Version 01.31.2013

ChromaLink™ Biotin One-Shot Antibody-Labeling Kit

Technical Manual

Cat. No. B-9007-009K

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- Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (e.g. safety glasses, gloves, or clothing). For additional safety guidelines consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's clean-up procedures as recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling and disposal.

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Chapter 1: Introduction

A. Product Description

The ChromaLink™ Biotin One-Shot Antibody Labeling Kit is designed to biotinylate a single 100 microgram quantity of antibody in about 2.0 hours. The One-Shot kit relies on a UV-traceable linker called ChromaLink™ Biotin to label the antibody **(Figure 1)**. This unique labeling reagent (patent pending) contains an aromatic, water-soluble N-hydroxy-succinimidyl ester functional group ***(a)*** *that* efficiently modifies antibody lysine residues under mild phosphate buffer conditions. The reagent also possesses an embedded bis-aryl hydrazone structure ***(b)*** which forms the linker's UV-traceable chromophore. The absorbance signature provided by the chromophore enables rapid and non-destructive quantification of incorporated biotin ***(c)***. The linker also features a long PEG3 spacer ***(d)*** which preserves streptavidin/biotin affinity and helps maintain antibody solubility (see kit disclaimer).

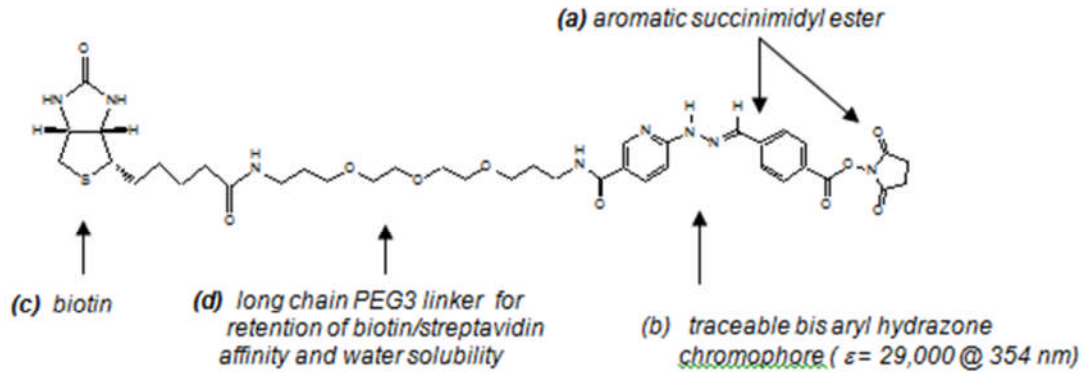


Figure 1. Structure of ChromaLink™ Biotin $C_{38}H_{50}N_8O_{10}S$; MW 810.92.

B. Features and Benefits

The ChromaLink™ Biotin One-Shot Kit is a simple, cost-effective way of incorporating a readily verifiable amount of biotin into a single 100 µg quantity of antibody. The kit conveniently features a pre-weighed amount of ChromaLink™ Biotin reagent that is readily resuspended directly into the antibody solution. ChromaLink™ Biotin can be used to label a variety of different antibodies, including mammalian IgG (monoclonal or polyclonal) and/or avian IgYs. Biotin incorporation is rapidly determined by means of a simple, non-destructive UV-scan (220–400 nm) of the sample after removal of excess labeling reagent. The kit features high antibody recovery (60–90 µg) and a consistent level of biotin incorporation (3–8 biotin molecules per antibody molecule) when used as directed.

C. ChromaLink™ One-Shot Process Diagram

The ChromaLink™ Biotin One-Shot antibody labeling procedure is illustrated in **Figure 2**.

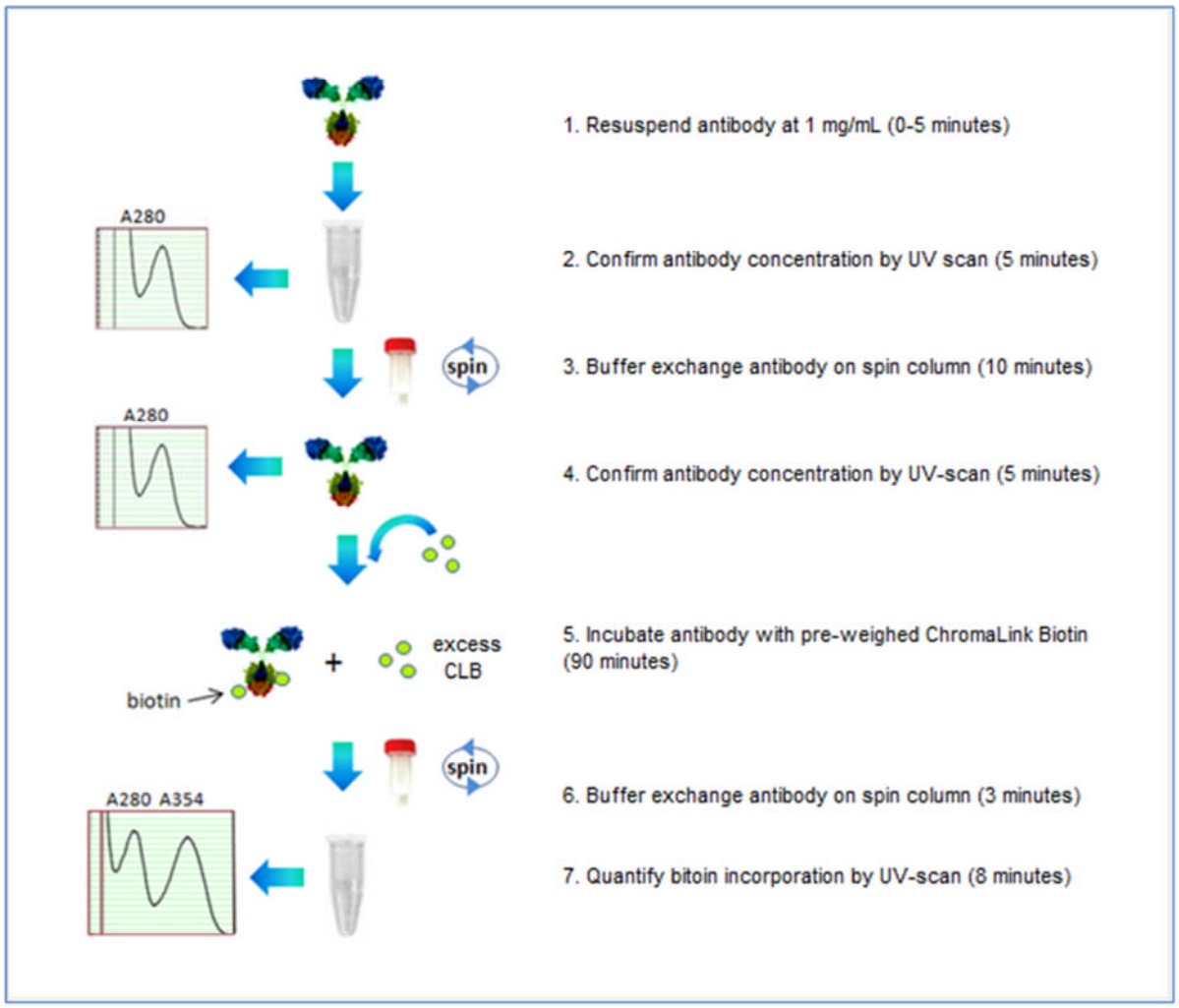


Figure 2. ChromaLink™ Biotin One-Shot antibody labeling procedure.

D. Process Summary

1. *Sample Preparation*: bring antibody to 1 mg/mL in 100 µL buffer
2. *1st Sample Analysis:* confirm antibody concentration using spectrophotometer
3. *1st Buffer Exchange:* equilibrate spin columns and buffer exchange antibody
4. *2nd Sample Analysis:* reconfirm antibody concentration using spectrophotometer
5. *Biotinylation:* label antibody with ChromaLink™ Biotin
6. *2nd Buffer Exchange:* remove excess labeling reagent
7. *Quantify Biotin Incorporation:* measure biotin incorporation using spectrophotometer

E. Important Labeling Parameters

The ChromaLink™ Biotin One-Shot Antibody Labeling Kit is designed to biotinylate a single 100 µg quantity of antibody resuspended in 100 µL buffer as indicated in Table 1.

Initial Antibody Concentration	Intitial Mass of Antibody	Initial Antibody Volume
1.0 ± 0.1 mg/ml	100 ± 10 µg	100 ± 5 µl

Table 1. Initial conditions required for the ChromaLink™ Biotin One-Shot labeling procedure.

