



ChromaLink™ Biotin One-Shot Antibody Labeling Kit

(Cat. # B-9007-009K)

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I. Introduction

a. Product Description

The ChromaLink™ Biotin One-Shot antibody labeling kit is specifically designed to biotinylate a single 100 microgram quantity of antibody resuspended in 100 µl of buffer. The kit comes complete with all the necessary components to label and rapidly quantify biotin incorporation from a small quantity of antibody in about 90 minutes (see kit disclaimer, p.28 for exceptions).

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The labeling process used in the One-Shot kit is based on a patented UV-traceable linker, Sulfo-ChromaLink™ Biotin (**Figure 1**). This advanced protein biotinylation reagent contains an aromatic water-soluble N-hydroxy-sulfosuccinimidyl ester functional group (**a**), which efficiently modifies protein lysine residues in an aqueous phosphate buffer system. The linker also possesses an embedded bis-aryl hydrazone structure (**b**), which forms the compound's UV-traceable chromophore. This traceable signature enables the non-destructive and sensitive quantification of biotin (**c**), attached to the antibody. All these diverse chemical elements are linked together through a long-chain PEG3 spacer (**d**), which preserves streptavidin/biotin affinity while simultaneously enhancing antibody solubility.

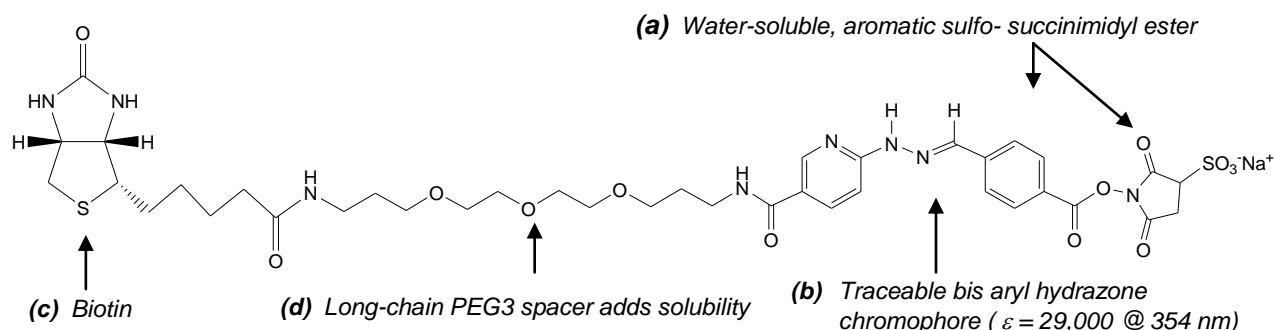


Figure 1. Structure of hBiotin (C₃₈H₄₉N₈NaO₁₃S₂) (M.W. 912.96)

b. Benefits and Features

The ChromaLink™ Biotin One-Shot kit is a convenient, cost-effective way of incorporating biotin into a small quantity (100 µg) of an antibody. After incorporation, biotin is rapidly quantified using a non-destructive UV-scan (220-400 nm). The kit features high antibody recoveries (50-80%) with consistent biotin incorporation. The degree of incorporation or molar substitution ratio (MSR) ranges from 3-8 biotin molecules per antibody when used as directed.

This kit can be used to label a diverse group of antibodies including all mammalian IgG, IgE, and avian IgY molecules. The One-Shot kit offers a robust method of both labeling and quantifying incorporated biotin starting from a small quantity of antibody. This “double-feature” provides researchers with greater confidence, consistency and reproducibility in all downstream applications.

c. Procedure Diagram

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

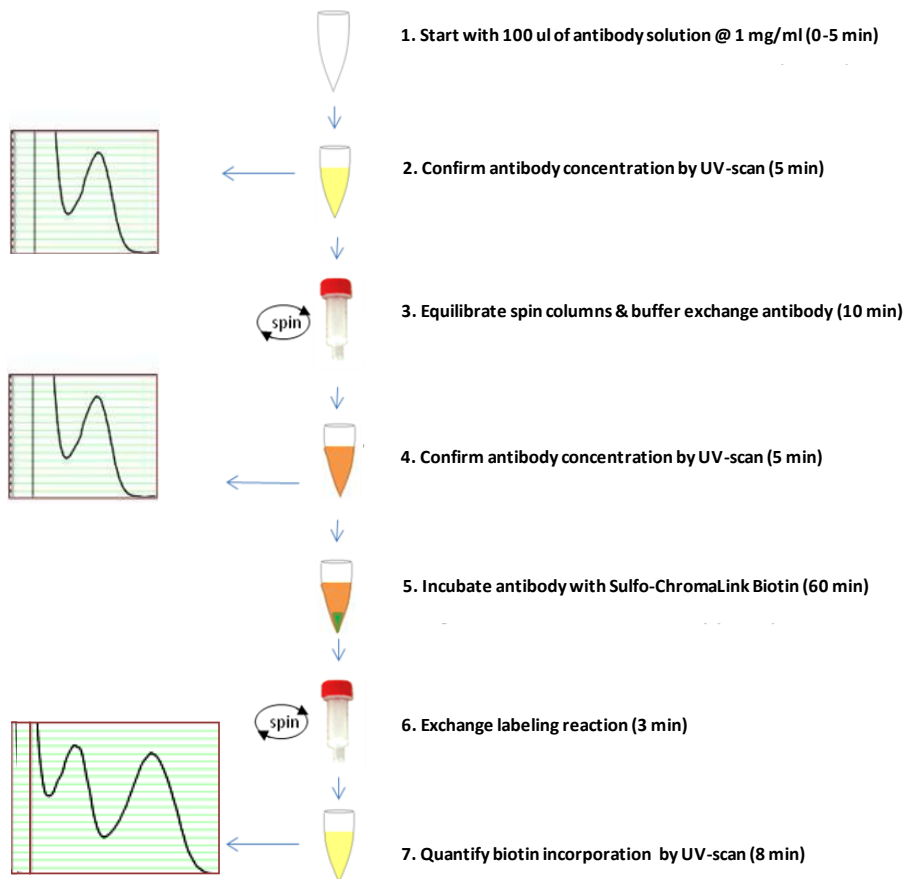


Figure 2. ChromaLink™ Biotin One-Shot antibody labeling procedure.

d. Process Summary

1. *Sample Preparation:* adjust antibody to 1 mg/ml in 100 µl buffer
2. *Sample Analysis:* confirm antibody concentration using a spectrophotometer
3. *First Buffer Exchange:* equilibrate spin columns & buffer exchange antibody
4. *Sample Analysis:* reconfirm recovered antibody concentration on a spectrophotometer
5. *Biotinylation:* label antibody with ChromaLink™ Biotin linker
6. *Second Buffer Exchange:* remove excess labeling reagent
7. *Biotin Incorporation:* quantify incorporation using a spectrophotometer

e. Important Labeling Parameters

The ChromaLink™ Biotin One-Shot antibody labeling kit is specifically designed to biotinylate a single 100 microgram quantity of antibody resuspended in 100 µl of a suitable buffer as indicated in **Table 1**.

Initial Antibody Concentration	Initial Mass of Antibody	Initial Antibody Volume
1.0 ± 0.1 mg/ml	100 ± 10 ug	100 ul

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

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The kit provides consistent and reliable biotinylation by controlling the following reaction variables:

- Initial antibody mass (100 µg) and volume (100 µl)
- Reaction buffer composition
- Reaction time (60 min)
- Reaction stoichiometry (12x equivalents)

Critical to biotin incorporation is the ability to accurately determine an antibody's initial concentration in a non-destructive manner with full recovery of the sample. The One-Shot procedure employs a spectral scan (220-400 nm) rather than a single wavelength measurement @ 280 nm to accomplish this task. Commercial antibodies often contain preservatives or other additives capable of masking or distorting the intrinsic UV spectrum of a sample. As a consequence, additives make it more difficult to **accurately** estimate protein concentration using a single wavelength (i.e. A280) measurement. A scan however, provides greater assurance that an antibody's concentration is correct since a spectrum often reveals the presence of interfering additives.

