Version 11.07.11

AP-Antibody All-In-One™ Conjugation Kit

Technical Manual

Catalog # A-9105-001

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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. Product Description

The AP-Antibody All-in-One™ Conjugation kit is designed to conjugate any user-supplied antibody (100 µg) with pre-activated, high-activity alkaline phosphatase (>7000 DEA U/mg) and to deliver a purified, ready to use conjugate. Any high quality monoclonal or polyclonal antibody can be conjugated to AP and purified in just over 4 hours (1 h hands-on). Best of all, All-in-One™ kits are specially designed for researchers with little or no conjugation experience.

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. 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 (figures 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 AP conjugate.

Complete conversion of antibody to conjugate greatly simplifies the purification process. A rapid spin column is used to trap any residual excess AP in the matrix, yielding a highly purified and ready-to-use conjugate. AP conjugates made with this All-in-One™ kit are compatible with many sensitive downstream applications including Westerns, ELISAs, and/or immunohistochemical detection (IHC). Each kit provides sufficient reagents to perform two conjugation reactions yielding between 40-60 µg of purified conjugate.

B. All-in-One™ Technology

Conjugation Chemistry

HydraLinK™ chemistry is based on the interplay between two heterobifunctional linkers; S-HyNic and Sulfo-S-4FB **(Figure 1).** S-HyNic (Succinimidly-6-hydrazino-nicotinamide) is used by a researcher to incorporate protected aromatic hydrazines (HyNic groups) on to their antibody through acylation of lysine residues. In a similar fashion, Sulfo-S-4FB linker (Sulfo-N-succinimidly-4-formylbenzamide) is used at Solulink to pre-incorporate stable formylbenzamide (4FB groups) on high activity alkaline phosphatase. Simple incubation of user-modified HyNic-antibody with pre-activated 4FB-AP in the presence of aniline catalyst leads to rapid and quantitative formation of a conjugate linked through a stable bis-arylhydrazone bond **(Figure 2)**.

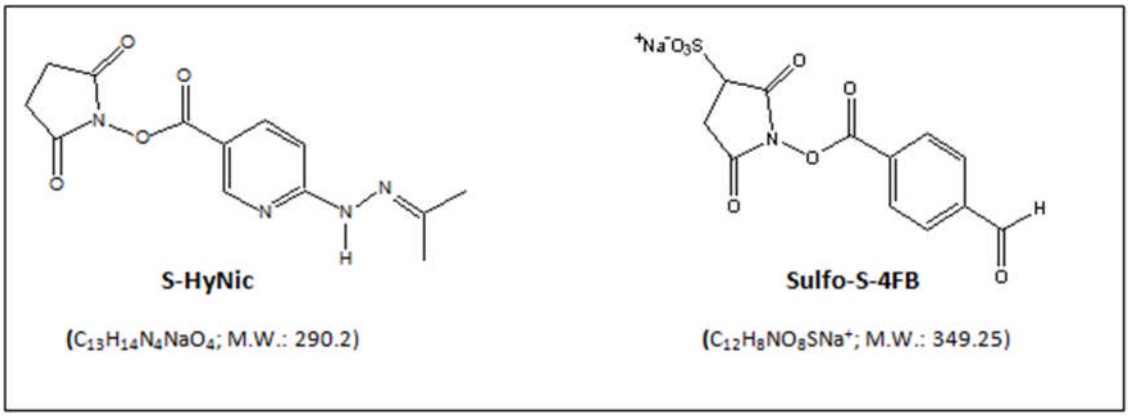


Figure 1. Molecular structure of S-HyNic and Sulfo-S-4FB; linkers used for conjugating AP to antibody.

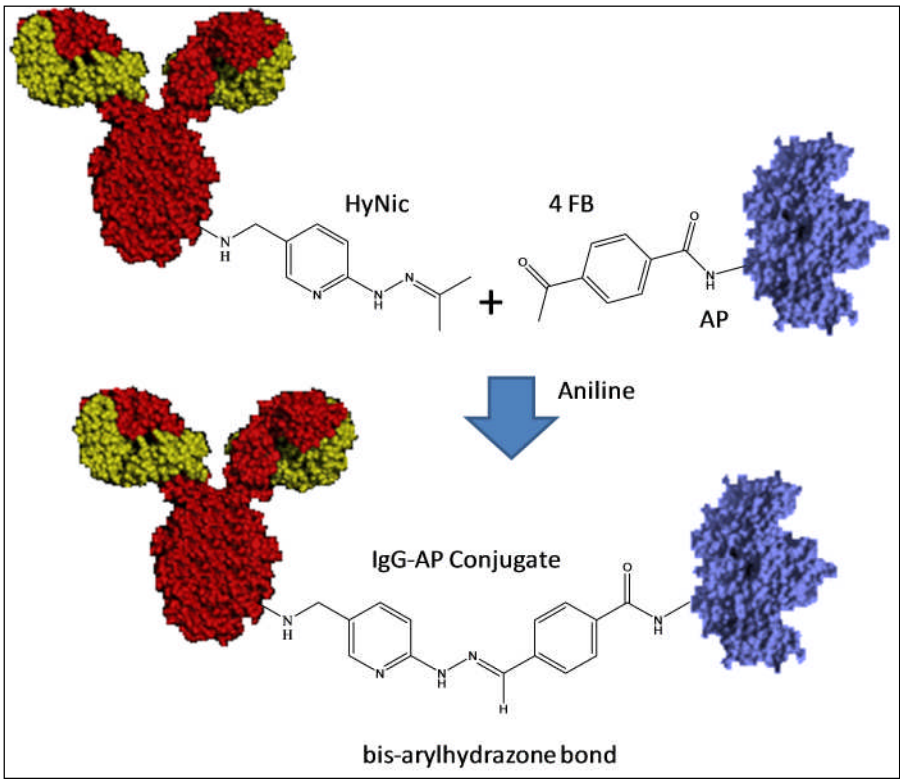


Figure 2. Catalyzed HydraLink™ chemistry used for conjugating HyNic-modified antibody with pre-activated 4FB-Alkaline phosphatase (AP).

