydrolysis of this NHS ester	-make sure to properly mix the antibody- HyNic reaction mixture -use a calibrated P-10 pipette to insure accuracy of small volumes -remove all non-protein amine contaminants such as glycine or Tris before modification -keep and store S-HyNic sealed in the aluminum pouch provided that

	-initial antibody concentration was too low or too high.	contains dessicant. measure the initial antibody concentration before proceeding (Bradford or NanoDrop) -concentrate or dilute the antibody sample into the recommend range (4-5 mg/ml and 25 µl) before proceeding
Low conjugate and/or antibody recovery	-low spin column recovery volume	-use a properly calibrated variable-speed centrifuge Follow recommended spin speed/time. Altered spin speeds can adversely compromise protein and/or volume recovery

J. Component Stability on Storage

Component	Stability	Storage Condition
Unopened Kit	1 yr	Refrigerated (2-8°C)
S-HyNic	1 yr 24 h after re-suspending S-HyNic in DMF	Keep in sealed aluminum pouch provided (2-8°C). Room temperature
HRP-Antibody Conjugate	9 month 1 yr	Refrigerated (2-8°C) in final conjugate solution. Refrigerated (2-8°C) (50% glycerol)
All other kit components	1 yr	Refrigerated (2-8°C)

K. References

1. **Dirksen, A., Hackeng, T., Dawson, P., (2007).** *Nucleophilic Catalysis of Oxime and Hydrazone Reactions by Aniline. ACS Poster*
2. **Dirksen, A., Hackeng, T., Dawson, P., (2006).** *Nucleophilic Catalysis of Oxime Ligations. Angew. Chem. Int. Ed. 45, 7581-7584*
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4. **Lim, S., Manus, H.P., Gooley, A.A., Williams, K. L., Rylatt D.B., (1998).** *Purification of monoclonal antibodies from ascitic fluid using preparative electrophoresis. Journal of Chromatography A. Vol. 827, Issue 2, 11, Pages 329-335.*
5. **Chiodi, F., Sidén, A., Ösby, E., (2005).** *Isoelectric focusing of monoclonal immunoglobulin G, A and M followed by detection with the avidin-biotin system. Electrophoresis, Vol. 6 Issue 3, 124-128.*