Commonly used additives may include preservatives such as sodium azide, thimerosal, protein stabilizers such as *BSA or *gelatin, or small molecule additives such as glycine and trehalose. If a commercial antibody sample contains any of these additives, please refer to the Troubleshooting guide in the Appendix to determine which materials will interfere with the labeling procedure. ***Note**-protein carriers such as BSA or gelatin must be removed before labeling can proceed

f. Materials Provided and Storage Conditions

Components	Size/Quantity	Storage Conditions
Sulfo-ChromaLink™ Biotin	8.0 µg /1	room temp
1x Modification Buffer (pH 7.4)	1.5 ml /1	room temp
1x PBS (pH 7.2)	1.5 ml /1	room temp
Collection tubes	1.5 ml /4	room temp
Zeba™ Spin Columns	0.5 ml /2	room temp
Biotinylated IgG Control	100 µg /1	room temp
1M Tris·HCL (pH 8.9)	100 µl /1	room temp
Flash drive	0.5 Gb/1	room temp

g. Additional Materials Required But Not Provided

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

II. ChromaLink™ Biotin One-Shot Antibody Labeling Protocol

a. Sample Preparation (0-5 min)

Antibodies are packaged in a variety of different physical forms including solids and liquids. Individual antibody samples vary greatly from vendor to vendor and are often sold in a variety of different sizes (e.g. 0.05 – 1 mg) and/or concentrations. Proceed as follows to prepare your sample.

Sample (Solid Form)

Initial Sample: 100 µg/vial

Resuspend the sample in 100 µl 1X Modification Buffer (pH 7.4) to yield a 1 ± 0.1 mg/ml solution and proceed directly to the Sample Analysis section (II-b).

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Initial Sample: > 120 µg/vial

Resuspend the sample in a sufficient volume of 1X Modification Buffer (pH 7.4) to yield a 1 mg/ml solution. Transfer a 100 µl volume to a new 1.5 ml microfuge tube and refrigerate the unused portion of the sample. Proceed directly to the Sample Analysis section (II-b).

Initial Sample: < 90 µg/vial

We do not recommend using the ChromaLink™ Biotin One-Shot labeling kit to label sample quantities less than 90 µg. Obtain additional material before proceeding to the Sample Analysis section (II-b).

Sample (Liquid Form)**Initial Sample: 1 mg/ml**

If the antibody sample is already in liquid form @ 1 mg/ml (100 µl) in a suitable buffer (e.g. PBS or TBS), proceed directly to the Sample Analysis section (II-b).

Initial Sample: > 1 mg/ml

Adjust the antibody concentration by diluting an aliquot to 1 mg/ml and 100 µl with 1X Modification Buffer (pH 7.4). Transfer this volume (100 µl) to a new 1.5 ml microfuge tube and refrigerate the unused portion of the concentrated sample. Proceed directly to Sample Analysis section (II-b).

Initial Sample: < 1 mg/ml

Concentrate the antibody sample to 1 mg/ml and 100 µl before proceeding to Sample Analysis section (II-b). Numerous commercial diafiltration spin filters are available for this purpose (e.g. Millipore/Amicon), Sartorius/VivaSpin). **Note**-a minimum volume equivalent to 100 µg of total antibody is required.

b. Sample Analysis (5-10 min)

After the antibody sample has been adjusted to 100 µl and 1 mg/ml, it is ready to be scanned on either a UV-VIS or NanoDrop™ spectrophotometer. This scan is used to confirm and validate initial sample protein concentration. Scan the sample as directed below for the corresponding instrument (e.g. either a UV-VIS or NanoDrop™ spectrophotometer).

Important- do not attempt to label or scan an antibody sample containing protein-based carriers such as BSA or gelatin (see Troubleshooting Guide).

b1. UV-VIS Spectrophotometer

1. Program a spectrophotometer to scan from 220-400 nm. Follow the manufacturer's instructions for using a particular instrument.
2. Using a clean semi-micro quartz cuvette, blank the instrument using the appropriate sample buffer (e.g. PBS or 1x Modification Buffer).
3. Discard the blank solution.
4. Transfer the antibody sample (100 µl @ 1 mg/ml) to the cuvette and scan.
5. Recover the sample from the cuvette by placing it into a clean 1.5 ml microfuge tube.
6. Calculate the initial antibody concentration as illustrated for the example that follows.

Example: Bovine IgG (100 µl @ 1 mg/ml) as illustrated in **Figure 3**.

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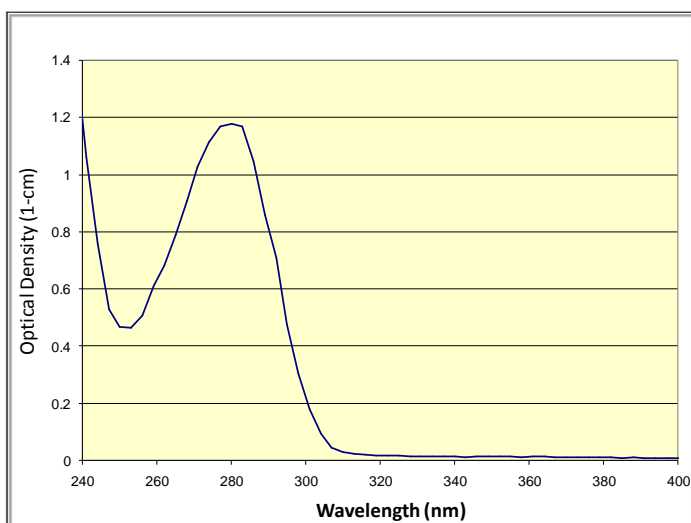


Figure 3. Scan of bovine IgG 100 µl @ 1 mg/ml in 1x Modification Buffer (100 mM sodium phosphate, 150 mM NaCl, pH 7.4) using a quartz semi-micro cuvette (50-100 µl)

Sample Calculation

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

*E1% (antibody 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. Antibody extinction coefficients (E1%) used to calculate antibody concentration. The E1% is the A280 of a 10 mg/ml solution using a 1-cm path length.

Sample: Bovine IgG 100 µl @ 1 mg/ml

A₂₈₀ (from scan in Figure 3) = 1.18

Antibody E1% (Table 2) = 12.40

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

$$[1.18 / 12.40] \times 10 \text{ mg/ml} = 0.95 \text{ mg/ml}$$

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7. After calculating a sample's concentration, adjust it to 1 ± 0.1 mg/ml and 100 μ l by addition of 1x Modification Buffer (if necessary) and proceed directly to the buffer exchange procedure (section II-c). **Note**-sample volume is important because the sample mass represented should be as close as possible to 100 μ g. The accuracy of this mass is critical to maintaining the ratio of labeling reagent to antibody. The volume should also be as close as possible to 100 μ l (e.g. ± 5 μ l) for optimal results.

Important-if a sample's spectrum does not appear similar to the example provided in **Figure 3** or if the spectrum appears altered, distorted, or contains a large baseline offset error, please refer to the Troubleshooting Guide found in the Appendix. A proper antibody spectrum is required to confirm protein concentration.

b2. NanoDrop™ Spectrophotometer

1. Turn on the NanoDrop™ spectrophotometer and click on the NanoDrop™ icon to launch the software.
2. Place a 2 μ l 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 the sample.
4. After the A280 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 (**from Table 2**) corresponding to your particular antibody sample type. For example, 14.00 for mouse IgG.
6. Blank the NanoDrop™ spectrophotometer by placing a 2 μ l drop of the appropriate sample buffer (e.g. PBS or 1x Modification) and with the mouse 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. **Note**-sometimes air bubbles can become trapped on the pedestal during pipetting and cause baseline offsets. Remove air bubbles and rescan to insure a proper baseline.
8. Transfer a 2 μ l volume of antibody solution @ 1 mg/ml to the pedestal, with the mouse click in the 'Measure' icon. Wait until the spectrum (220-350 nm) appears in the window. **Note**-there is no need to recover the 2 μ l aliquot after a scan.
9. Record the antibody concentration directly from the NanoDrop™ display window [mg/ml]. Alternately, calculate the antibody concentration (manually) as illustrated for the following example.