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 AP and conjugate. Conjugate purification then simply involves the use of a rapid gel filtration spin column that quantitatively traps any remaining free AP enzyme **(Figure 3)** but excludes the much larger conjugate from the matrix allowing it to flow through. The final result is a highly purified AP-antibody conjugate.

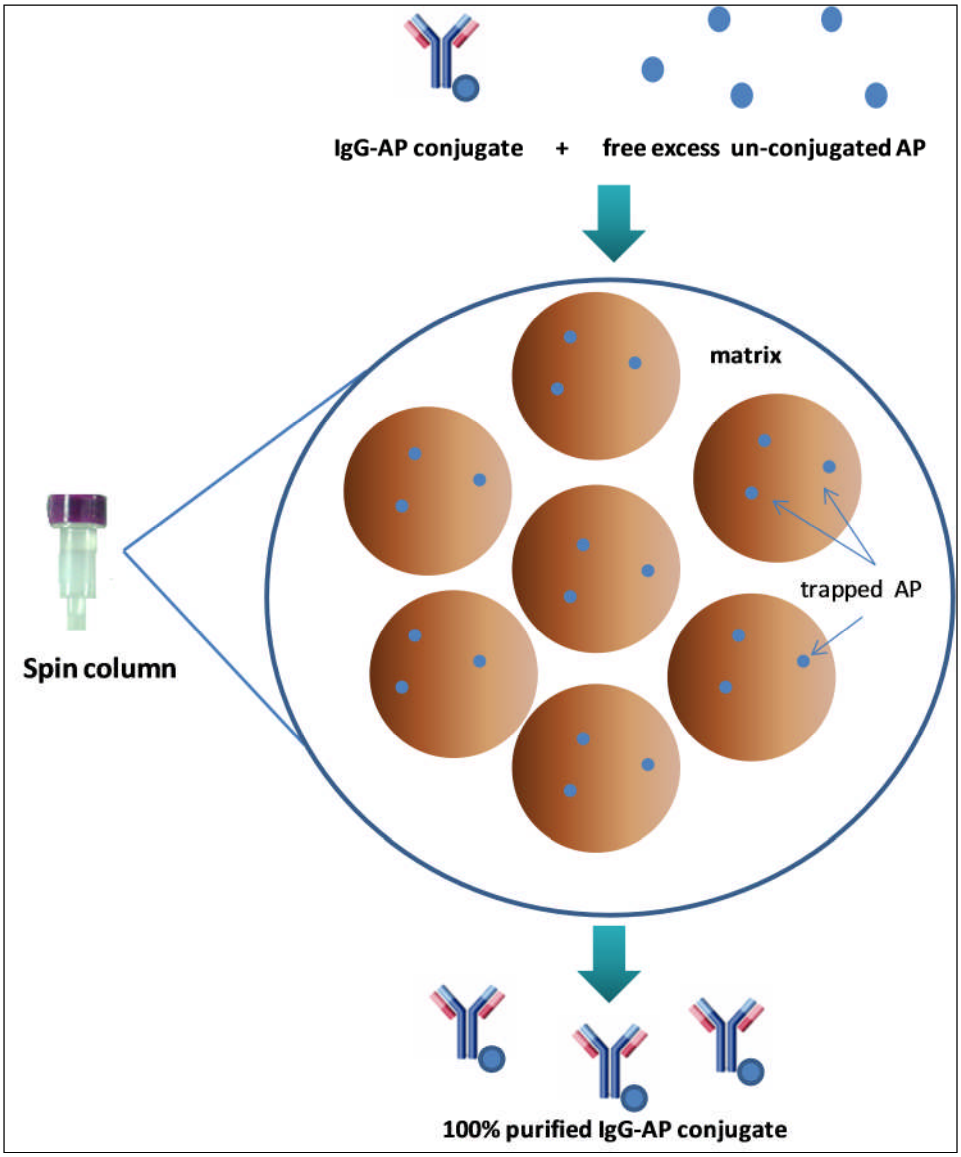


Figure 3. Gel filtration-based spin column purification of All-in-One™ AP-IgG conjugates.