This kit provides a consistent and reliable degree of biotinylation by controlling the following reaction variables:

- Fixed antibody mass (100 µg) and volume (100 µL)
- Optimized reaction buffer
- Controlled and fixed reaction time (90 min)
- Consistent reaction stoichiometry (12X mole-equivalents) (12-fold molar excess of biotin reagent)

Critical to consistent biotin incorporation is the ability of a user to accurately determine the antibody's **initial protein concentration** in a non-destructive manner that allows full or nearly full recovery of the precious sample. The One-Shot procedure recommends using a scan (220–400 nm) on a spectrophotometer rather than the traditional A_{280} to estimate antibody concentration. The rationale for using a scan rather than a single wavelength (A_{280}) is that many commercial antibodies contain preservatives and/or other additives that mask or distort the intrinsic A_{280} of a sample. The presence of an additive can make it more difficult or sometimes impossible to accurately estimate protein concentration via A_{280}. A scan however contains greater **spectral information,** often revealing the presence of spectrum-altering additives that are known to interfere with the accurate estimate of protein concentration. Altered spectra are a warning that the A_{280} of the sample will not yield an accurate estimate of protein concentration. Commonly used additives include preservatives such as sodium azide, thimerosal, protein stabilizers such as *BSA or gelatin and/or small molecule additives such as glycine or trehalose. If a commercial antibody sample contains any of these additives, please refer to the Troubleshooting section in the Appendix to determine how to proceed. For example, protein carriers such as BSA or gelatin must be removed before labeling can proceed whereas the presence of trehalose will not alter either the spectrum or the A_{280} of a sample and is therefore perfectly compatible with the One-Shot labeling procedure.

F. Materials Provided and Storage Conditions

Components	Amount	Storage Conditions
ChromaLink Biotin	6.49 µg	Room temperature
1X Modification Buffer	1.5 mL	Room temperature
1X PBS	1.5 mL	Room temperature
Collection Tubes	4	Room temperature
7K MWCO Zeba Columns	2 x 0.5 mL	Room temperature
Biotinylated Bovine IgG Control	100 µg	Room temperature
1M Tris HCl	100 µL	Room temperature
Anhydrous DMF	500 µL	Room temperature

Zeba™ is a registered trademark of Pierce/ThermoScientific..

G. Additional Materials Required But Not Provided

UV-VIS or NanoDrop™ Spectrophotometer
Semi-micro quartz cuvette (50–100 µL capacity) (not required w/NanoDrop™ instrument)
Variable speed microcentrifuge (e.g., Eppendorf 5415D, IEC MicroMax, or similar)
1.5 mL microcentrifuge tubes
Molecular grade water
P-10, P-200, P-1000 pipettes

Chapter 2: ChromaLink™ Biotin One-Shot Labeling Protocol

A. Sample Preparation (0–5 min)

Antibodies come packaged in two different physical forms, solids or liquids. Individual antibody samples vary greatly from vendor to vendor and are often sold in a variety of different sizes and/or concentrations. In all cases, Solulink recommends starting with a high- quality/high-purity antibody from a reputable vendor. Depending on which form your sample is in (solid or liquid); proceed as directed below.

Initial Antibody Sample is in Solid Form (e.g., lyophilized)

Initial Sample: 100 µg/vial

Resuspend the antibody in 100 µL 1X Modification Buffer (provided) to yield a 1 ± 0.1 mg/mL solution. Proceed directly to 1st Sample Analysis.

Initial Sample: >100 µg/vial

Resuspend the antibody in a sufficient volume of 1X Modification Buffer (provided) to yield a 1 mg/mL solution. Transfer a volume equivalent to 100 µL to a new 1.5 mL microcentrifuge tube and store the unused portion of the sample. Proceed directly to 1st Sample Analysis.

Initial Sample: <100 µg/vial

If the initial sample contains less than 100 µg/vial (e.g., 50 µg/vial) reconstitute the requisite number of vials equivalent to 100 µg in 100 µL 1X Modification Buffer (provided) to yield a 1 ± 0.1 mg/mL

solution. Proceed directly to 1st Sample Analysis. **Note: We do not recommend using the One-Shot kit to label samples containing less than 90 µg antibody.**

Initial Antibody Sample is in Liquid Form (e.g., PBS OR TBS)

Initial Sample Concentration: 1 mg/mL

Transfer 100 µL to a new 1.5 mL microcentrifuge tube. Proceed directly to 1st Sample Analysis.

Initial Sample Concentration: >1 mg/mL

Transfer a volume equivalent to 100 µg into a new 1.5 mL microcentrifuge tube and dilute the sample using 1X Modification Buffer (provided) to obtain 100 µL at 1 mg/mL. Proceed directly to 1st Sample Analysis.

Initial Sample: <1 mg/mL

Concentrate dilute samples to 1 mg/mL and transfer 100 µL to a new 1.5 mL microcentrifuge tube. Proceed to 1st Sample Analysis. Commercial diafiltration spin filters are available for this purpose (e.g., Sartorius or Amicon) but are not included with the kit. **Note: for dilute solutions, a volume equivalent to 100 µg total antibody is required.**

B. 1st Sample Analysis (5–10 min)

To confirm that the initial antibody sample is at 1 mg/mL and 100 µL, scan the sample using either a conventional UV-VIS (requires quartz micro-cuvette) or a micro-volume spectrophotometer such as a NanoDrop™ spectrophotometer (ND-1000) using the instructions that follow as a guide.

Determine Antibody Concentration (Conventional UV-VIS Spectrophotometer)

1. Program the spectrophotometer to scan from 220–400 nm. Follow the manufacturer's instructions for each instrument.
2. Using a clean semi-micro quartz cuvette (e.g., 50–100 µL), blank the instrument using the appropriate sample buffer (e.g., PBS, TBS, or 1X Modification Buffer).
3. Discard the blank solution.
4. Transfer the antibody sample (100 µL @ 1 mg/mL) to the clean cuvette and scan.
5. Inspect the scan and record the A_{280} value from the spectrum.
6. Recover the sample from the cuvette.
7. If the initial antibody spectrum has a normal shape and baseline as illustrated in **Figure 3**, then proceed to calculate the initial antibody concentration as illustrated in the following example; otherwise, refer to the Troubleshooting Guide in the Appendix.

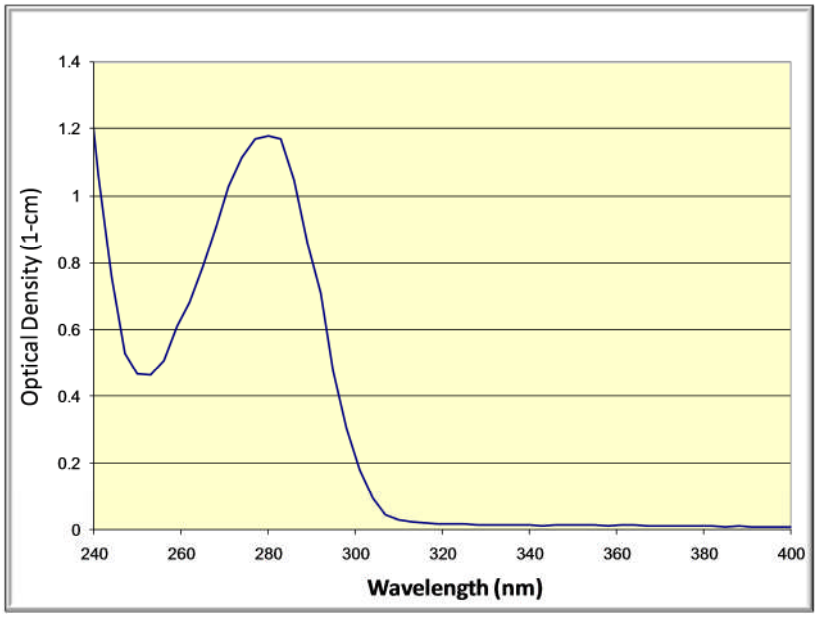


Figure 3. Normal UV-scan of bovine IgG sample (100 µg) resuspended in 100 µL 1X Modification Buffer (100 mM sodium phosphate, 150 mM NaCl, pH 8.0). The scan was taken in a quartz semi-micro cuvette (100 µL volume, 1-cm pathlength).

Sample Calculation

Use the following equation to estimate initial protein concentration

[A_{280} /E1%] x 10 mg/mL = protein concentration (mg/mL)

E1% = mass extinction coefficient (see Table 2)

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
Avian IgY	12.76

Table 2. A list of known mass extinction coefficients (E1%) for antibodies derived from different animal species. The E1% is used to calculate antibody concentration. The E1% of an antibody represents the A_{280} of a 10 mg/mL solution as measured in a 1-cm path length cuvette.

Sample calculation (continued)

A_{280} (from scan in Figure 3) = 1.18
E1% (from Table 2) = 12.40

[A_{280} / E1% of bovine IgG] x 10 mg/mL = protein concentration (mg/mL)

Therefore, [1.18/12.40] x 10 mg/mL = 0.95 mg/mL

8. Record the antibody concentration and total sample volume. If the antibody concentration is 1 ± 0.1 mg/mL and approximately 100 µL proceed to 1st Buffer Exchange in the next section.

   Note: Obtain additional antibody if a sample contains less than 90 µg (e.g., 0.85 mg/mL and 100 µL). If the measured antibody concentration is too high (e.g., 1.2 mg/mL and 100 µL) adjust the concentration by diluting with 1X Modification Buffer then transfer an aliquot equivalent to 1 mg/mL and 100 µL to a new tube and proceed to 1st Buffer Exchange in the next section.