Example: Mouse IgG (100 μ l @ 1 mg/ml)

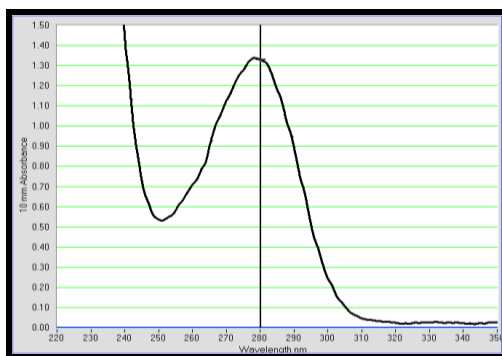


Figure 4. Mouse IgG 100 μ l @ 1 mg/ml in 1x Modification Buffer (100 mM sodium phosphate, 150 mM NaCl, pH 7.4) scanned on a NanoDrop™ (220-350 nm).

Sample Calculation

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

*E1% (antibody extinction coefficient, see Table 2)

Example: Mouse IgG @ 1 mg/ml (Fig. 4)

A_{280} (from scan in Figure 4) = 1.34

Antibody E1% value (Table 2) = 14.00

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

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

- After calculating the sample's concentration, adjust it to $1 \pm 0.1 \text{ mg/ml}$ and 100 μ l by small additions of 1x Modification Buffer (if necessary) and proceed directly to the buffer exchange procedure section (II-c). **Note**-sample volume is important because the sample mass represented should be as close as possible to 100 μ g. The accuracy of this mass is critical to maintaining the ratio of labeling reagent to antibody. The volume should also be as close as possible to 100 μ l (e.g. $\pm 5 \mu$ l) for optimal results.

Important-if a sample's spectrum does not appear similar to the example provided in Figure 4 or if the spectrum appears altered, distorted, or contains a large baseline offset error, please refer to the Troubleshooting Guide found in the Appendix. A proper antibody spectrum is required to confirm protein concentration.

c. First Buffer Exchange Procedure (10 min)

After adjusting a sample to $1 \pm 0.1 \text{ mg/ml}$ and 100 μ l, buffer exchange the antibody with the following procedure.

- Prepare the two spin columns provided by twisting off the bottom closures and loosening the red caps (do not remove caps).
- Place each spin column into separate 1.5 ml microcentrifuge collection tubes (provided).

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3. Place the spin columns opposite each other in the microcentrifuge and spin @ 1,500 x g for 1 minute to remove the storage solution from the resin. After centrifugation the column matrix will appear dry and white in color.
4. Remove the columns from the centrifuge and discard the solutions from the bottom of the collection tubes. **Note**-do not discard the collection tube.
5. Using a marker pen, place a mark on the side of each spin column where the compacted resin is slanted upward.
6. Using the same marker pen, mark the top of one cap with the letter **A** and the other spin column cap with the letter **B**.
7. Add 300 µl 1x Modification Buffer (pH 7.4) to the top of the resin bed **A** and 300 µl 1x PBS to the top of the resin bed **B**. Loosely recap the lids. **Note**-when loading the buffer or sample, do not disturb the resin bed with the pipette tip.
8. Place the spin columns back into their used collection tubes, centrifuge at 1,500 x g for 1 minute to remove the buffer. **Important**-always orient the spin column with the pen mark aiming outward and away from the center of the rotor.
9. Repeat steps 7 and 8 two additional times, discarding the flow-through buffer each time.
10. After the last spin, transfer the equilibrated spin column **A** into a new 1.5 ml collection tube (provided). **Note**-do not transfer the spin column labeled **B** into a new 1.5 ml collection tube at this time.
11. Buffer exchange the antibody sample (100 µl @ 1mg/ml) by loading the contents to the top of the equilibrated spin column **A**.
12. Add 100 µl 1x PBS buffer to the top of the equilibrated spin column **B**. Recap the lid loosely. **Note**-this column will serve as a balance tube.
13. Centrifuge the columns @ **1500 x g for 2 minutes** to collect the eluate at the bottom of the collection tubes.
14. Remove the spin column tube assembly **A** containing the antibody sample from the centrifuge and set aside. **Important**-do not discard this eluate!
15. Now remove the spin column tube assembly **B** from the centrifuge and discard the bottom eluate. Add an additional 300 µl **1x PBS** to the top of resin bed **B** to rehydrate the resin, cap and set aside for later use. **Important**-300 µl 1xPBS is necessary to keep the resin hydrated for later use.

d. Sample Analysis (5 min)

1. Using a spectrophotometer or NanoDrop™ scan the buffer exchanged antibody sample from collection tube **A** to confirm the amount of recovered antibody. **Note**- refer to previous Sample Analysis instructions (section IIb-1 or IIb-2) to calculate the recovered antibody concentration.
2. If the recovered antibody is at a concentration of **1 ± 0.2 mg/ml** in a volume of **100 ± 5 µl**, proceed directly to the biotinylation procedure below (II-e). **Note**-a small loss of antibody mass is sometimes seen after buffer exchange. This loss is generally 10% or less. If the antibody concentration at this juncture is higher than the required range (e.g. 1 ± 0.2 mg/ml), adjust the sample to 100 µl and 1 mg/ml by addition of 1X Modification Buffer before proceeding. If the antibody concentration is less than 1 ± 0.2 mg/ml, do not attempt to label the sample. See TroubleShooting Guide found in the Appendix.

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e. Biotinylation Procedure (60 min)

1. Transfer the desalted antibody solution from the bottom of collection tube **A** (100 µl @ 1 mg/ml) directly to a vial of Sulfo-ChromaLink™ Biotin labeling reagent (vial with yellow cap). **Note**-do not discard the empty collection tube, set aside for later use.
2. Mix the solution thoroughly by pipetting the solution up and down 10-20 times, vortex for a few seconds. Briefly centrifuge the contents of the tube (e.g. 5 seconds) to collect the entire reaction mixture at the bottom of the tube.
3. Allow the reaction to proceed for 60 min at room temperature.
4. After the reaction is complete, quench the reaction by addition of 10 µl 1M Tris (pH 8.7), mix well. Set the reaction aside. **Important**-do not skip addition of 1M Tris.
5. Place the previously hydrated **B** spin column assembly containing 300 µl 1x PBS (section c-15) into the centrifuge. Loosely recap the lid and properly orient in the centrifuge.
6. Now add 300 µl molecular grade water to the previously used **A** spin column, and place the properly oriented assembly opposite **B** in the centrifuge. **Note**- the **A** assembly now serves as a balance tube.
7. Centrifuge at 1,500 x g for 1 minute. Discard the flow through from each spin column assembly.
8. Transfer the **B** spin column only to a new 1.5 ml collection tube (provided). Proceed immediately to the second buffer exchange procedure (see below).

f. Second Buffer Exchange Procedure (3 min)

1. Add the entire contents of the quenched biotinylation reaction (step e-4) to the center of the compacted **B** resin spin column. Recap the column loosely.
2. Apply 100 µl of 1x Modification Buffer to the center of **A** spin column. Recap loosely. **Note**-this column now serves as the balance tube.
3. Orient the columns in the microcentrifuge and spin at 1,500 x g for **2 minutes**. **Note**-approximately 100 µl will be recovered from the bottom of each collection tube.
4. Transfer the biotinylated antibody from the bottom of **B** collection tube to a new 1.5 ml microfuge tube and cap. Proceed to determine biotin incorporation as directed in the following section.

g. Determining Biotin Incorporation (10 min)

Biotin incorporation is determined by scanning the final biotinylated antibody sample on either a UV-VIS or NanoDrop™ spectrophotometer. Proceed as directed below for the corresponding instrument.

g1. UV-VIS Spectrophotometer

1. Program the spectrophotometer to scan from 220-400 nm.
2. Using a clean semi-micro quartz cuvette (50-100 µl), blank the instrument with 1x PBS buffer.
3. Discard the blank solution.
4. Transfer the biotinylated antibody sample to the cuvette and scan.