C. All-in-One™ Conjugation Process Summary

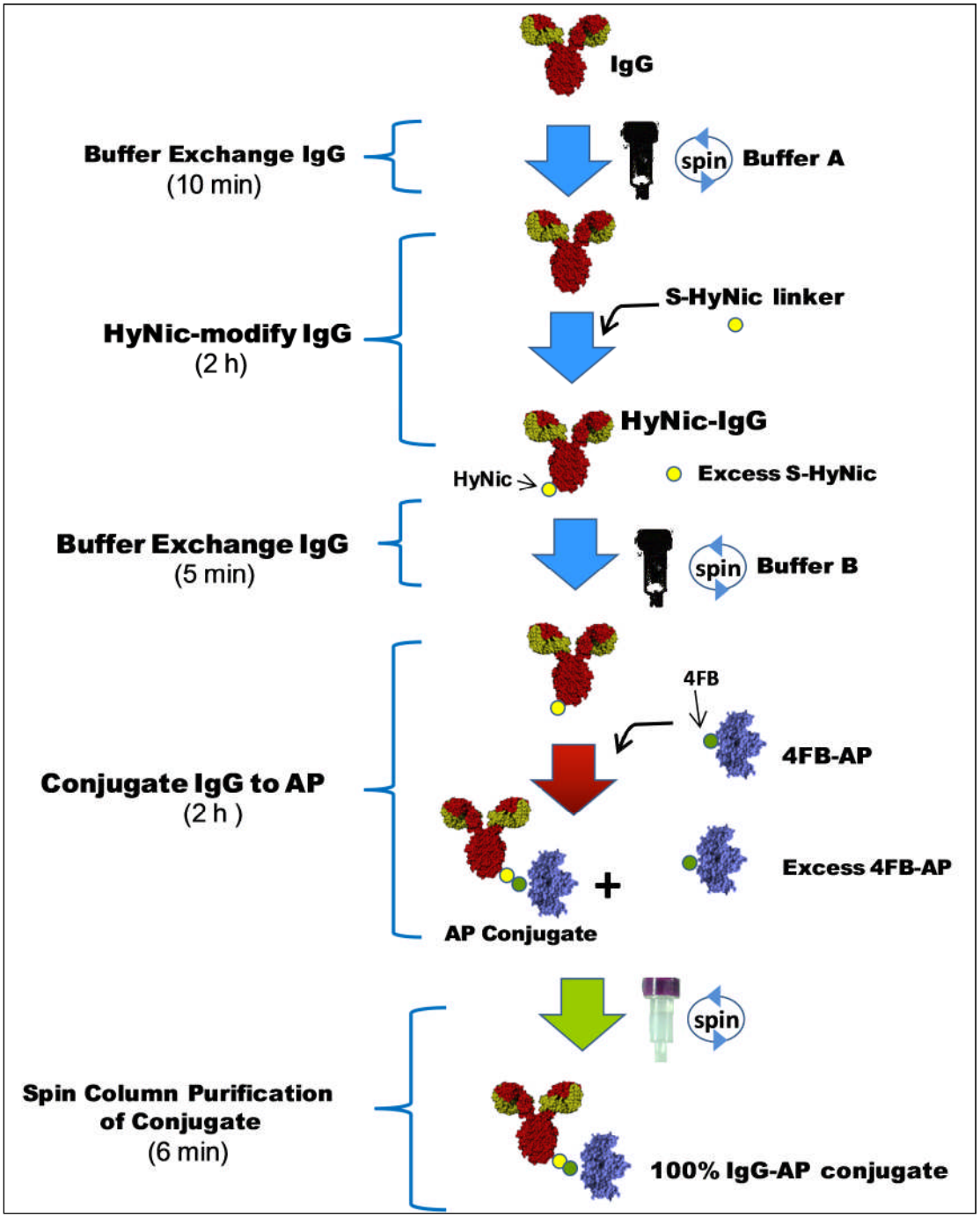


Figure 4. AP-Antibody All-in-One™ conjugation process.

D. Components Provided and Storage Conditions

S-HyNic	2 x 100 µg	Keep sealed in aluminum pouch provided (2-8°C).
4FB- AP	2 x 10 µL	Keep refrigerated (2-8°C)
Buffer A	2 x 1.5 mL	Keep refrigerated (2-8°C)
Buffer B with Aniline	2 x 1.5 mL	Keep refrigerated (2-8°C)
50 mM Tris-HCL (pH 7.4)	2 x 1.5 mL	Keep refrigerated (2-8°C)
Conjugation Additive	20 µL	Keep refrigerated (2-8°C)
Anhydrous DMF	500 µL	Keep refrigerated (2-8°C)
0.5 mL Zeba Spin Column	4	Keep refrigerated (2-8°C)
Blue Cap Conjugate Spin Column	2	Keep refrigerated (2-8°C)
1.5 mL Collection tubes	12	Keep refrigerated (2-8°C)
30K MWCO Diafiltration Spin Filters	2	Keep refrigerated (2-8°C)

Zeba™ is a registered trademark of Pierce/ThermoFisher

E. Additional Materials Required But Not Provided

BCA Protein Assay Reagents (verification of initial IgG concentration)
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 2: AP-Antibody All-in-One™ Conjugation Protocol

A. Antibody Preparation (0-10 min)

Antibodies come in two physical forms, solids and 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 by either a BCA protein assay or A_{280} measurement before proceeding. The All-in-One™ conjugation protocol requires that antibody samples be free of protein carriers such as BSA or gelatin. A mass of antibody (100 µg) dissolved in 25 µl buffer to a final concentration of 4 mg/ml is required. Depending on the state of your initial sample (solid or liquid), proceed as follows:

Solid Form (e.g. lyophilized powder)

If the antibody sample to be conjugated is packaged as a lyophilized powder (100 µg) free of protein additives such as gelatin or BSA, simply resuspend the sample in 25 µl **Buffer A** to yield a 4 mg/ml solution. Proceed directly to 1st Buffer Exchange.

If the antibody sample to be conjugated is packaged at less than 100 µg IgG per vial (e.g. 50 µg), simply resuspend the requisite number of vials equivalent to 100 µg in a final volume of 25 µl **Buffer A** to obtain 4 mg/ml solution. Proceed directly to 1st Buffer Exchange.

If the antibody sample to be conjugated is packaged at greater than 100 µg IgG per vial, simply resuspend in a suitable volume **Buffer A** to yield a 4 mg/ml solution. Transfer 25 µl to a new microfuge tube and store the remainder. Proceed directly to 1st Buffer Exchange.

Liquid Form (e.g. PBS or TBS Buffer)

If the antibody sample to be conjugated is packaged in liquid form at 4 mg/ml, simply transfer 25 µl to a new microfuge tube (100 µg). Proceed directly to 1st Buffer Exchange.

If the antibody sample to be conjugated is in liquid form at a concentration greater than 4 mg/ml, simply transfer a volume equivalent to 100 µg into another microfuge tube and dilute to a final volume of 25 µl by adding the requisite volume Buffer **A** to obtain a 4 mg/ml solution. Proceed directly to 1st Buffer Exchange.

If the antibody sample to be conjugated is packaged at less than 4 mg/ml, the sample must be concentrated before proceeding. Concentrate the sample to 25 µl and 4 mg/ml as directed in the Appendix. After concentrating the sample, proceed directly to 1st Buffer Exchange.

B. 1st Buffer Exchange (10 min)

1. For each conjugation reaction, prepare two spin columns by twisting off the bottom closures. Loosen the red caps (do not remove caps) and place each spin column into an empty collection tube (provided).