   Note: Sample volume is important because the total amount (mass) of antibody represented by that volume should be 100 µg ± 10 µg. The total amount of antibody in a sample is critical to maintaining the appropriate ratio between pre-weighed ChromaLink Biotin reagent and the antibody during the labeling procedure.

   Important: If the antibody spectrum appears altered, distorted, or contains a large baseline offset error, refer to the Troubleshooting Guide in the Appendix.

Determine Antibody Concentration (NanoDrop™ Spectrophotometer)

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 and click OK to initiate the instrument.

3. When the main menu appears, select the Protein A_{280} menu option. **Note:** Do not use the UV-VIS menu option to read a sample.

4. When the A_{280} menu appears, click off the 340 nm normalization option using the mouse. **Note:** The 340 nm normalization option is not found on all instrument models and can be ignored in those instances.

5. In the window labeled Sample Type, select 'Other protein E1%' option from the pull-down menu. Enter the appropriate E1% value (from Table 2) corresponding to your particular antibody type (i.e., species). For example, enter 14.00 for mouse IgG.

6. Place a 2 µL drop of the appropriate buffer on the clean pedestal and blank the instrument. Re-click the 'Measure' icon to validate a flat baseline has been obtained. Clean the pedestal and repeat the procedure if necessary until a suitable baseline is obtained. **Note:** Sometimes air bubbles become trapped on the pedestal causing a baseline offset. Remove air bubbles and rescan to ensure a proper baseline is obtained.

7. Clean the pedestal and place a 2 µL volume of antibody solution on the pedestal. Click the 'Measure' icon. When the spectrum (220–350 nm) appears in the window, read the resulting concentration directly from the [mg/mL] window display. **Note:** It is not necessary to recover the 2 µL sample aliquot from the pedestal after the scan.
8. Record the antibody concentration and total sample volume. If the antibody concentration is 1 ± 0.1 mg/mL and approximately 100 µL proceed to 1st Buffer Exchange in the next section.

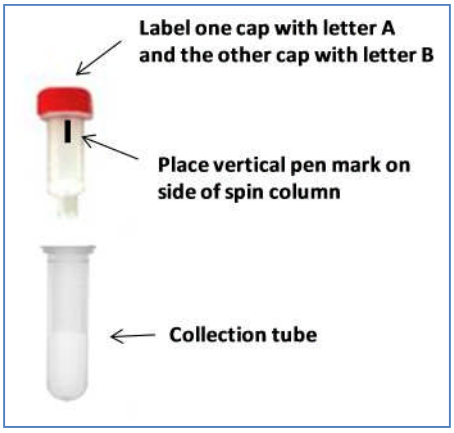
   Note: Obtain additional antibody if a sample contains less than 90 µg (e.g., 0.85 mg/mL and 100 µL). If the measured antibody concentration is too high (e.g., 1.2 mg/mL and 100 µL), adjust the concentration by diluting with 1X Modification Buffer then transfer an aliquot equivalent to 1 mg/mL and 100 µL to a new tube and proceed to 1st Buffer Exchange in the next section.

   Note: Sample volume is important because the total amount (mass) of antibody represented by that volume should be 100 µg ± 10 µg. The total amount of antibody in a sample is critical to maintaining the appropriate ratio between pre-weighed ChromaLink™ Biotin reagent and the antibody during the labeling procedure.
9. **Important:** If the antibody spectrum appears altered, distorted, or contains a large baseline offset error refer to the Troubleshooting Guide in the Appendix.

C. 1st Buffer Exchange (10 min)

Once a sample is confirmed to be at 1 ± 0.1 mg/mL and 100 µL, buffer exchange the sample as follows:

1. Prepare two Zeba™ spin columns (red caps) by twisting off the bottom closures and loosening the red caps (do not remove). Place each spin column into a collection tube (provided).
2. Mark the top of one cap with the letter **A** and the other cap with the letter **B** using an indelible pen. 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.
4. Centrifuge at **1,500 x g for 1 min**. Discard the flow through from the collection tube. The column matrix (resin bed) will appear white in color. Place the column back into the empty collection tube.

> **Important:** Rotor speed must be set to 1,500 x g (RCF) and not 1,500 x rpm (RPM). The volume recovered from the spin column should always be approximately the same volume that was originally loaded (e.g., 100 ± 10 µL). If the recovered volume is low, the centrifuge may need recalibration.

5. Add 300 µL **1X Modification Buffer** to the top of resin bed **A** and 300 µL 1X PBS to top of resin bed **B**. Loosely recap.
6. Place the assembly in the centrifuge and orient the vertical mark as before. Centrifuge at **1,500 x g for 1 min**. Discard the flow through from the bottom of the collection tube.
7. Repeat steps 5 and 6 **two (2)** additional times; discarding the flow through each time. The matrix in tube **A** now contains Modification Buffer.
8. Add 300 µL **1X PBS** to the top of resin bed **B**, recap and set this spin column aside on the bench.
9. Transfer the **A** spin column to a new empty collection tube (provided). Add 100 µL at 1 mg/mL antibody to the top of the dry resin bed **A**; loosely recap.
10. Place the **A** spin column assembly in the centrifuge and orient the vertical mark on the spin column aiming **outward and away** from the center of the rotor. Place an appropriately water-filled tube opposite the **A** spin column to properly balance the rotor.
11. Centrifuge the **A** spin column at **1,500 x g for 2 min**. Transfer the **flow-through containing the antibody** from the bottom of the collection tube to a new, labeled 1.5 mL tube. Set the dry **A** spin column assembly aside as a balance tube for later use.

D. 2nd Sample Analysis (5 min)

1. Using a conventional or NanoDrop™ spectrophotometer as previously described in the 1st Sample Analysis section, measure the antibody concentration to confirm antibody recovery. **Note:** Remember to recover the antibody sample from the cuvette after a measurement.
2. If the antibody concentration is at 1 ± 0.2 mg/mL in a volume of approximately 100 µL, proceed to Biotinylation of Antibody.

   Note: A small loss of antibody is always unavoidable after buffer exchange. This loss is ~10–12% (refer to Appendix, Section F). If the antibody concentration at this juncture is ≥0.8 mg/mL in a volume 100 µL, then proceed to Biotinylation of Antibody. If the amount of antibody recovered is below this amount, **do not attempt to biotinylate the sample**.

Biotinylation of smaller quantities over-modifies the antibody, potentially leading to aggregation/precipitation or even loss of binding affinity.

E. Biotinylation of Antibody (90 min)

1. Resuspend ChromaLink Biotin reagent (reagent may not be visible) in 5 µL of anhydrous DMF. Mix.
2. Add the antibody solution (100 µL at 1 ± 0.2 mg/mL) directly to the vial of resuspended ChromaLink™ Biotin reagent (vial with purple cap).
3. Mix the solution thoroughly by pipetting up and down 10–20 times; vortex for a few sec. Briefly flash spin the contents in the centrifuge for 5 sec to collect the reaction mixture at the bottom of the tube.
4. Allow the reaction to proceed for 90 min at room temperature.
5. When the reaction is complete, quench by adding 10 µL 1 M Tris (pH 8.7). Set the quenched reaction aside. The basic pH stops the modification reaction.
6. Place the previously set-aside **B** spin column assembly containing 300 µL 1X PBS (Section C, step 8) in the centrifuge and orient the vertical mark as before.
7. Add 300 µL molecular grade water to **A** spin column (Section C, step 11) to use as a balance tube opposite the **B** spin column.
8. Centrifuge the two spin columns at **1,500 x g for 1 min**. Discard the flow-through from the bottom of each collection tube.
9. Transfer the **B** spin column to a new collection tube (provided) and proceed to 2nd Buffer Exchange.

F. 2nd Buffer Exchange (3 min)

1. Add the quenched biotinylation reaction (Section E, step 4) to the center of the dry **B** resin spin column. Recap the column loosely and place the spin column into its new collection tube.
2. Add 100 µL of molecular grade water to the **A** spin column, recap loosely and use it as a balance assembly opposite the **B** spin column in the centrifuge.
3. Orient the two spin columns in the centrifuge and spin at **1,500 x g for 2 min**. **Note:** Approximately 100 µL will be recovered from the bottom of each collection tube.
4. Transfer the biotinylated antibody (~100 µL) from the bottom of the **B** collection tube to a new 1.5 mL microfuge tube. Label appropriately and proceed to measure the degree of biotin incorporation.

G. Degree of Biotin Incorporation (10 min)

Biotin incorporation is determined by scanning the biotinylated sample in a conventional UV-VIS or NanoDrop™ spectrophotometer. Proceed as directed for the corresponding spectrophotometer.