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- Record the A280 and A354 from the spectrum.
- Recover the biotinylated sample from the cuvette by transferring the solution back to a clean 1.5 ml microfuge tube. Label the sample and store refrigerated.
- Input the A280 and A354 values along with the corresponding E1% into the Biotin MSR calculator (located on the flash drive) to automatically calculate the biotin molar substitution ratio (MSR). **Note**-typical MSR values generally range from 3-8 biotins per antibody. The amount of biotinylated antibody (mass) recovered can range from 50 to 100 µg depending on the exact starting mass and type of antibody being labeled.

g2. NanoDrop™ Spectrophotometer

- Turn on the NanoDrop™ spectrophotometer and click on the NanoDrop™ icon to launch the software.
- Place a 2 µl drop of molecular grade water on the clean pedestal, click 'OK'.
- When the main menu appears, select the A280 menu option. **Note**- do not use the UV-VIS menu option on the NanoDrop™ to read the sample.
- After the A280 menu appears, **click-off the 340 nm normalization** option using the mouse.
- 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 sample type.
- Blank the NanoDrop™ spectrophotometer by placing a 2 µl drop of 1x PBS on the pedestal and using the mouse click 'Blank'.
- Re-click the 'Measure' icon to validate that the baseline is flat and near zero. Clean the pedestal and repeat the procedure if necessary until a flat baseline is observed.
- Transfer a 2 µl volume of the antibody solution @ 1 mg/ml to the pedestal, and click 'Measure'. Wait until the spectrum (220-350 nm) appears in the window. **Note**-there is no need to recover the 2 µl aliquot after a scan.
- Record the absorbance @ 280 nm from the λ absorbance window.
- Record the absorbance @ 354 nm by typing the numeric value '354' into the λ window.
- Input the A280 and A354 and the corresponding E1% value into the Biotin MSR calculator (located on the flash drive) to automatically calculate the biotin molar substitution ratio (MSR). **Note**-typical MSRs range from 3-8 biotins per antibody. The amount of biotinylated antibody (mass) recovered can range from 50 to 100 µg depending on the exact starting mass and type of antibody being labeled.

III. Biotinylating 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 7.4) to yield a 1 mg/ml solution. The sample was scanned using a NanoDrop™ spectrophotometer (**Figure 5**). 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 spin column buffer exchange the sample was scanned to yield a 0.87 mg/ml solution (**Figure 6**). The sample was then labeled with ChromaLink™ Biotin reagent for 60 minutes,

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quenched and excess reagent removed with a second spin column (1x PBS). The labeled sample was then rescanned (220-350 nm) as shown in **Figure 7**. The calculated molar substitution ratio was 7.0 with 62 µg of recovered antibody.

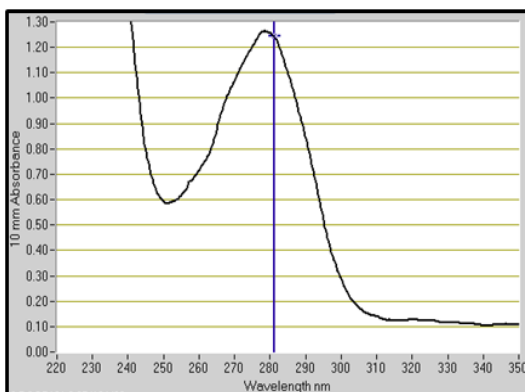


Figure 5. Antibody before buffer exchange

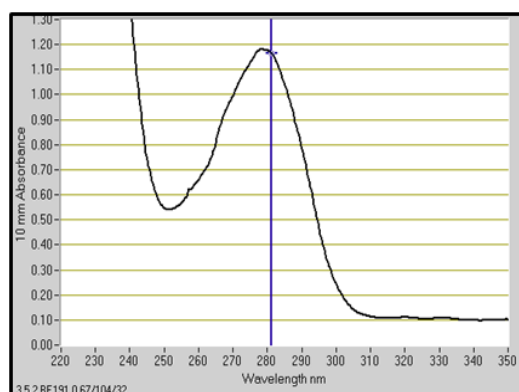


Figure 6. Antibody after buffer exchange

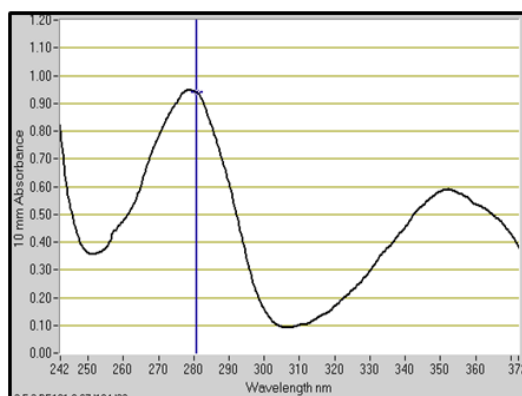


Figure 7. Antibody after biotinylation

One-Shot Biotinylation of Rat Anti-Mouse IgG₁ (Example 2)

A commercial rat anti-mouse IgG₁ (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 NanoDropTM spectrophotometer (**Figure 8**). The shape of the spectrum appeared 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 yield a sample concentration of 0.99 mg/ml solution (**Figure 9**). The sample was then labeled with ChromaLinkTM Biotin reagent for 60 minutes, quenched and excess reagent removed with a second spin column (1x PBS). The labeled sample was then rescanned (220-350 nm) as shown in **Figure 10**. The calculated molar substitution ratio was 6.4 with 89 µg of recovered antibody.