2. Using a permanent marker pen, mark one red cap with the letter **A** and the other with the letter **B** to differentiate the two spin columns.

3. Centrifuge the spin columns @ **1,500 x g for 1 minute.** Discard the flow through buffer from the bottom of each collection. After centrifugation the column matrix should appear dry and white in color.

4. After centrifugation, use a permanent marker pen to place a mark on the side of each spin column where the compacted resin has slanted upward. **Note**-orient the spin columns in all subsequent centrifugation steps with this mark aiming outward and away from the center of the rotor.

5. Add 300 µl Buffer **A** to the top of the **A** resin and 300 µl Buffer **B** with Aniline to the top of the **B** resin; loosely recap columns. **Note-**when loading the buffer, do not disturb the resin bed with the pipette tip.

6. Centrifuge @ **1,500 x g for 1 minute**. Once again, discard the flow-through buffer from the bottom of each collection tube.

7. Repeat steps 5 and 6 **two (2**) additional times.

8. Transfer the dry **A** spin column to a new collection tube and set aside.

9. Add an additional 300 µl Buffer **B** with aniline to the dry **B** spin column resin. Set this rehydrated spin column aside for later use (Section D).

10. Immediately load the prepared antibody solution (25 µl @ 4 mg/ml) to the top of the dry **A** spin column resin. Loosely recap; and centrifuge at **1,500 x g for 2 minutes.**

11. After centrifugation, transfer the solution from the bottom of the **A** collection tube to a new microfuge tube. Use a calibrated pipette to check the recovered volume (30 ± 5 µl). **Note**-if significantly less volume is recovered the centrifuge may need to be recalibrated.

C. Modification of Antibody with S-HyNic (2 h)

1. Add 20 µl DMF (anhydrous) to a vial containing S-HyNic reagent. Pipette up and down to resuspend the reagent completely. **Note-**this may take a couple of minutes. Make sure the visible pellet in the vial is completely dissolved before proceeding.

2. Add 1.0 µl dissolved DMF/S-HyNic reagent with a calibrated P-2 or P-10 pipette to the antibody solution from step B-11. Gently pipette the mixture up and down several times to mix; spin the tube briefly (5 seconds @ 1000 x g) to collect the reaction contents at the bottom of the tube.

3. Incubate the reaction for 2 h at room temperature.

D. 2nd Buffer Exchange (5 min)

1. After HyNic modification of the antibody, centrifuge the hydrated **B** spin column at **1,500 x g for 1 minute** to remove residual hydration buffer. Transfer the spin column to a new collection tube. **Note-**the **A** spin column can now be used as a balance tube.

2. Load the entire volume of the completed HyNic modification reaction (from step C-3) to the top of the dry B spin column resin; loosely recap and centrifuge at **1,500 x g for 2 minutes**.

3. Transfer the contents from the bottom of the collection tube to a new 1.5 ml microfuge tube; label appropriately (e.g. HyNic-IgG) and proceed to the next section.

E. Conjugation of Antibody to AP (2 h)

1. Add 8 µl Conjugation Additive to the HyNic-IgG mixture from step D-3; gently pipette up and down to mix; incubate for 5-10 minutes.

2. Add 40 µl **Buffer B with Aniline** to the HyNic-IgG mixture.

3. Briefly spin the vial containing 4FB-AP (5 seconds @ 1000 x g) to collect the contents at the bottom of the tube; pipette up and down to mix.

4. Transfer 10 µl of 4FB-AP to the HyNic-modified antibody containing conjugation additive. Gently pipette up and down to mix; briefly spin (5 seconds @ 1000 x g) to insure the liquid contents are at the bottom of the tube.

5. Incubate for 2 h at room temperature.

F. Spin Column Purification (6 min)

1. Prepare a Conjugate Spin Column by twisting off the bottom closure. Use a permanent marker pen to label the top of the purple cap to identify the conjugate. Loosen the cap (do not remove cap) and place the spin column into a previously used collection tube.

2. Centrifuge **@ 1,500 x g for 1 minute**; discard the flow-through buffer from the bottom of the collection tube. **Note-** a previously used spin column can serve to balance the rotor.

3. Add 300 µl 50 mM Tris-HCl (pH 7.4) to the top of the resin; loosely recap the spin column.

4. Centrifuge @ **1,500 x g for 1 minute**. Once again, discard the flow-through buffer from the bottom of the collection tube.

5. Repeat steps 3 and 4 **two (2)** additional times to complete the equilibration of the spin column.

6. Add the contents of the AP-IgG conjugation reaction (~ 80-100 µl from step E-5) to the top of the dry resin; loosely recap and transfer to a new collection tube (provided).

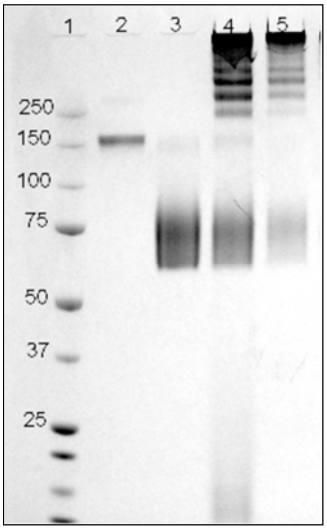
7. Centrifuge @ **1,500 x g for 2 minute**.

8. After centrifugation, transfer the purified AP-IgG conjugate from the bottom of the collection tube to a new microfuge tube. Verify the recovered volume (usually 100 ± 15 µl).