Instructions for Spectrophotometer (Conventional UV-VIS)

1. Program the spectrophotometer to scan from 220–400 nm.
2. Using a clean semi-micro quartz cuvette (50–100 µL), blank the instrument using 1X PBS buffer.
3. Discard the blank solution from the cuvette.
4. Transfer the biotinylated antibody sample to the cuvette and scan.
5. Record the A_{280} and A_{354} from the scan.
6. Recover the biotinylated antibody sample back from the cuvette. Label the sample and store refrigerated (2–8°C).
7. Enter the A_{280} and A_{354}, E1% (see Table) and MW of the antibody into the **ChromaLink Biotin MSR Calculator**. The calculator automatically determines the biotin molar substitution ratio (MSR).

   Note: Typical MSR values range from **3–8 biotin molecules per antibody molecule**. The amount of labeled antibody recovered can range from **60 to 100 µg** and often depends on the exact starting mass.

Instructions for Spectrophotometer (NanoDrop™)

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 a clean pedestal and click 'OK' to initiate the instrument.
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 determine biotin MSR.
4. When the A_{280} menu appears, click off the 340 nm normalization option using the mouse. **Note:** The 340 nm normalization option is not found on all instrument models and can be ignored in those instances.
5. In the window labeled Sample Type, select 'Other protein E1%' option from the pull-down menu. Enter the appropriate E1% value (see Table 2) corresponding to your antibody sample type (e.g., mouse IgG = 14.00).
6. Clean the pedestal and blank the NanoDrop™ spectrophotometer with 2 µL 1X PBS.
7. Re-click the 'Measure' icon to validate that a flat baseline has been obtained. Clean the pedestal and repeat the blank procedure if necessary until a flat baseline is observed.
8. Place 2 µL biotinylated antibody solution (Section F, step 4) on the clean pedestal and click the 'Measure' icon. Wait until the spectrum (220–350 nm) appears in the window.
9. Record the A_{280} directly from the Abs. window.
10. Obtain the A_{354} from the scan by manually entering the numeric value '354' into the λ window and reading the resulting value displayed in the Abs. window. Record the value.

11. Enter the A_{280} and A_{354} along with the E1% and the M.W. of your antibody into the **ChromaLink Biotin MSR Calculator**. The calculator automatically determines the biotin molar substation ratio (MSR).

    Note: Typical MSR values range from 3–8 biotin molecules per antibody molecule. The amount of labeled antibody recovered can range from 60 to 100 µg and often depends on the exact starting mass.

Chapter 3: Appendix

A. Biotinylated IgG Control

The ChromaLink™ Biotin One-Shot Antibody Labeling Kit comes with a pre-labeled **biotinylated antibody control**. This control consists of a lyophilized biotinylated bovine IgG at a precisely known biotin molar substitution ratio (see Certificate of Analysis). This control can be used to validate the accuracy of a given spectrophotometer or NanoDrop™, and to validate MSR measurements. To use the control, proceed as follows:

1. Using a semi-micro quartz cuvette (50–100 µL), record a "blank" buffer spectrum using 1X PBS (220–400 nm). Discard the blank buffer solution from the cuvette. **Note:** If using a NanoDrop™, follow the instructions in the manual.

2. Resuspend the control (lyophilized biotin-IgG, 100 µg) using 100 µL molecular grade water and pipetting the solution up and down for at least a min to fully dissolve the antibody to 1.0 mg/mL.

3. Centrifuge the control very briefly at low speed (30 sec at 1,500 x g).

4. Remove an aliquot (2.0 µL) and scan the biotinylated antibody.

5. Record the A_{280} and A_{354} values obtained from the scan.

6. Enter those values along with the E1% (12.40) and M.W. of the antibody (150 kDa) into the **ChromaLink Biotin MSR Calculator** to determine the biotin molar substitution ratio of the control.

7. Confirm the value obtained with the MSR found in the certificate of analysis.

B. HABA Assay vs. ChromaLink™ Spectrophotometric Assay

Comparisons between the HABA assay (2-4'-hydroxyazobenzene-2-carboxylic acid dye-binding assay) and the ChromaLink™ spectrophotometric assay are summarized in **Table 3**. These results reveal significant differences between the two biotin assays. For example, we find that the biotin molar substitution ratio using the HABA dye-binding assay is typically 33%, the value obtained using the ChromaLink™ assay.

ChromaLink Biotin Reagent (mole-equivalents in labeling reaction)	Number biotins/IgG (HABA Assay)	Number biotins/IgG (ChromaLink™ Assay)
5x	1.03	2.45
10x	1.60	4.71
15x	2.22	6.25

Table 3. Comparison of HABA and ChromaLink™ assay for determination of biotin molar substitution ratio (MSR). Bovine IgG samples were biotinylated at different reagent equivalents. Biotin incorporation was determined using each method on the same sample and the results tabulated.

As shown in the table, the HABA dye-binding assay tends to **underestimate** the true biotin molar substitution ratio. This occurs because the HABA assay measures the number of biotin molecules available for binding to streptavidin and not the absolute number of biotin molecules attached to the antibody surface. For example, two biotin molecules in proximity to each other will bind to only one streptavidin molecule, under-representing the number of biotin labels. Other differences include the fact that the ChromaLink™ method is a non-destructive assay whereas HABA is destructive. The HABA assay consumes and destroys up to 75 micrograms of biotinylated antibody per assay whereas the ChromaLink™ method uses as little as 1 µg (NanoDrop™) of recoverable antibody. The HABA assay also requires an external streptavidin/biotin calibration curve whereas the ChromaLink™ method requires no such calibration curve.

C. Biotinylation of Antibodies: Some Examples

One-Shot Biotinylation of Goat Anti-Mouse IgG (Example 1)

A goat anti-mouse IgG (100 µg lyophilized solid) was dissolved in 100 µL 1X Modification Buffer (100 mM sodium phosphate, 150 mM NaCl, pH 8.0) to yield a 1 mg/mL solution. The sample was scanned using a NanoDrop™ spectrophotometer **(Figure 4).** The shape of the spectrum appears ideal with a flat baseline at 350 nm. The initial concentration was calculated to be 0.93 mg/mL (E1% value of 13.60). After the 1st buffer exchange, the sample was scanned to yield a 0.87 mg/mL solution **(Figure 5).** The sample was then labeled with ChromaLink™ Biotin reagent for 60 min, quenched, and excess reagent removed with a second spin column (1X PBS). The labeled sample was then rescanned (220–350 nm) (**Figure 6)**. The calculated molar substitution ratio was 7.0 with 62 µg of recovered antibody.

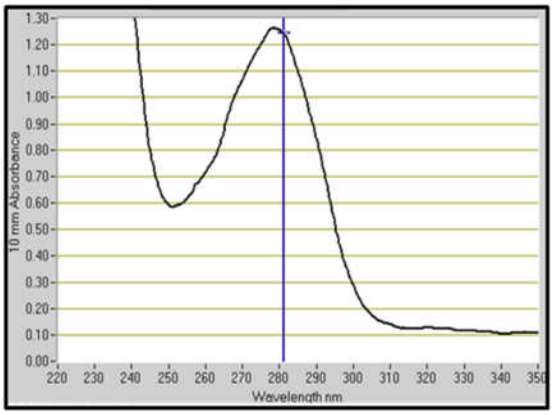


Figure 4. Antibody before buffer exchange.

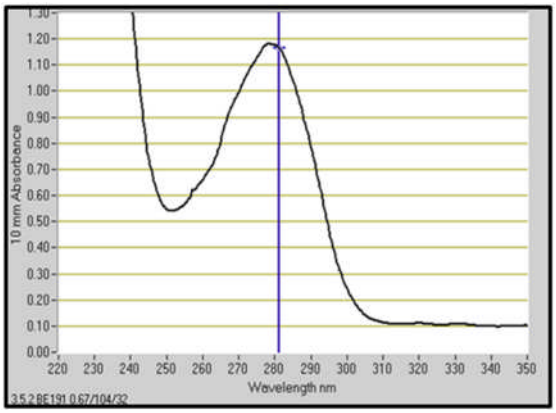


Figure 5. Antibody after buffer exchange.

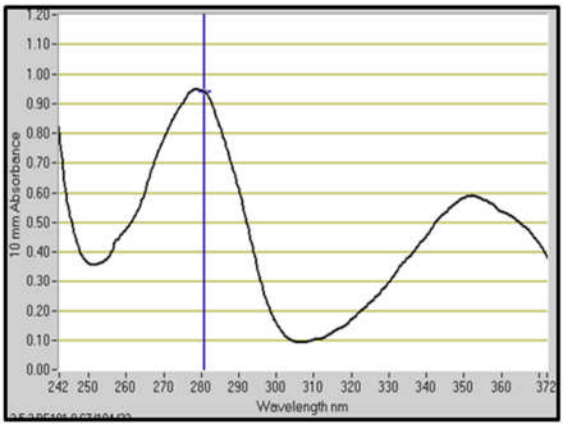


Figure 6. Antibody after biotinylation.