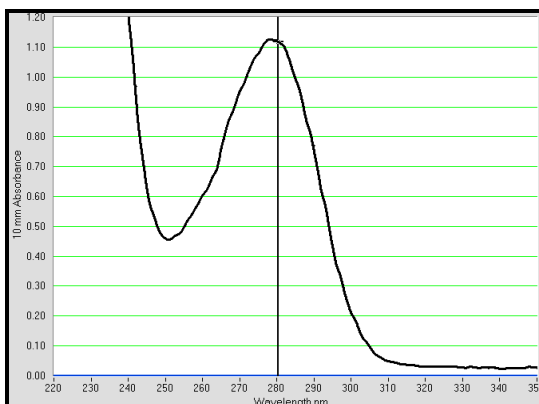


Figure 8. Antibody before buffer exchange

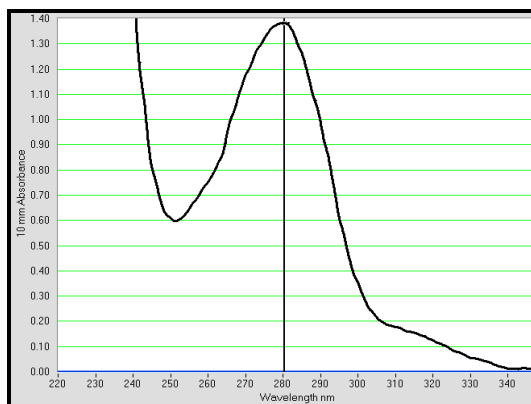


Figure 9. Antibody after buffer exchange

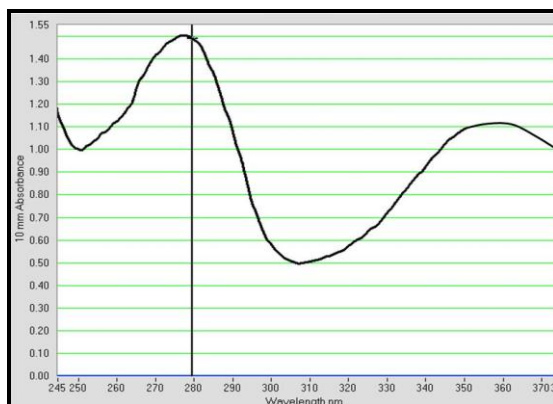


Figure 10. Antibody after biotinylation

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

A commercial rabbit anti-bovine IgG @ 1mg/ml in 100 μ l PBS was scanned using a NanoDropTM spectrophotometer to confirm the initial protein concentration (scan not shown). After the 1st spin column the concentration was determined to be 0.95 mg/ml (E1% value of 13.5) **Figure 11**. The sample was then labeled with ChromaLinkTM Biotin reagent for 60 minutes, quenched and excess reagent removed with a second spin column (1x PBS). The labeled sample was then rescanned (220-350 nm) as shown in **Figure 12**. The final biotinylated rabbit IgG sample had a calculated molar substitution ratio of 6.6 with 82 μ g of recovered antibody.

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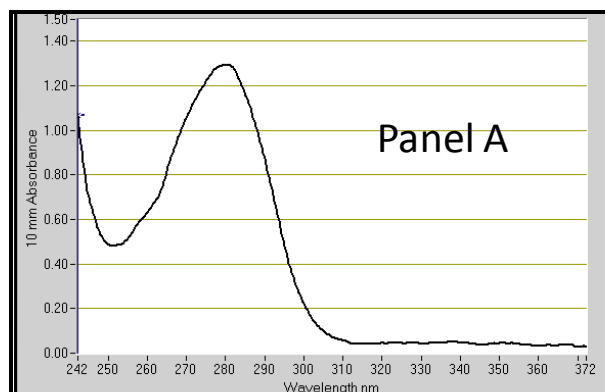


Figure 11. Antibody after buffer exchange

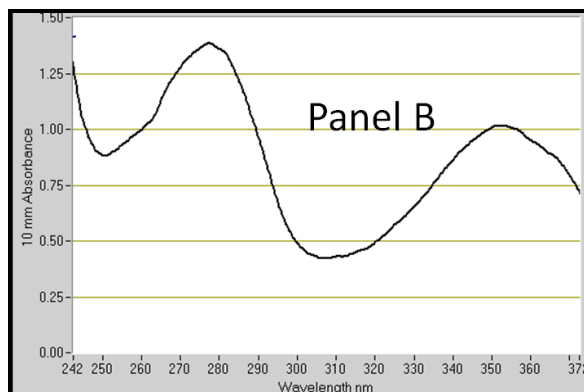


Figure 12. Antibody after biotinylation

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IV. Appendix

a. *Biotinylated IgG Control*

The ChromaLink™ Biotin One-Shot Antibody Labeling Kit comes complete with a biotinylated antibody control. This control consists of a lyophilized biotinylated bovine IgG at a precisely known biotin molar substitution ratio (refer to lot specific product data sheet on flash drive). This control is used to validate the accuracy of a given spectrophotometer or NanoDrop™ and to validate MSR calculations. To use the control proceed as follows:

1. Using a semi-micro quartz cuvette (50-100 µl) record a “blank” buffer spectrum using 1x Modification Buffer (220-400 nm). Discard the blank buffer solution from the cuvette. **Note-** follow the instructions on page 9 for the NanoDrop™.
2. Resuspend the control (lyophilized biotin-IgG, 100 µg) using 100 µl molecular grade water at 1.0 mg/ml and close the lid.
3. Centrifuge the control very briefly at low speeds (30 seconds at 1500xg).
4. Remove an aliquot and scan the biotin labeled antibody.
5. Record the A280 and A354 values generated from the spectrum.
6. Input these values along with the E1% (12.40) and M.W. (150 kD) into the biotin MSR Calculator located on the flash drive to determine the biotin molar substitution ratio.
7. Confirm the value obtained with the lot specific MSR found on the product data sheet.

b. *HABA Assay vs. ChromaLink™ Spectrophotometric Assay (CSA)*

Comparisons between the HABA assay (2-4'-hydroxyazobenzene-2-carboxylic acid dye-binding assay) and the ChromaLink™ spectrophotometric assay (CSA) are summarized in Table 3. These results reveal significant differences between the two biotin assays.

For example, the biotin molar substitution ratio using the HABA dye-binding assay is generally 1/3 the value obtained using the ChromaLink method. As seen in the table,

ChromaLink Biotin (mole-equivalents)	Biotin/IgG HABA	Biotin/IgG A ₃₅₄
5X	1.03	2.45
10X	1.60	4.71
15X	2.22	6.25

Table 3. Comparison of HABA and ChromaLink™ assays for determination of biotin molar substitution ratios (MSRs). Bovine IgG samples were biotinylated at different reaction equivalents and results tabulated.

the HABA dye-binding assay generally underestimates the true biotin molar substitution ratio because that assay measures the number of moles of biotin 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 are likely only to bind to one streptavidin molecule.

Additionally, the ChromaLink™ assay is a non-destructive assay whereas the HABA assay is destructive. HABA consumes and destroys a minimum of 75 micrograms of biotinylated antibody per assay whereas the ChromaLink™ method requires as little as 25 µg and the labeled sample is fully recoverable. Finally, the HABA assay requires an external spectrophotometric calibration curve whereas the ChromaLink™ method requires no such external calibration and measures incorporation directly from the sample's spectrum.

c. Troubleshooting Guide

This section of the manual is intended to be used as a technical troubleshooting guide. Most of the problems associated with labeling antibodies using the One-Shot procedure arise from inaccuracies in measuring initial antibody concentration. For this reason, the One-Shot protocol employs a scan (220-400 nm) to estimate antibody concentration rather than a single wavelength measurement @ 280 nm.

A scan provides greater assurance that a sample's concentration is accurate since a spectrum will often reveal the presence of A280 altering additives. These spectral aberrations are often revealed when scanning a sample but not from single point, A280 measurement. Distortions to an antibody's intrinsic spectrum or other spectral aberrations such as baseline offset errors lead to problems when estimating sample concentration. Errors of this type are likely to lead to poor labeling results because the One-Shot protocol requires precise control of the stoichiometry between antibody mass and labeling reagent (ChromaLink Biotin).

Spectral errors can also be associated with antibody preparations that contain additives or preservatives. A host of factors can affect the accuracy of the expected concentration including:

- Antibody sample contains preservatives (e.g. sodium azide, or thimerosal)
- Antibody sample contains protein-based additives (e.g. BSA or gelatin)

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Antibody sample contains an unknown concentration of some additive
Antibody (protein) becomes degraded during storage
Buffer blank is unknown or cannot be reproduced (baseline offset errors)
Antibody sample is under-filled by vendor
Antibody sample is over-filled by vendor
Improperly calibrated spectrophotometer (e.g. lamp output may be low)

In the examples that follow, we provide various spectra for reference and comparison purposes. These are intended to aid the troubleshooting process. Suggested corrective actions are also included for each type of sample spectrum problem.

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

A commercial mouse IgG (100 µg solid) was resuspended in 100 µl 1x Modification Buffer @ 1 mg/ml solution and the sample scanned as illustrated in **Figure 13**. 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 this 'ideal' sample.

Corrective action: None. The theoretically expected A280 value for this mouse IgG is 1.35 vs. the experimentally measured value of 1.34 (see **Table 4**). The measured value is well within the acceptable labeling range (1 ± 0.1 mg/ml).