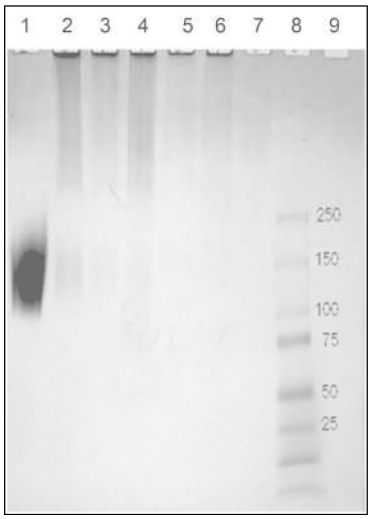
9. Label and store the purified AP-IgG conjugate at 4°C. Conjugate concentrations are generally between 0.4 and 0.6 µg/µL. **Note**-exact conjugate concentrations can be determined using a BCA assay. Never freeze AP conjugates.

Chapter 3: AP-Antibody All-in-One™ Conjugate: An Example

A. Monoclonal IgG-AP Conjugate



Panel A



Panel B

Panel A

1. Protein M.W. marker
2. HyNic-modified Mouse Anti-FITC monoclonal IgG (1 µg)
3. 4FB-modified alkaline phosphatase (5 µg)
4. Mouse Anti-FITC IgG-AP (crude reaction) (10 µg)
5. Mouse Anti-FITC IgG-AP (purified) (5 µg)

Panel B

1. Alkaline phosphatase-4FB modified (5 ug)
2. All-in-One Mouse Anti-FITC purified conjugate reaction #1 (2 µg total protein)
3. All-in-One Mouse Anti-FITC purified conjugate reaction #2 (1 µg total protein)
4. All-in-One Mouse Anti-FITC purified conjugate reaction #3 (2 µg total protein)
5. All-in-One Mouse Anti-FITC purified conjugate reaction #4 (0.5 µg total protein)
6. All-in-One Mouse Anti-FITC purified conjugate reaction #5 (0.75 µg total protein)
7. All-in-One Mouse Anti-FITC purified conjugate reaction #6 (0.5 µg total protein)
8. All-in-One Mouse Anti-FITC purified conjugate reaction #7 (0.5 µg total protein)
9. Blank lane

Figure 5. Coomassie-stained (4-12% SDS-PAGE) of a typical AP-IgG conjugate is illustrated in Panel A. Conjugate was heat denatured before loading gel. Panel B is a native protein gel containing a series of 7 separate conjugation reactions using the same monoclonal antibody. In both panels, the vast majority of Coomassie-stained conjugate is a high molecular weight species that barely migrates into the gel. **Note-** alkaline phosphatase is a 140 kD glycosylated dimer that migrates as a broad 70 kD on denaturing SDS gels. In denaturing gels, a portion of AP can re-dissociate from the conjugate due to the dimeric form of this enzyme.

B. Direct ELISA Using an IgG-AP Conjugate

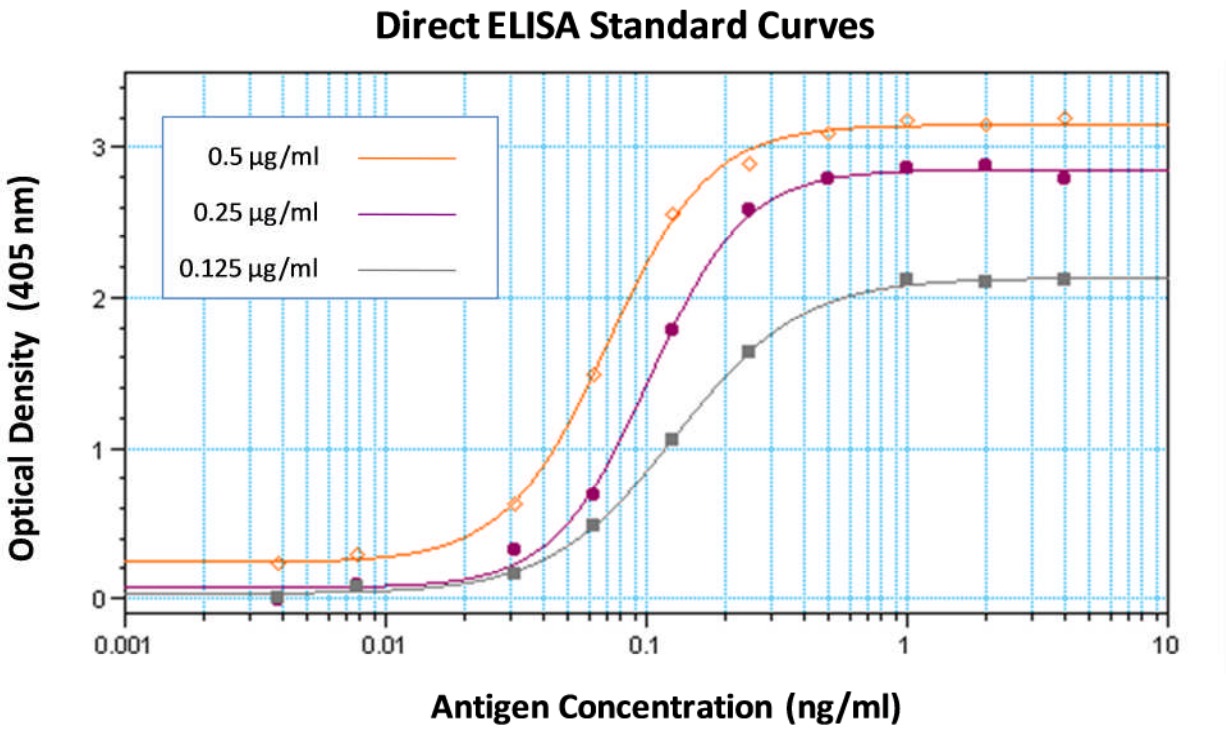


Figure 6. Direct *ELISA* curves generated using an AP conjugate made with the All-in-One kit. A mouse anti-FITC monoclonal antibody was conjugated to AP as described in the manual. Antigen consisting of FITC-labeled BSA (FITC MSR = 2) was coated on plates in a 2-fold dilution series (100 µl per well @ 4000, 2000, 1000, 625, 312.5, 156.25, 78.0, 39.0, and 19.5 ng/ml) using standard methods. Immobilized antigen was then detected at 3 different conjugate concentrations (0.5 µg/ml. 0.25 µg/ml. 0.125 µg/ml) using pNPP substrate (20 minutes @ 405 nm) on a Molecular Devices plate reader.