One-Shot Biotinylation of Rat Anti-Mouse IgG_1 (Example 2)

A commercial rat anti-mouse IgG_1 (500 µg lyophilized solid) was dissolved in 500 µL PBS to obtain a 1 mg/mL solution. An aliquot (2 µL) of the sample was scanned on a NanoDrop™ spectrophotometer **(Figure 7).** The shape of the spectrum appears ideal with a flat baseline at 350 nm. The initial concentration was determined to be 0.80 mg/mL (E1% value of 14.00). After the 1st spin column, the sample was rescanned to obtain a sample concentration of 0.99 mg/mL solution **(Figure 8).** The sample was then labeled with ChromaLink™ Biotin reagent for 60 min, quenched and excess reagent removed with a second spin column (1X PBS). The labeled sample was then rescanned (220–350 nm) (**Figure 9).** The calculated molar substitution ratio was 6.4 with 89 µg of recovered antibody.

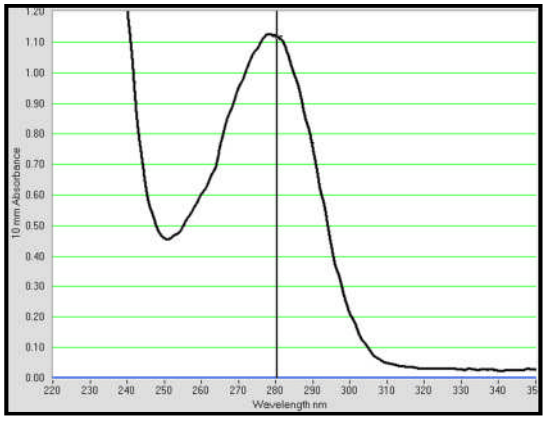


Figure 7. Antibody before buffer exchange.

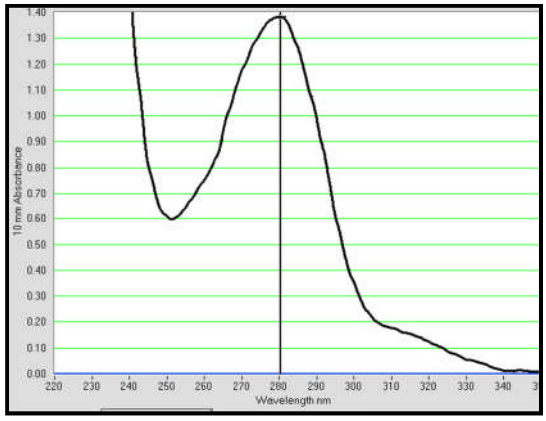


Figure 8. Antibody after buffer exchange.

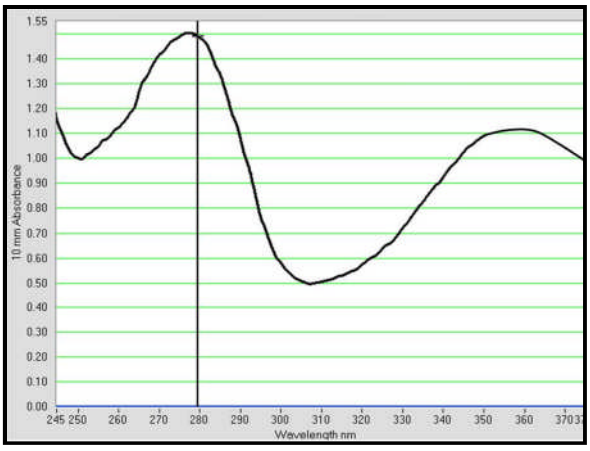


Figure 9. Antibody after biotinylation.

One-Shot Biotinylation of Rabbit Anti-Bovine IgG (Example 3)

A commercial rabbit anti-bovine IgG @ 1mg/mL in 100 µL PBS was scanned using a NanoDrop™ spectrophotometer to confirm the initial protein concentration (scan not shown). After the 1st spin column to exchange the sample into 1X Modification Buffer, the concentration was determined to be 0.95 mg/mL (E1% value of 13.5) **Figure 10**. The sample was then labeled with ChromaLink™ Biotin reagent for 60 min, quenched, and excess reagent removed with the second spin column (1X PBS). The biotin-labeled sample was then rescanned (220–350 nm) as shown in **Figure 11**. The final biotinylated rabbit IgG sample had a calculated molar substitution ratio of 6.6 with 82 µg of recovered antibody.

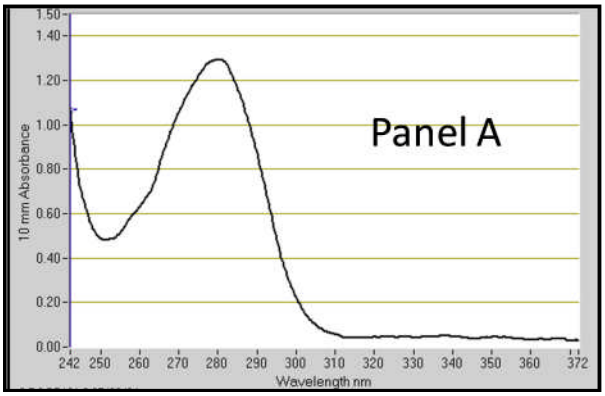


Figure 10. Antibody after 1st buffer exchange.

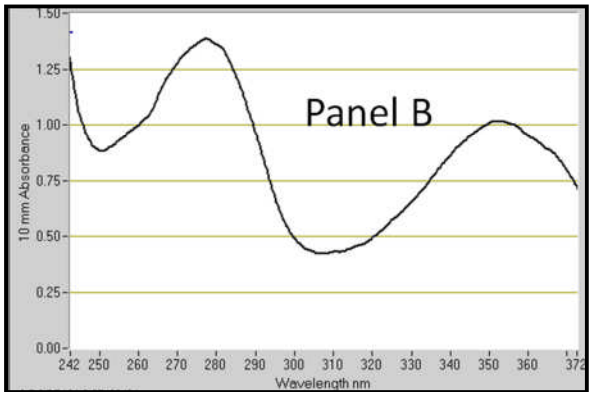


Figure 11. Antibody after biotinylation.

D. Troubleshooting Guide

Many of the problems associated with labeling antibodies arise from an inaccurate or incorrect antibody concentration at the start of the procedure. In order to avoid common problems with estimating protein concentration, the One-Shot protocol recommends using a UV-scan (220–400 nm) of the antibody sample to be labeled rather than a single wavelength measurement @ 280 nm.

A scan provides greater information (and assurance) that sample concentration is accurate, because a spectrum can reveal the presence of undesirable additives that interfere with the sample's true A_{280}. Distortions to an antibody's intrinsic spectrum or other spectral aberrations (e.g., a baseline offset) can lead to problems estimating true antibody concentration. Inaccurate or unknown protein concentrations are likely to lead to poor labeling results because it impacts the final stoichiometry between antibody and ChromaLink™ reagent equivalents during the labeling reaction.

Concentration errors are often associated with antibody preparations that contain additives or preservatives. A host of additional factors can also affect the accuracy of a measured concentration, including:

Antibody samples containing preservatives (e.g., sodium azide or thimerosal)

Antibody samples containing protein-based additives (e.g., BSA or gelatin)
Antibody samples containing unknown concentrations of some additive
Antibody samples containing protease contamination
Buffer blank is unknown or cannot be reproduced (baseline offset)
Antibody samples are under-filled by the vendor or improperly resuspended back into solution by the user
Antibody samples are sometimes over-filled by a vendor
Spectrophotometer is not calibrated properly (e.g., lamp output may be low)

In the following examples we provide reference spectra for comparison to your samples. These are intended to aid the troubleshooting process. Suggested corrective actions are included for each type of problem encountered.

Example 1: High-purity mouse IgG (lyophilized solid, no additives or preservatives)

A commercial mouse IgG (100 µg lyophilized solid) was resuspended in 100 µL 1X Modification Buffer @ 1 mg/mL solution and the sample scanned as illustrated in **Figure 12**. This sample was free of all preservatives, protein-stabilizers, and any other interfering additives. Note the "ideal" shape of the spectrum confirming both purity and concentration of the sample.

Corrective action: None. The theoretically expected A_{280} value for this mouse IgG was 1.35 vs. the experimentally measured value of 1.34 **(see E1% in Table 2**). The measured value is well within the acceptable labeling range (1 $\pm$ 0.1 mg/mL).

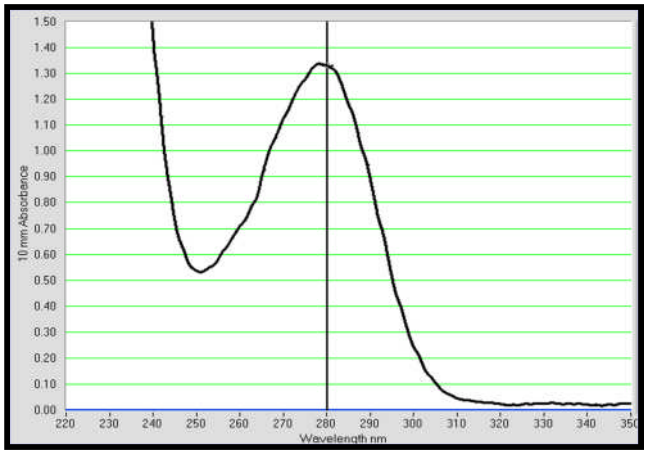


Figure 12. UV spectrum (220–350 nm) of a highly purified mouse IgG antibody @ 1 mg/mL without any additives or preservatives. Note the uniform shape and flat baseline @ 350 nm.