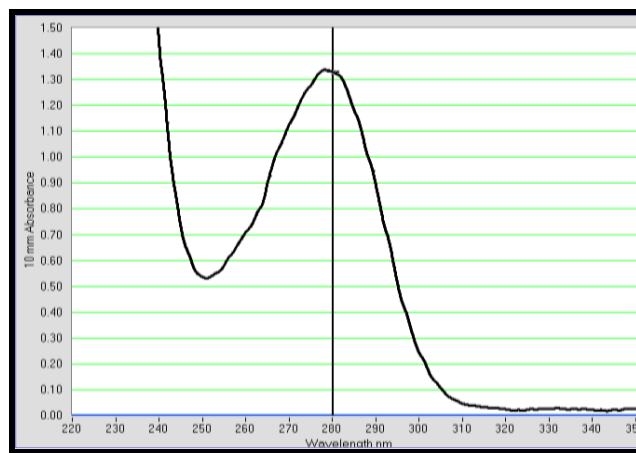


Figure 13. UV-spectrum (220-350 nm) of a highly purified mouse IgG antibody @ 1 mg/ml without any additives or preservatives. Note the uniform shape and flat baseline @ 350 nm.

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Antibody Source	Antibody A280 (1-cm path)
Human IgG	1.36 \pm 0.136
Human IgE	1.53 \pm 0.153
Rabbit IgG	1.35 \pm 0.135
Donkey IgG	1.50 \pm 0.150
Horse IgG	1.50 \pm 0.150
Mouse IgG	1.40 \pm 0.140
Rat IgG	1.40 \pm 0.140
Bovine IgG	1.24 \pm 0.124
Goat IgG	1.36 \pm 0.136
Avian IgY	1.27 \pm 0.127

Table 4. Expected A280 value of various antibody solutions (1 ± 0.1 mg/ml) for the listed antibodies (1 cm pathlength).

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 14**. 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 14**. These samples contain no significant baseline offset errors or other spectral distortions other than attenuation of the spectrum's valley and a general shift from 250 to 260 nm.

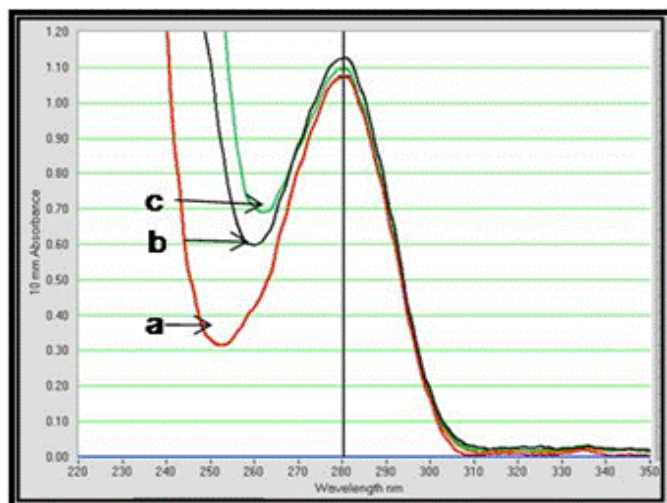


Figure 14. Superimposed spectra of purified bovine IgG @ 0.90 mg/ml with a) no sodium azide, b) spiked with 0.05% sodium azide, or c) spiked with 0.1% sodium azide.

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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 accurate estimates of initial protein concentration. Two examples are illustrated in **Figure 15**.

Panel A illustrates the presence of high concentrations of sodium azide leading to both a positive baseline offset error and a distorted antibody spectrum. Panel B illustrates a severe negative baseline offset error caused by the presence of an unknown quantity of the preservative. Both examples make an accurate estimate of initial protein concentration impossible.

Corrective Action: In both Panel A and Panel B, we recommend passing each sample through the 1st spin column and rescanning to confirm antibody concentration. After the spin column exchanges the sample into 1x Modification Buffer, rescan the sample and recalculate the resultant protein concentration. . If the resultant sample spectrum confirms a concentration of 1 ± 0.1 mg/ml, proceed to the biotinylation procedure (II-e). If the sample contains significantly less or more than 1 mg/ml than we recommend contacting the antibody vendor and requesting additional information on how product concentration was determined.

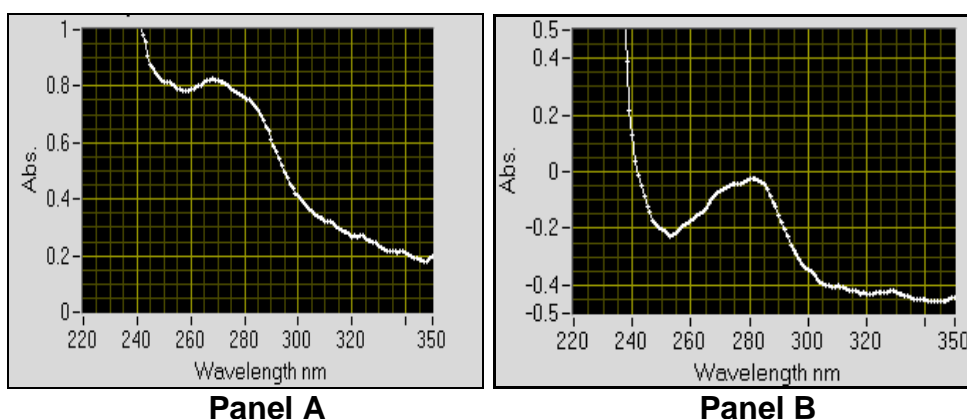


Figure 15. Two commercial rat monoclonal antibody preparations are illustrated in Panel A&B. The sample illustrated in Panel A contains a high concentration of sodium azide. The sample in Panel B contains an unknown quantity of sodium azide. Note the baseline offset errors and distortions to the spectra.

Example #4: Rabbit polyclonal IgG @ 1.0 mg/ml (improper blank solution)

At times, a scan of a commercial antibody preparation can generate a large baseline offset error. These offset errors generally occur when an improper buffer solution is used to blank the spectrophotometer. **Figure 16** is a scan of one such commercial preparation where the buffer blank could not be accurately matched. Although the spectrum may be normal in shape, it nonetheless contains a rather large 0.6 abs. unit offset @ 350 nm. Acceptable offsets typically range from 0 to 0.1 A units @ 350 nm. As a consequence, the estimated protein concentration from such a preparation is significantly higher (1.4 mg/ml) than the actual concentration (1.0 mg/ml) based on A280. When using a NanoDropTM, offsets can also occur when air bubbles get trapped on the pedestal.

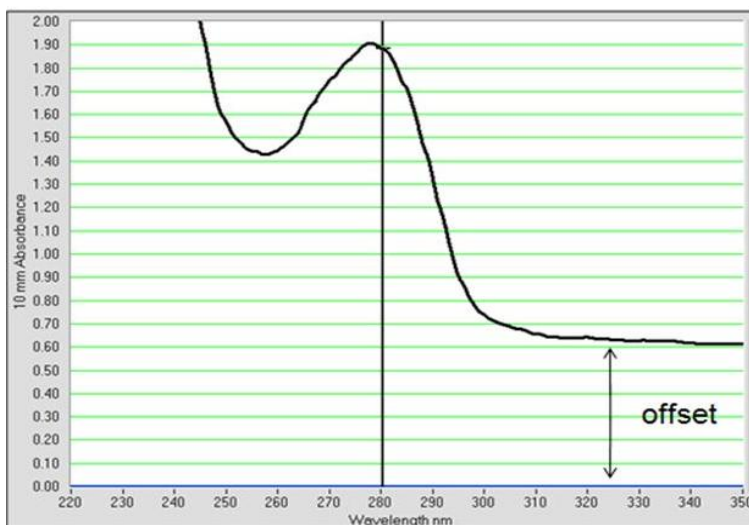


Figure 16. Baseline offset in a commercial rabbit polyclonal antibody preparation (5 mg/ml) diluted to 1 mg/ml in 1x Modification Buffer. Based on the A280 value the estimated concentration was 1.40 mg/ml. The actual protein concentration after desalting the sample on a spin column to remove the buffer blank offset was 1 mg/ml.