Chapter 4: Appendix

A. BCA Protein Assay

Solulink highly recommends (when IgG is not limiting or its concentration, source, or quality are unknown) that antibody samples be assayed for initial protein concentration using the BCA™ Protein Assay (Pierce, Cat. #23225, BCA™ is a registered trademark ThermoScientific/Pierce) prior to conjugation. The starting quality and quantity of an antibody is critical to the success of the procedure. A reference assay protocol is provided for measuring antibody or conjugate protein concentrations using the BCA™ Protein Assay (BCA reagents are not provided in the kit).

BCA™ Microplate Procedure

Required Materials (sufficient for ~25 protein assays)

BCA Reagent A- 5 ml	96-well polystyrene plate
BCA Reagent B-100 µl	40° C water bath
Bovine IgG standard: 2 mg/ml	1X PBS (10 ml)
Molecular grade water	P-2, P-100 pipettes

1.) Prepare a working solution of BCA reagent just prior to use by adding 5 ml of BCA Reagent A to a clean 15 ml conical tube followed by addition of 100 µl of BCA Reagent B. Mix the two solutions until a clear green solution forms. Note-Prepare the BCA working reagent fresh daily.

2.) For each antibody to be measured, place the indicated volume of 1x PBS (see Table 2) into a microplate well and add the appropriate aliquot of the protein sample to the PBS (see table below). The final volume of each sample in the plate must be 20 µl. Record the dilution factor.

Protein Concentration (mg/ml)	Sample Volume Required (ul)	Volume PBS (ul)	Final Volume (ul)	Dilution Factor
2-10 mg/ml	2	18	20	10
≤ 1 mg/ml	10	10	20	2

Table 2. Preparation of protein samples for BCA assay.

3.) Prepare a BCA protein standard curve by making a 2-fold serial dilution of a 2 mg/ml bovine IgG standard (e.g. Pierce Chemical, Product Number 23212) or into individual wells of a microplate as illustrated on the next page.

Well #1 – Add 50ul PBS and 50ul bovine IgG standard (2 mg/ml) to a well (1 mg/ml)
Well #2 – Add 50ul PBS and 50ul from the 1st well to a 2nd well (0.5 mg/ml)
Well #3 – Add 50ul PBS and 50ul from the 2nd well to a 3rd well (0.25 mg/ml
Well #4 – Add 50ul PBS and 50ul from the 3rd well to a 4th well (0.125 mg/ml
Well #5 – Add 50ul PBS and 50ul from the 4th well to a 5th well (0.0625 mg/ml)
W ell #6 – Add 50ul PBS to the 6th well (Buffer blank)

4.) Transfer 20 µl aliquots from each of the 2-fold serially diluted IgG standards to six empty microplate wells, preferably adjacent to wells containing 20 µl of the protein sample to be assayed (from step 2 above).

5.) Add 150 µl freshly prepared BCA reagent to each well containing 20 µl of each dilution standard and sample to be assayed; mix well.

6.) Seal the wells using clear adhesive film or scotch tape and incubate the plate at 37-40^{o}C in a water bath for 15-20 minutes.

7.) Remove the plate from the water bath, dry the bottom of the plate and read the plate in a suitable reader (e.g. Molecular Devices) at 562 nm. A typical BCA assay result is depicted in Figure 7.

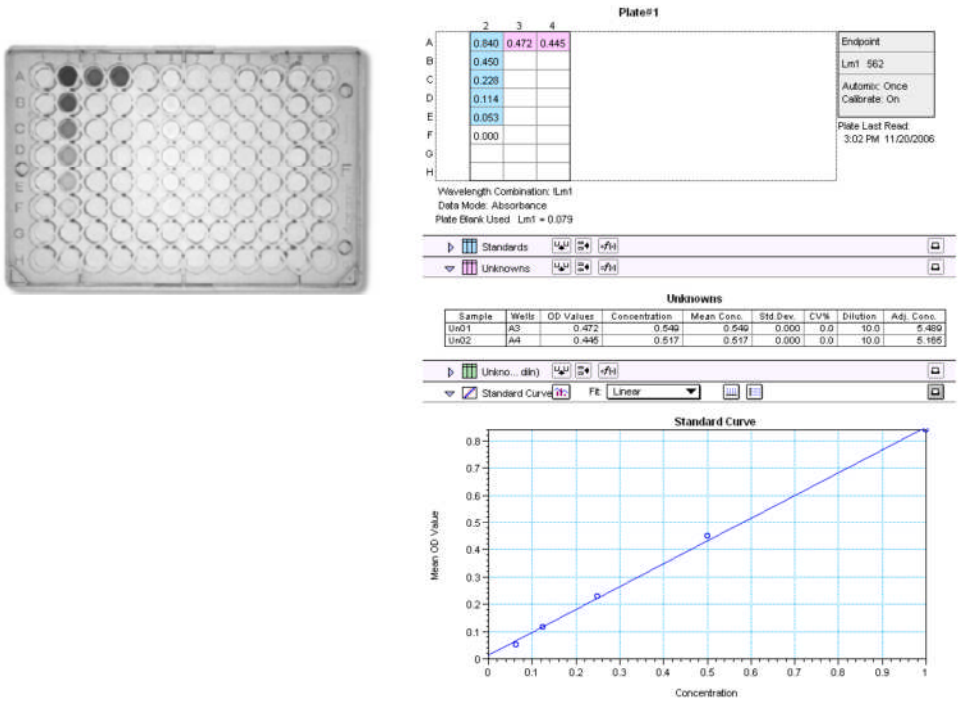


Figure 7. BCA protein microplate assay result. On the left is a plate containing a dilution series of IgG standards (wells A2-F2) along with two protein samples (A3, A4). On the right is the plate output from a Molecular Devices UV-VIS microplate reader illustrating BCA assay result.