Example 2: Bovine IgG containing sodium azide preservative

The presence of sodium azide @ 0.05 or 0.1% in a sample of bovine IgG @ 0.9 mg/mL is illustrated in **Figure 13.** The presence of this additive primarily alters the shape of the antibody's spectrum. As seen in the figure, the presence of this additive does not alter the measurement of the sample's true

protein concentration. However, this is not always the case at high or unknown concentrations of additive. Uncertainties in the concentration of this additive may sometimes lead to large baseline offset errors when blanking the spectrophotometer making it difficult to estimate protein concentration.

Corrective action: None if sample spectra are similar to those in **Figure 13**. These samples contain no significant baseline offset errors or other spectral distortions other than attenuation and a shift of the spectrum's valley from 250 to 260 nm. Passage through the 1st spin column removes all traces of azide and allows an accurate estimation of protein concentration during the 2nd Analysis scan.

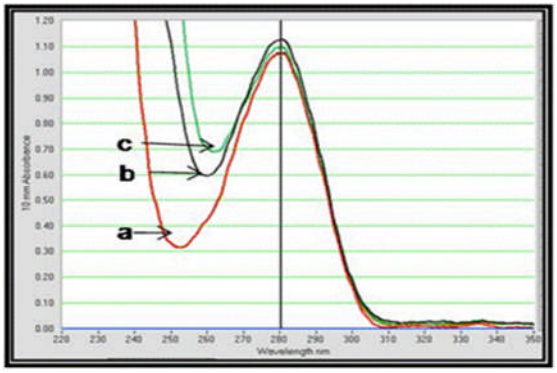


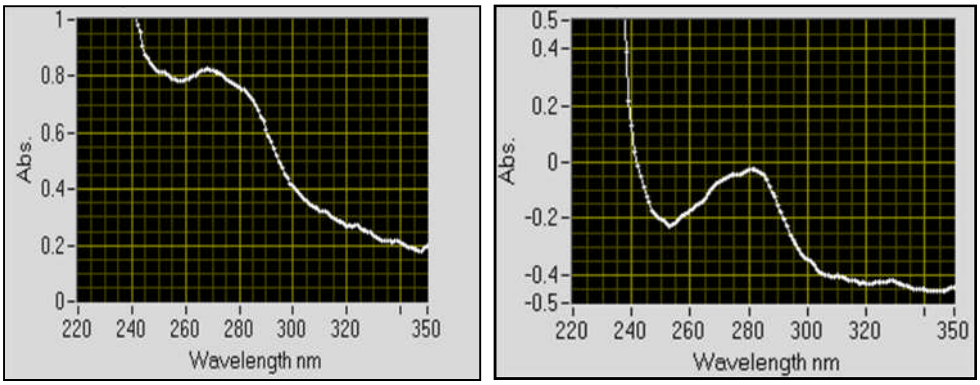
Figure 13. Superimposed spectra of purified bovine IgG @ 0.90 mg/mL with (a) no sodium azide, (b) spiked with 0.05% sodium azide, or (c) spiked with 0.1% sodium azide.

Example 3: Rat IgG containing an unknown concentration of sodium azide

Sometimes antibody samples contain high or unknown amounts of sodium azide. If the quantity of azide is not precisely known, it becomes difficult to properly blank a sample on the spectrophotometer. Unknown or high concentrations of this additive often contribute to large baseline offset errors that preclude an accurate estimate of initial protein concentration using a spectrophotometer. Two examples are illustrated in **Figure 14**.

Panel A illustrates the presence of high concentrations of sodium azide leading to both a positive baseline offset error and a distorted spectrum in a commercial antibody preparation. **Panel B** illustrates a severe negative baseline offset error caused by the presence of an unknown quantity of the preservative making true baseline normalization using a blank impossible. Both of these examples make accurate estimation of initial protein concentration impossible.

Corrective action: For samples like those illustrated in **Panel A** and **Panel B**, we recommend passing the sample through the 1st spin column and rescanning to confirm antibody concentration. If the resulting buffer-exchanged sample spectrum confirms a concentration of 1 ± 0.1 mg/mL, proceed to the biotinylation procedure. If the sample contains significantly less or more than 1 mg/mL, we recommend contacting the antibody vendor and requesting additional information on how product concentration was determined.



Panel A **Panel B**

Figure 14. Two commercial rat monoclonal antibody preparations are illustrated in Panels A and B. Panel A contains a high concentration of sodium azide. Panel B contains an unknown quantity of sodium azide. Note the baseline offset errors and distortions to the antibody spectra.

Example 4: Rabbit polyclonal IgG @ 1.0 mg/mL (incorrect blank solution)

At certain times, a scan of a commercial antibody preparation can generate a large baseline offset error. An offset error generally occurs when an incorrect buffer blank is used to blank the spectrophotometer. **Figure 15** is a scan of one such commercial preparation, where the buffer blank could not be accurately matched due to an unknown sample buffer composition. Although the spectrum may be nearly normal in shape, it nonetheless contains a large 0.6 abs. unit offset @ 350 nm. Acceptable offsets typically range from 0 to 0.1 A units @ 350 nm. In this example, the estimated protein concentration from the A_{280} was significantly higher (1.4 mg/mL) than the actual concentration (1.0 mg/mL). When using the NanoDrop™ spectrophotometer, an offset can also occur when air bubbles get trapped on the pedestal during a read.

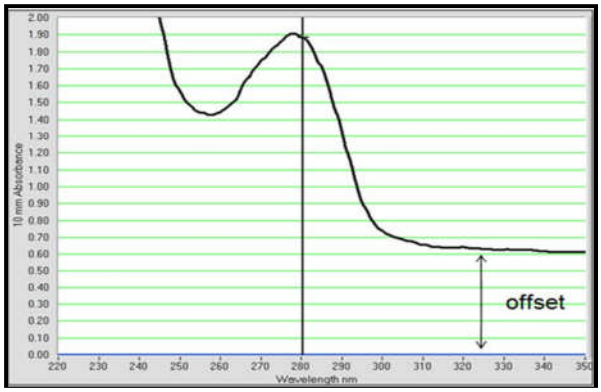


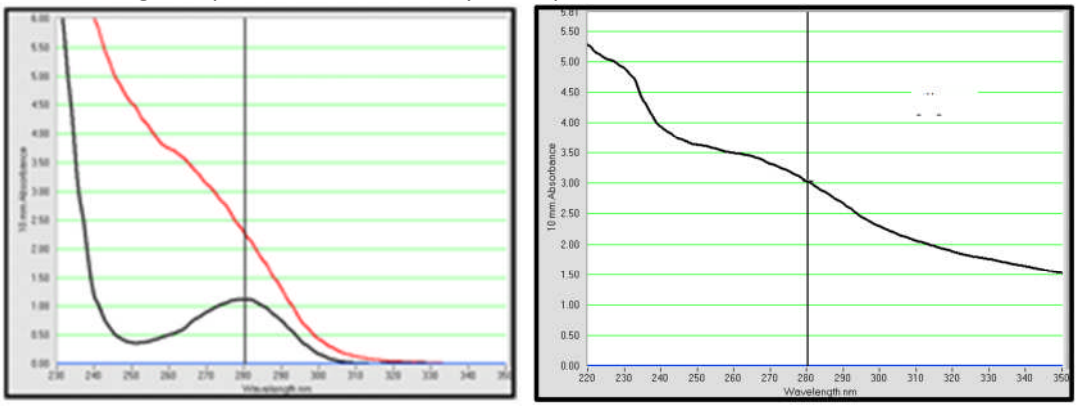
Figure 15. Baseline offset in a commercial liquid rabbit polyclonal antibody preparation sold at 1 mg/mL. Based on the measured A_{280} value from this scan, the estimated concentration was 1.40 mg/mL. The actual protein concentration after desalting the sample after the 1st spin column allowed for a proper baseline blank to be obtained and the true concentration to be determined (1 mg/mL).

Corrective action: For samples containing large baseline offset errors, we recommend passing the sample through the 1st spin column and rescanning. The 1st spin column exchanges the antibody into a known buffer composition (1X Modification Buffer), which allows for a proper blank to be established.

Example 5: Bovine IgG containing thimerosal preservative

A bovine IgG sample was spiked with thimerosal preservative as illustrated in **Figure 16** (Panel A). In this example, thimerosal was introduced @ 0.01% into 100 µL of highly purified bovine IgG sample @ 0.9 mg/mL. Note the dramatic masking influence (red trace) of the preservative over the intrinsic antibody spectrum (black trace). The presence of this preservative makes it impossible to properly blank a sample on a spectrophotometer. Small changes in this preservative's concentration create large baseline offset errors. A second thimerosal-containing sample is illustrated in Panel B. This sample contains a commercial monoclonal IgG solution @ 1 mg/mL in PBS with an undetermined quantity of thimerosal. Note the large masking effect created by the preservative.