Corrective Action: For samples containing large baseline offset errors, we recommend passing the sample through the 1st spin column and rescanning to obtain a properly blanked sample spectrum. Since the spin column exchanges the antibody into a known buffer solution (1x Modification Buffer) it should readily remove any offset error originating from the buffer blank. After confirming sample concentration proceed to the biotinylation procedure (II-e). If after buffer exchange, it is determined that a sample contains significantly less or more than 1 mg/ml than we recommend contacting the antibody vendor and requesting additional information on how protein and package concentration/quality was determined. Always adjust sample concentration to 1 mg/ml and 100 μ l before proceeding.

Example #5: Bovine IgG containing Thimerosal preservative

A bovine IgG sample was spiked with thimerosal preservative as illustrated in **Figure 17** (Panel A). In this example, thimerosal was introduced @ 0.01% into 100 μ l of highly purified bovine IgG sample @ 0.9 mg/ml. Note the dramatic masking influence of this preservative over the intrinsic antibody spectrum. This preservative makes it impossible to properly blank a sample on the spectrophotometer. Small errors 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 @ 1mg/ml in PBS with an undetermined quantity of thimerosal. Note the large masking effect and baseline offset error created by this preservative.

Corrective action: We recommend passing the sample through the 1st spin column, and rescanning to confirm concentration. If the resultant sample spectrum confirms a concentration of 1 ± 0.1 mg/ml, proceed to the biotinylation procedure (II-e). If the sample concentration is greater than 1 mg/ml, the sample must be adjusted with 1x Modification Buffer to 1 mg/ml and 100 μ l before proceeding. If the sample contains significantly less or more than 1 mg/ml than we recommend that you contact your antibody vendor and request additional information on how product concentration was determined.

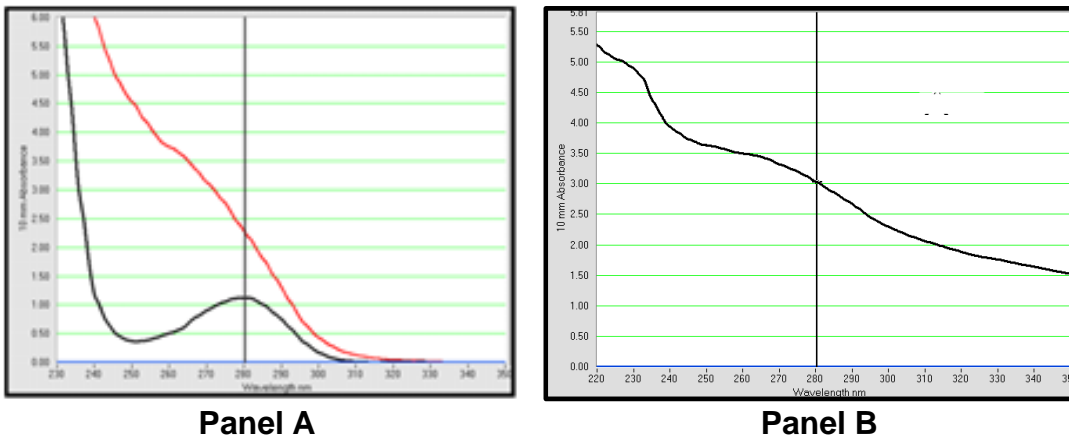


Figure 17. Superimposed spectra (Panel A) of purified bovine IgG @ 0.9 mg/ml spiked with a) no thimerosal or b) 0.01% Thimerosal. Note the masking effect of the preservative. Panel B is a commercial antibody preparation at 1 mg/ml with an unknown concentration of the preservative.

Example #6: Low Initial Antibody Concentration (Bovine IgG).

In rare cases, the amount of antibody packaged by the vendor may be lower than expected. **Figure 18** illustrates an example of a commercial preparation of bovine IgG (100 µg solid) that 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 is significantly less than the expected concentration (1 mg/ml.).

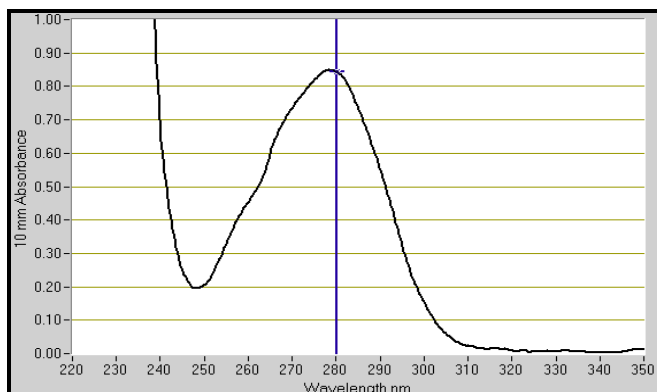


Figure 18. A commercial bovine IgG sample containing significantly less antibody (68 µg) than expected from the original package label.

Corrective action: If a commercial antibody sample contains significantly less mass or concentration than expected, we recommend you contact the vendor and request additional product information. Do not attempt to label less than 80 µg of buffer exchanged 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 A_{280} is much higher than anticipated while the sample retains its 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, insure that the final protein-free antibody is desalted to remove any excess glycine buffer used for elution of the antibody from the affinity column.

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). 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 must be removed.

Corrective Action: Remove all traces of glycine and/or other amine-containing buffers by exhaustive dialysis or properly desalting the sample into a 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 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 any 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 actions in the Trouble-shooting Guide
Complete failure of biotin labeling reaction	-improper mixing of reaction components -improper operation of the spectrophotometer -presences of amine contaminants	-make sure to mix the antibody-ChromaLink™ reaction mixture completely -use the biotinylated IgG positive control provided to validate the spectro-photometer

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	-improper storage of the ChromaLink™ Biotin reagent may have caused it to hydrolyze -failure to follow the recommended protocol	-remove all amine contaminants such as glycine before labeling -store ChromaLink™ Biotin dessicated at room temp -follow the recommended labeling protocol
Molar substitution ration was out of recommended range (3-8 biotins/antibody)	-initial antibody concentration used was too low or too high. -antibody may have precipitated due to over-modification of available lysine residues	-make sure to properly estimate the initial antibody concentration -concentrate or dilute the antibody sample into the recommend range (1 mg/ml in 100 µl) before proceeding -follow the recommended protocol
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 1M Tris quench buffer to the labeled sample before final desalting -follow the recommended guidelines -always use a calibrated variable-speed centrifuge and spin at exactly 1.5xg for the indicated time, spins at lower or higher speeds can compromise recovery

d. 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. This level of incorporation was evaluated in both direct and sandwich ELISA assays.

Direct ELISA

A goat anti-bovine IgG antibody was biotinylated using ChromaLink™ Biotin across a multitude of different molar substitution ratios. The biotinylated antibodies were then used to detect immobilized antigen (bovine IgG) using a standard ELISA procedure. Purified bovine IgG was immobilized (2-fold dilution series) (0.5 - 5,000 ng/ml). After immobilization (4 hr @ RT), the wells were blocked with 1% casein/PBS and subsequently washed. The immobilized antigen was then incubated with streptavidin-HRP @ 1 µg/ml for 60 minutes. After washes, TMB substrate (3,3',5,5'- tetramethylbenzidine) was added for 20 minutes. Signals were measured on a Molecular Devices SpectraMax Plus plate reader @ 650 nm. Direct ELISA dose response curves were plotted as illustrated in Figure 19.

Results: Signal/noise increased approximately 2.9-fold (linear portion of the curve) as the MSR increased from 1.3 to 6.1. Background controls were constant across the various MSRs (data not shown).