B. 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 the concentration saving the trouble of conducting a Bradford protein assay to confirm concentration. To estimate antibody concentration using a NanoDrop™ spectrophotometer, 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 A_{280} menu option. **Note-** do not use the UV-VIS menu option on the NanoDrop™ to read an antibody sample.

4. After the A_{280} menu appears, **click-off the 340 nm normalization option** using the mouse.

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 a flat baseline. Clean the pedestal and repeat (if necessary) until a flat baseline is obtained.

8. Transfer a 2 µl volume of antibody solution to the pedestal and click the 'Measure' icon. Wait until the spectrum (220-350 nm) appears in the window.

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 µl) was scanned as described and its concentration confirmed using equation #1 below.

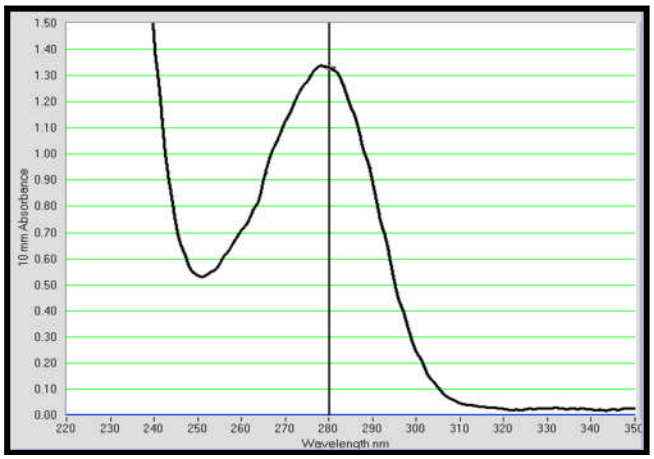


Figure 8. A mouse IgG sample 100 µl @ 1 mg/ml in PBS pH 7.2, scanned on the NanoDrop™ as described in the text.

Equation #1: [A_{280} /E1% value] x 10 mg/ml = 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% bovine IgG] x 10 mg/ml = protein concentration (mg/ml)

[1.34 / 14.00] x 10 mg/ml = 0.96 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.

C. AP Absorption Spectrum (Unmodified Alkaline phosphatase)

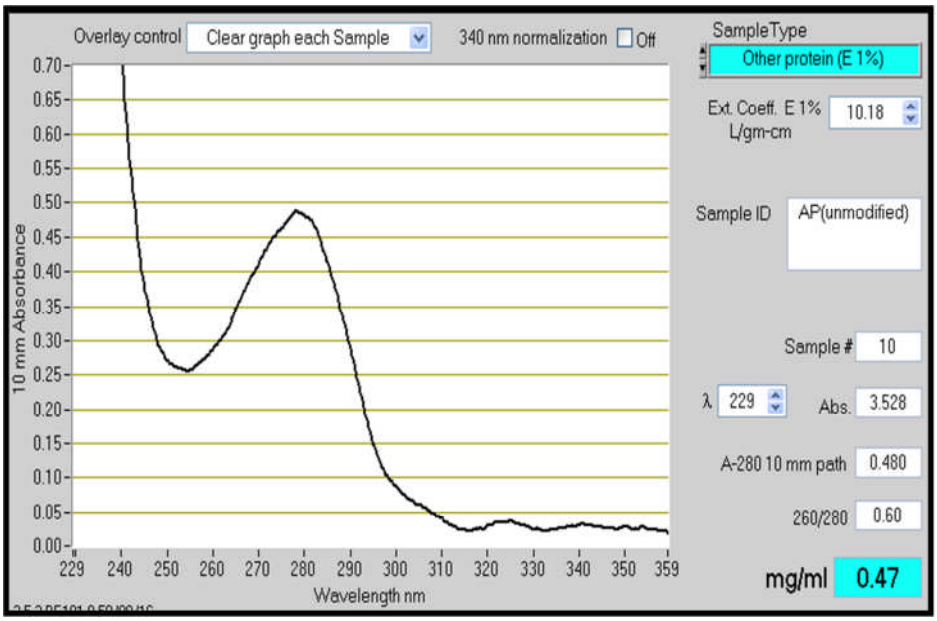


Figure 9. NanoDrop™ absorption spectrum of unmodified alkaline phosphatase (229-359 nm) @ 0.5 mg/ml (50 mM Tris-HCL pH 8.0, 1 cm-path length equivalence)

D. 4FB-modified AP Absorption Spectrum

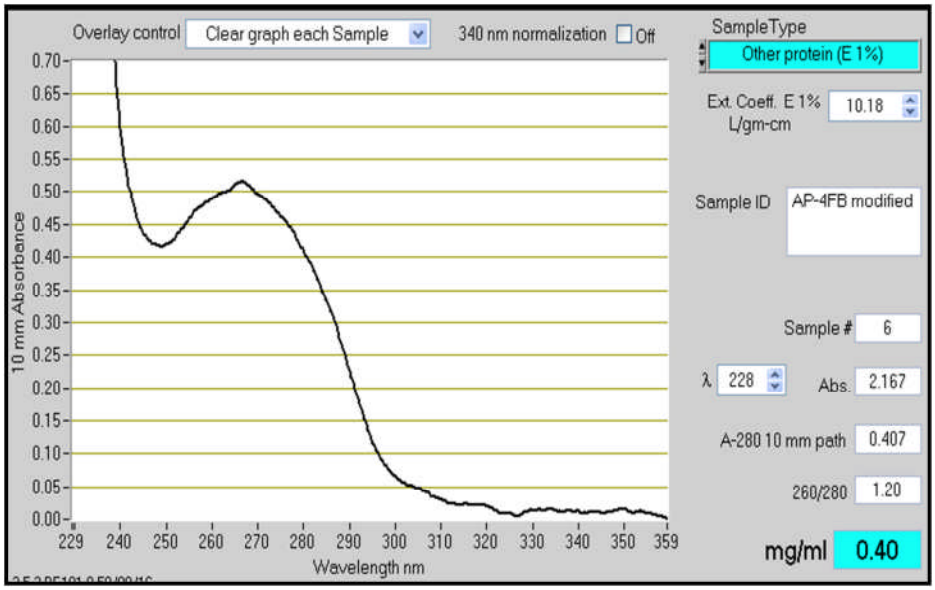


Figure 10. NanoDrop™ absorption spectrum of 4FB-modified alkaline phosphatase (229-359 nm) @ 0.4 mg/ml (50 mM Tris-HCL pH 8.0, 1 cm-path length equivalence)