Corrective action: We recommend passing such samples through the 1st spin column, and rescanning to confirm concentration. If the resultant spectrum appears normal and the resulting concentration is at 1 ± 0.1 mg/mL, proceed to the biotinylation procedure.



Panel A **Panel B**

Figure 16. Superimposed spectra (Panel A) of purified bovine IgG @ 0.9 mg/mL spiked with (a) no thimerosal and (b) 0.01% thimerosal. Note the masking effect of the preservative. Panel B is a commercial antibody preparation at 1 mg/mL with an unknown concentration of the preservative.

Example 6: Low Initial Antibody Concentration (Bovine IgG)

In rare cases, the amount of antibody packaged by a vendor may be lower than expected. **Figure 17** illustrates one such example. A commercial bovine IgG (100 µg solid) was dissolved in 100 µL 1X Modification Buffer. The sample spectrum indicated a concentration of 0.68 mg/mL (A_{280} = 0.84, E1% = 12.40), which was significantly lower than the expected concentration of 1 mg/mL after reconstituting in modification buffer.

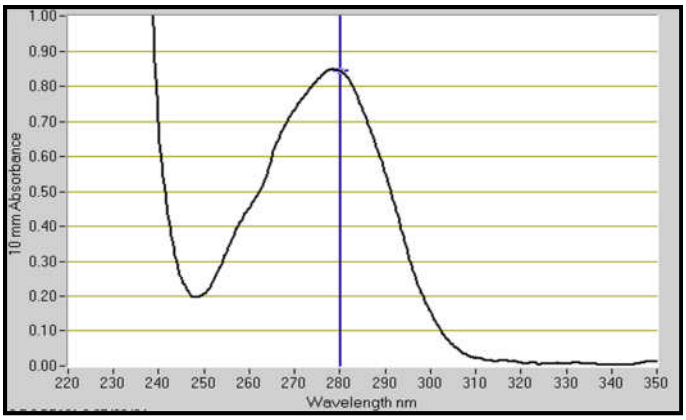


Figure 17. Scan of a commercial bovine IgG sample containing less material (68 µg) than was expected (100 µg) from the product label.

Corrective action: If a commercial antibody sample contains significantly less mass or concentration than expected, we recommend contacting the vendor and requesting additional product information. Do not attempt to label less than 90 µg of antibody using this procedure.

Example 7: Protein-Based Carriers or Additives (BSA or Gelatin)

The ChromaLink™ Biotin One-Shot antibody labeling procedure is not compatible with the presence of protein-based carriers such as BSA or gelatin. Their presence is generally detected when the expected A_{280} of an antibody preparation is much higher than anticipated, while the sample retains a normal protein spectrum or profile.

Corrective action: Contact the vendor to confirm the presence or absence of a protein additive. Do not attempt to label any product containing protein-based additives. Additives can be removed using affinity chromatography (e.g., Pierce's NAb™ Spin Columns) or other suitable methods. After affinity purification, ensure that the final protein-free antibody is desalted to remove residual glycine buffer used to elute affinity-purified material.

Example 8: Saccharide-Based Carriers (5% trehalose)

The ChromaLink™ Biotin One-Shot antibody labeling procedure is fully compatible with the presence of 5% trehalose. This additive does not interfere in any way with either a sample's spectrum or its concentration.

Corrective action: None. Proceed as directed in the procedure.

Example 9: Glycine-Based Buffers

The ChromaLink™ Biotin One-Shot antibody labeling procedure is not compatible with high concentrations of glycine buffer. This amino acid additive is sometimes found in high concentrations (e.g., 100 mM) from affinity-purified (Protein A or G) resins. Although its presence does not interfere with a sample's spectrum, it can overwhelm the exchange capacity of the spin column. Glycine is an amine contaminant that competes with the labeling reaction and therefore should be removed.

Corrective action: Remove all traces of glycine and/or other amine-containing buffers by exhaustive dialysis or proper desalting into a suitable buffer (e.g., phosphate-based buffer).

Problem	Possible Cause	Recommended Action
Poor biotin modification of the antibody	Initial protein concentration was incorrect.	Follow the recommended procedures only. Concentrate or dilute the antibody sample into the required range (i.e., 1 mg/mL and 100 µL).
	A large excess of non-protein amine contaminants are present in the antibody preparation (e.g., Tris or glycine buffer).	Before labeling, remove all amine contaminants. Some samples are so overly contaminated that exhaustive dialysis or two desalting steps may be required.
	Presence of protein carrier (e.g., BSA or gelatin) contaminated the sample.	Remove and purify away all protein carriers such as BSA or gelatin by affinity or other chromatographic methods. Re-adjust the initial antibody concentration to 1 mg/mL.
	Presence of preservative or other additive may be interfering with an accurate determination of the starting protein concentration.	Do not attempt to label an antibody containing thimerosal. First, remove the preservative, then re-measure and adjust the antibody concentration to 1 mg/mL and 100 µL.
	Presence of residual sodium azide interferes with the labeling reaction.	Refer to recommended Troubleshooting Guide.
Complete failure of biotin labeling reaction		
ChromaLink Biotin was hydrolyzed	Wet or poor quality DMF/DMSO hydrolyzed the NHS ester	Use a good quality anhydrous DMF/DMSO to solubilize ChromaLink Biotin.
labeling reaction	Improper mixing of reaction components.	Make sure to properly mix the antibody-ChromaLink™ reaction mixture.
	Spectrophotometer lamp output may be low.	Use the biotinylated IgG positive control provided to validate that the spectro-photometer is functioning properly.
	Presences of amine contaminants.	Remove all amine contaminants such as glycine before labeling.
	Improper storage of ChromaLink™ Biotin reagent may have caused it to hydrolyze.	Keep ChromaLink™ Biotin dessicated at room temp. Follow all recommended procedures.
Molar substitution ratio was out of recommended range (3–8	Initial antibody concentration used was too low or too high.	Make sure to properly estimate the initial antibody

biotins/antibody)	Antibody may have precipitated due to over-modification of available lysine residues.	concentration. Concentrate or dilute the antibody sample into the recommended range, (1 mg/mL in 100 µL), before proceeding. Insufficient mass of antibody.
Low antibody recovery and/or sample precipitation	Antibody may have aggregated/ precipitated during labeling. Incorrect antibody concentration antibody was over-modified. Zeba™ column recovery problem.	Make sure to add 1 M Tris quench buffer to the labeled sample before final desalting. Follow the recommended guidelines. Use a calibrated variable-speed centrifuge and spin at recommended speeds and times.

E. Relationship Between Molar Substitution Ratio & ELISA Sensitivity

The ChromaLink™ Biotin One-Shot Antibody Labeling Kit was optimized to incorporate between 3 and 8 biotins per antibody molecule. These and other levels of biotin incorporation were evaluated in direct and sandwich ELISA assays.

Direct ELISA

A goat anti-bovine IgG antibody was biotinylated using ChromaLink™ Biotin to generate a series of different molar substitution ratios. Biotinylated antibodies were then used to detect plate immobilized antigen (bovine IgG) using a standard direct ELISA procedure. Purified bovine IgG was immobilized (4 hr @ RT) in a 2-fold dilution series (0.5–5,000 ng/mL) on the plate. After immobilization, the wells were blocked with 1% casein/PBS and subsequently washed. The complex was then incubated with streptavidin-HRP @ 1 µg/mL for 60 min. After 3 washes, TMB substrate (3,3',5,5'-tetramethylbenzidine) was added for 20 min. Signals were measured on a Molecular Devices SpectraMax Plus plate reader @ 650 nm. Resulting dose response curves are illustrated in **Figure 18.**

Results: Signal/noise increased approximately 2.9 fold (linear portion of the curve) as the MSR increased from 1.3 to 6.1. No antigen controls were constant across the various MSRs (data not shown).

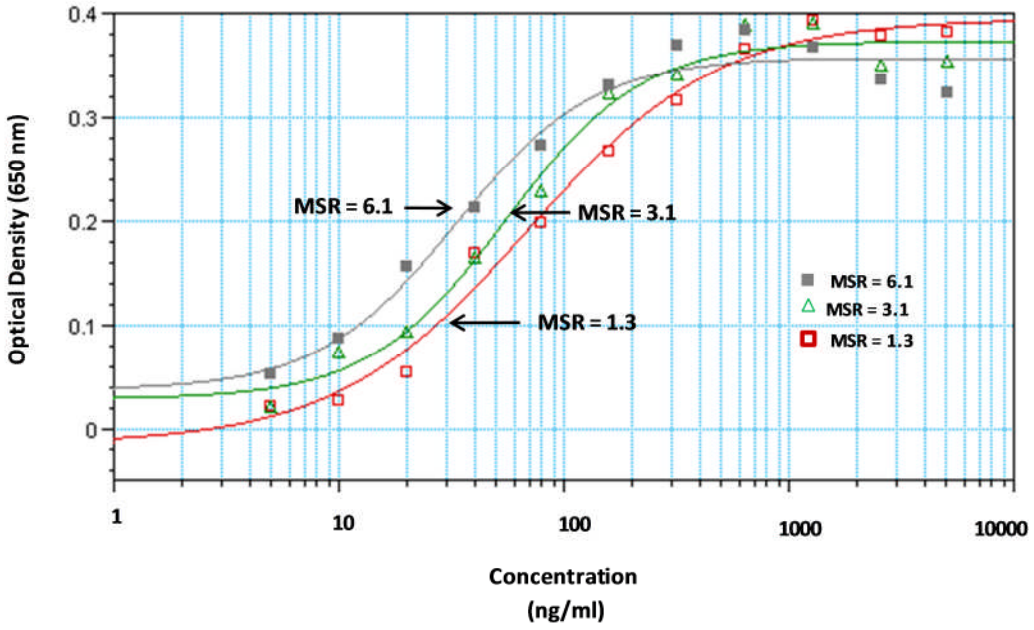


Figure 18. Dose response curves illustrating the relationship between antigen concentration and signal (650 nm) at three separate biotin molar substitution ratios.