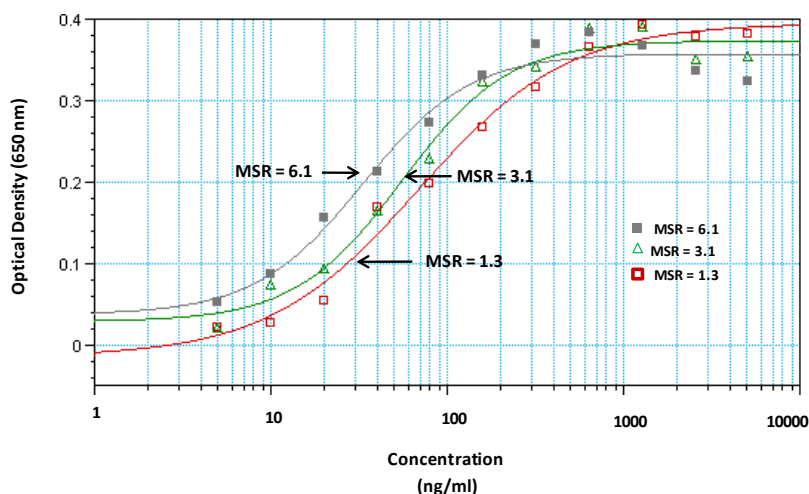


Figure 19. Direct ELISA response curves illustrating the relationship between biotin molar substitution ratio and direct ELISA signals @ 650 nm.

To further illustrate the Signal vs. MSR relationship, plots were generated at a single fixed antigen concentration (e.g. 2 ng/well) across a range of molar substitution ratios (Figure 20).

Results: Measured signal/noise increases almost 2.9-fold as the MSR goes from 1.3 to 6.1. Note the slight reduction in signal as the MSR goes beyond 6.1 probably due to over-modification of the antibody.

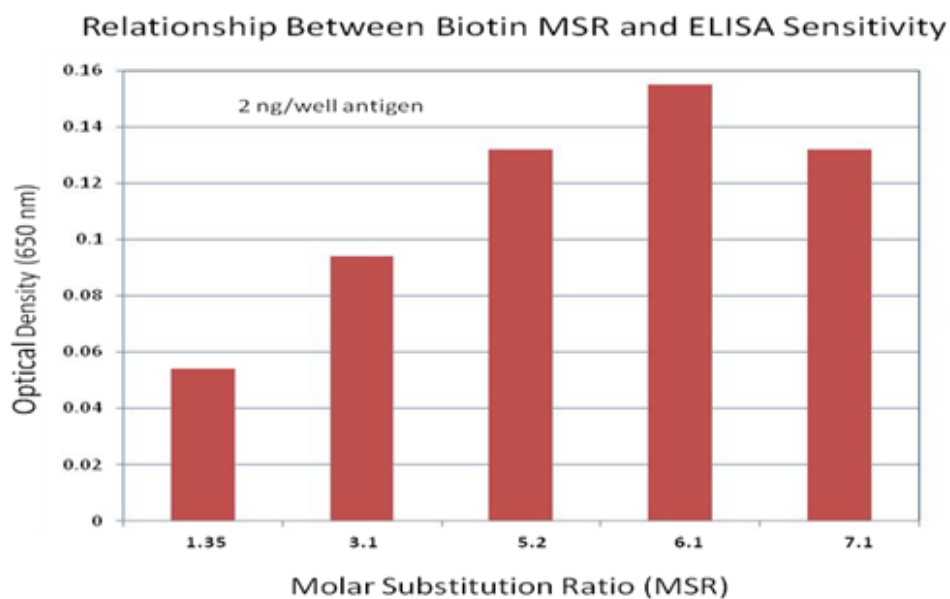


Figure 20. Background corrected direct ELISA signals at a fixed quantity of immobilized antigen (i.e. 2 ng per well) vs. MSR. Note the gradual increase in S/N (~ 2.9-fold) as the MSR increases from 1.3 to 6.1.

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Sandwich ELISA

Sandwich ELISA assays were performed by immobilizing IL-2 capture antibody in a 96-well plate. After blocking and wash steps, each well was incubated with a 2-fold dilution of analyte for 1 hour along with negative controls. After washes, a second monoclonal antibody (biotinylated at the indicated MSRs) was incubated @ 1 µg/ml for 1 hour. After washes, wells were incubated with streptavidin alkaline phosphatase for 30 minutes. Substrate (pNPP) signals were read @ 405 nm after 20 minutes. Sandwich ELISA response curve for IL-2 is summarized in Figure 21.

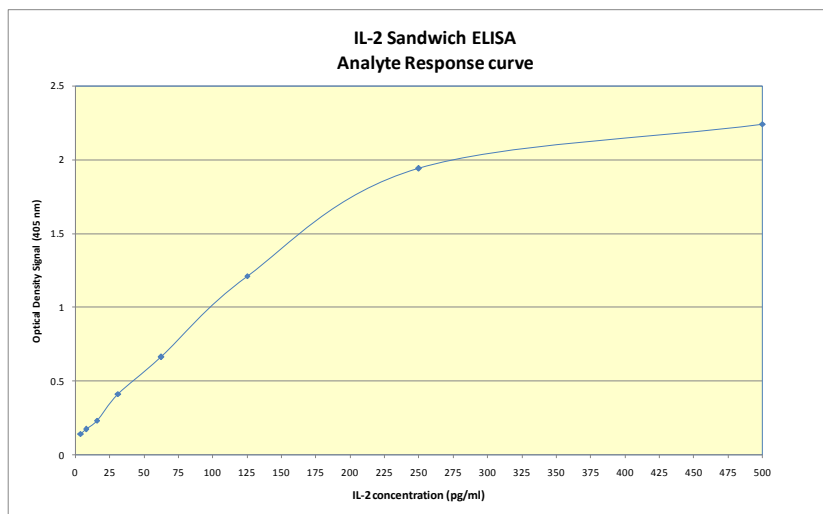


Figure 21. IL-2 specific sandwich ELISA response curve at an MSR = 6.35

e. Spin Column Antibody Recovery Yields

Table 5 summarizes typical antibody recovery yields using the ChromaLink™ Biotin One-Shot procedure. Highly purified goat anti-mouse IgG samples (80-100 µg) were resuspended in 100 µl 1x Modification Buffer at 1 mg/ml. Each sample was processed as described in the One-Shot procedure using the NanoDrop™ spectrophotometer (220-350 nm). Recovery yields 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 5. Spin column protein recovery yields.

Kit Disclaimer

Although Solulink and Neuromics have extensively optimized the ChromaLink™ Biotin One-Shot Antibody labeling kit and used it to label many different antibodies successfully, it is nonetheless still possible that antibody binding affinity may be compromised or even lost during the biotinylation process.

Although rare, this phenomenon occurs because the antibody in question possesses one or more critical lysine amino acid residues directly at the antigen-binding site (ABS) and whose function for binding is compromised during biotinylation. Additionally, Solulink and Neuromics have observed (also in rare instances) that some antibodies possess critical lysine residues (not necessarily at the bind site) that are absolutely critical for maintaining antibody stability/solubility. Once modified, these critical residues (usually only one or two) cause complete loss of antibody recovery due to precipitation. This phenomenon is sometimes observed even at very low biotin substitution levels and is not related to over-modification, namely, a high biotin molar substitution ratio (MSR)).

These rare situations are beyond the control of this kit and any other NHS-ester based labeling kit used to biotinylate antibodies in a non site-selective manner. Use of this labeling kit does not come with an implied guarantee or warranty for either loss of antibody binding affinity or precipitation in these rare cases. We feel our customers should be made aware that the biotin labeling process used in this kit can in some instances compromise the biological function of the antibody being modified.

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