E. Bovine IgG-AP Conjugate Absorption Spectrum (All-in-One Purified)

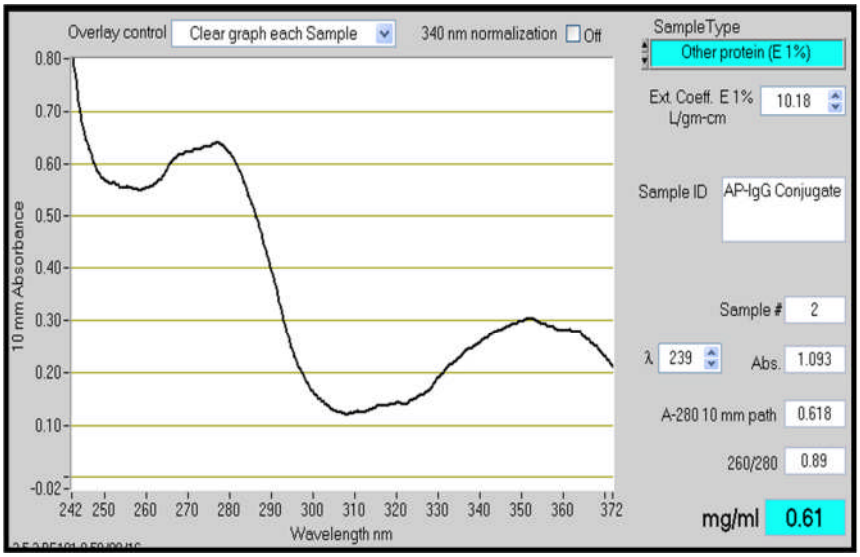


Figure 11. NanoDrop™ absorption spectrum of All-in-One AP-IgG conjugate (242-372 nm) @ 0.5 mg/ml (sodium phosphate buffer, pH 6.0, 1 cm-path length equivalence). Note the absorbance signature centered around 354 nm. This tell-tale signature is generated through hydrazone bond formation when conjugating with HydraLink™ chemistry.

F. Concentrating Dilute Antibody Solutions

The AP-Antibody All-in-One™ Conjugation protocol requires that initial antibody protein concentration be at 4 mg/ml and 25 µl. 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 require concentration to 4 mg/ml and 25 µl 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 the instructions below to avoid loss of antibody on the filter surface.

> **Note -** dilute antibody solutions require 100 µg of starting antibody (e.g. 500 µl @ 0.25 mg/ml) most diafiltration filters recover ~80% of input antibody. When samples are not limiting, 125 µg can be used to compensate for this unavoidable loss, we recommend all dilute antibody concentrations be confirmed using a Bradford protein assay before proceeding.

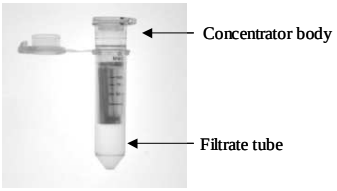


Figure 12. Diafiltration spin filter used for concentrating dilute antibody samples.

Protocol

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

1) Open the lid of a diafiltration spin filter device.

2) Transfer 500 µl (or less) of dilute protein solution (equivalent to 100-125 µg 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 check the remaining volume. If the volume remaining in the concentrator body is greater than 25 µl, 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 µl mark. Once the final volume reaches 25 µl, do not pipette up and down to avoid sample loss.

7) Carefully transfer the concentrated IgG solution (25 µl) to a new 1.5 ml microfuge tube and proceed with the conjugation procedure (1st Buffer Exchange).

G. 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 BCA or Bradford protein assay. Remove all interfering preservatives such as thimerosal or proclin before confirming protein concentration using a BCA or 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	-make sure to properly mix the antibody- HyNic reaction mixture -use a calibrated P-10 pipette to insure accuracy of small volumes
	-presences of amine contaminants	-remove all non-protein amine contaminants such as glycine or Tris before

	-improper storage of S-HyNic reagent can lead to hydrolysis of this NHS ester -initial antibody concentration was too low or too high.	modification -keep and store S-HyNic sealed in the aluminum pouch provided that 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 Incorrect speeds can impact protein and/or volume recovery

H. Component Stability and Storage Conditions

Component	Stability	Storage Condition
Unopened Kit	6 months from date of receipt	Refrigerated (2-8°C)
S-HyNic	6 months from date of receipt 24 h after re-suspending S-HyNic in DMF	Keep sealed in aluminum pouch provided (2-8°C). Room temperature
AP-Antibody Conjugate	1 month	Refrigerated (2-8°C) in final conjugate solution (50 mM Tris-HCL pH 7.4 containing 5 mM $MgCl_2$, 100 µM $ZnCl_2$.

	1 yr	50% glycerol, 50 mM Tris-HCL (7.4), 5 mM $MgCl_2$, 100 µM $ZnCl_2$
All other kit components	>1 yr	Refrigerated (2-8^{o}C)

I. 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*

3. ***Dirksen, A., Dirksen, S., Hackeng, T., Dawson, P (2006).*** *Nucleophilic Catalysis of Hydrazone Formation and Transimination: Implications for Dynamic Covalent Chemistry. JIAICIS Communications.*