To further illustrate the effect of biotin MSR on background-corrected signal in a direct ELISA format, fixed antigen concentrations (i.e., 2 ng/well) were detected with the same detector antibody labeled at 5 different molar substitution ratios (**Figure 19)**.

Results: Background-corrected signal increased almost 2.9 fold as the MSR went from 1.3 to 6.1. Note the slight reduction in signal as the MSR went beyond 6.1.

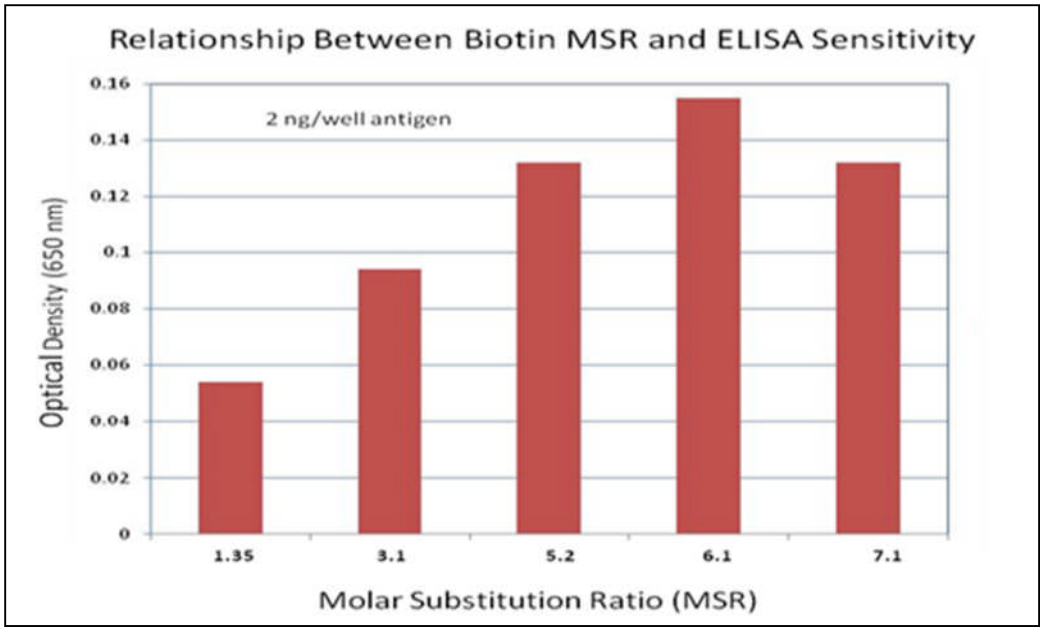


Figure 19. Background-corrected direct ELISA signals generated from the same detector antibody labeled at five different biotin molar substitution ratios. Each well was coated with the same fixed quantity of immobilized antigen (i.e., 2 ng per well). Note the gradual increase in signal (~ 2.9 fold) as the MSR went from 1.3 to 6.1.

Sandwich ELISA

A sandwich ELISA assay was performed by immobilizing IL-2 capture antibody in a 96-well plate. After blocking and washing wells, each was incubated with a 2-fold serial dilution of recombinant antigen for 1 hour along with appropriate negative controls (not shown). After washes, an IL-2 detector monoclonal antibody (biotinylated at a biotin MSR = 6.35) was incubated at 1 µg/mL for 1 hr. After additional washes, the biotinylated immune complex was incubated with streptavidin alkaline phosphatase (1 µg/mL for 30 min). After washes to remove excess unbound conjugate, the dose response curve was generated using pNPP substrate. Optical densities were read on a plate reader at 405 nm after 20 min in substrate **(Figure 20)**.

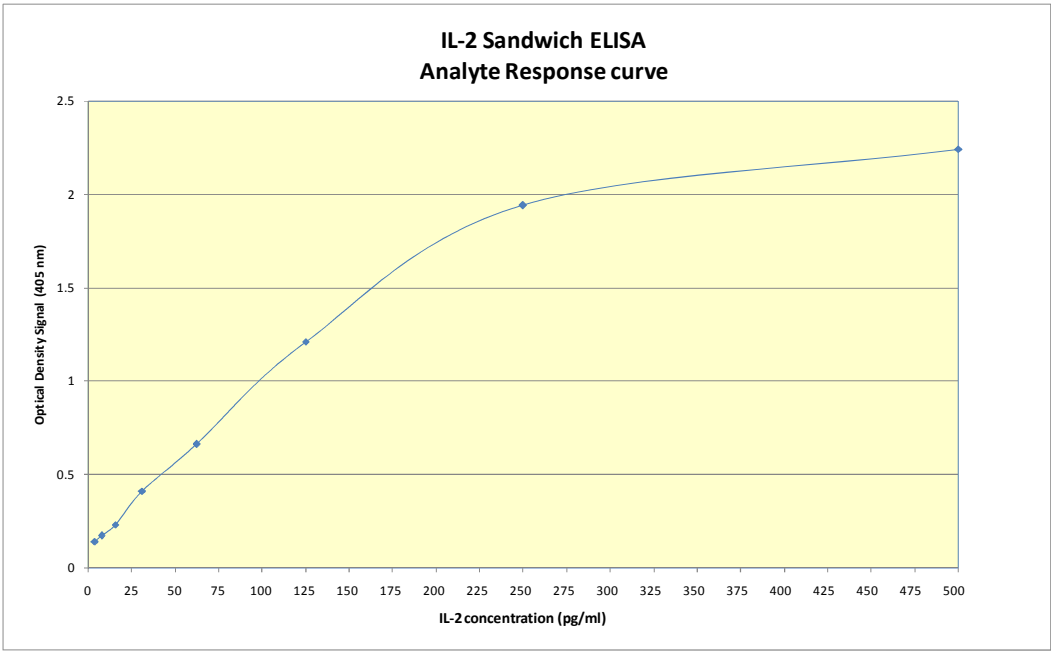


Figure 20. IL-2 specific sandwich ELISA response curve when using a biotinylated detector antibody at a biotin MSR of 6.35.

F. Spin Column Antibody Recovery Yield

Table 4 below summarizes typical antibody recovery yields obtained after two consecutive Zeba™ spin columns as per the One-Shot protocol. Three highly purified goat anti-mouse IgG samples (ranging from 80–100 µg) were resuspended in 100 µL 1X Modification Buffer. Each sample was processed using two consecutive Zeba™ spin columns as described in the procedure. Recovered protein concentrations were measured on the NanoDrop™ spectrophotometer (220–400 nm) before and after each spin column. Recovery yield averaged 86.7%.

	Initial	After 1st Spin Column	After 2nd Spin Column	% Recovery
	Concentration (mg/ml) NanoDrop™	Concentration (mg/ml) NanoDrop™	Concentration (mg/ml) NanoDrop™	
Goat IgG #1	0.80	0.75	0.65	81.25
Goat IgG #2	0.82	0.89	0.74	90.20
Goat IgG #3	0.98	0.95	0.87	88.78

Table 4. Zeba™ spin column protein recovery yield.

G. Kit Disclaimer

Although the ChromaLink™ Biotin One-Shot Antibody Labeling Kit has been used to label many types of antibodies successfully, we have observed that antibody binding affinity can sometimes be compromised or even lost as a result of the labeling process. Although rare, this phenomenon occurs because certain antibodies possess one or more critical lysine amino acid residues at the antigen-binding site (ABS) and once these are modified with biotin, they compromise binding affinity. In addition, from time to time Solulink has observed that certain monoclonal antibodies possess critical lysine residues (not necessarily at the ABS site) that are absolutely necessary for maintaining antibody stability/solubility in aqueous media. Once modified, these residues (often just one or two) lead to antibody precipitation and complete loss of sample. This phenomenon has only been observed with certain monoclonal antibodies and never with a polyclonal. Precipitation is not related to over-modification of the antibody. Our customers should be made aware that although rare, these phenomena do occur. Use of this labeling kit does not come with an implied guarantee or warranty that compromised binding affinity or precipitation will not occur.