Version 10.21.11

Chromalink Digoxigenin One-Shot Antibody-Labeling Kit

Technical Manual

Catalog # B-9014-009K

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Safety Information

WARNING – CHEMICAL HAZARD. Some chemicals used can be potentially hazardous, and can cause injury or illness.

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

Table of Contents

I. Introduction

a. Product Description

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

Digoxigenin has long been used as an alternative hapten reporter system to the streptavidin/biotin system (1). This system is based on a steroid isolated from the blossoms and leaves of the plant Digitalis purpurea, the only known natural source of digoxigenin. High affinity antibodies raised against digoxigenin thus exhibit virtually no cross-reaction with other biological molecules found in higher organisms. Both the specificity and sensitivity of this "parallel" hapten-reporter system often gives digoxigenin a major advantage over biotin-based detection systems. In more recent times, this reporter system has also been used in conjunction with the biotin-streptavidin system to detect multiplexed antigens (2). Unlike the biotin/streptavidin system, the digoxigenin label has never had a method for directly quantifying incorporation, until now.

The labeling process used in the One-Shot kit is based on a patented UV-traceable linker called Sulfo-ChromaLink™ Digoxigenin **(Figure 1).** This advanced digoxigenin labeling reagent contains an aromatic water-soluble N-hydroxy-sulfosuccinimidyl ester functional group ***(a***), which efficiently modifies protein lysine residues in a 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 direct quantification of digoxigenin ***(c),*** attached to the antibody. These diverse chemical elements are linked together through a long-chain PEG3 spacer ***(d),*** which preserves antibody-digoxigenin binding affinity while simultaneously maintaining antibody solubility.

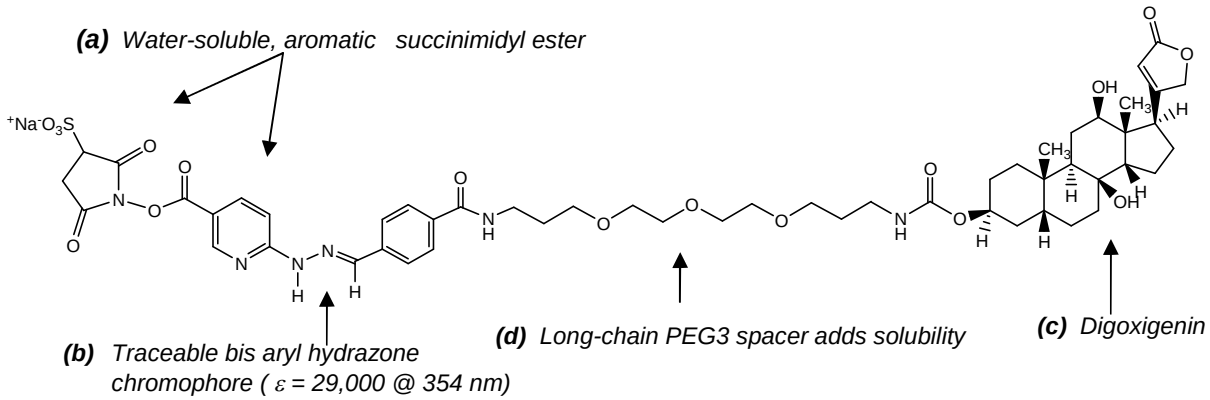


Figure 1. Structure of ChromaLink Digoxigenin $C_{52}H_{67}N_6NaO_{17}S$; M.W. 1103.17

b. Benefits and Features

The ChromaLink™ Digoxigenin One-Shot kit is a convenient, cost-effective way of incorporating digoxigenin into a small quantity (100 µg) of an antibody. After incorporation, digoxigenin is rapidly quantified using a non-destructive UV-scan (220-400 nm). The kit features high antibody recoveries (50-80%) with consistent digoxigenin incorporation. The degree of incorporation or molar substitution ratio (MSR) ranges from 2-8 digoxigenin molecules per antibody when used as directed.

The One-Shot 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 digoxigenin with a simple protocol. This "double-feature" provides researchers with greater confidence, consistency and reproducibility in all downstream applications.

c. Procedure Diagram

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

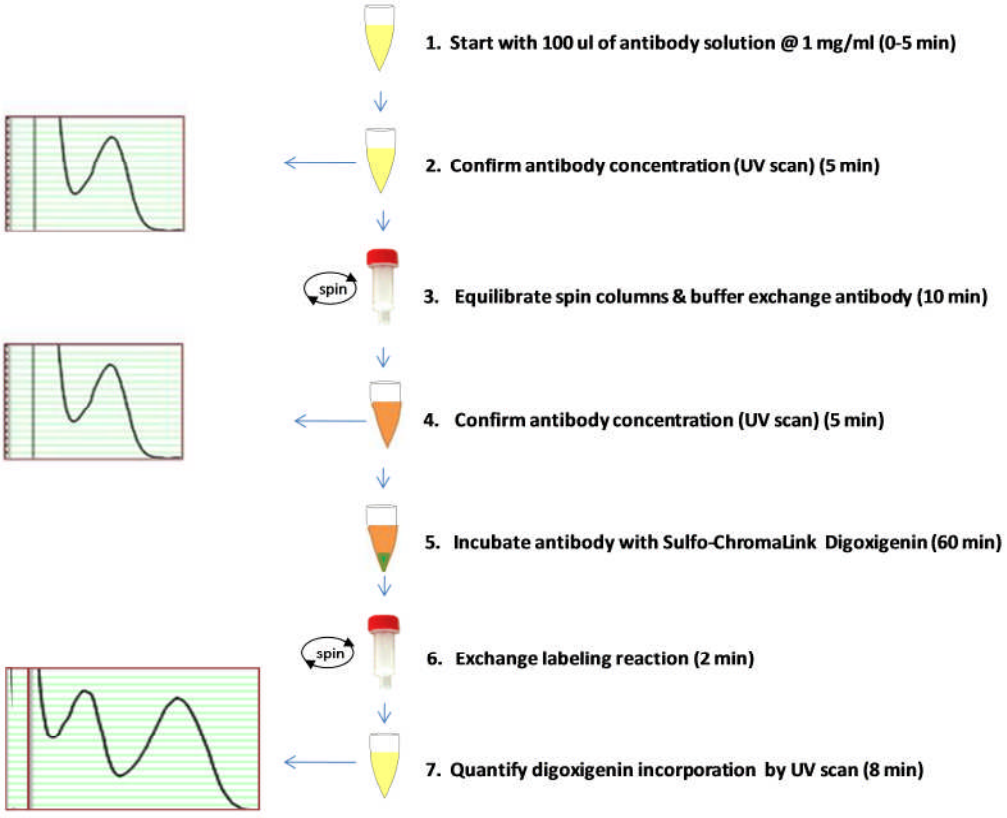


Figure 2. ChromaLink™ Digoxigenin One-Shot antibody labeling procedure**.**

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. *Digoxigenin Labeling:* label antibody with ChromaLink™ Digoxigenin linker
6. *Second Buffer Exchange:* remove excess labeling reagent with spin column
7. *Digoxigenin Incorporation:* quantify incorporation using a spectrophotometer

e. Important Labeling Parameters

The ChromaLink™ Digoxigenin One-Shot antibody labeling kit is specifically designed to incorporate digoxigenin into 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 µg	100 µl

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

The kit provides consistent and reliable incorporation of digoxigenin by controlling the following reaction variables:

- Initial antibody mass (100 µg) and volume (100 µl)
- Reaction buffer composition
- Reaction time (60 min)
- Reaction stoichiometry (15-fold mole-equivalents of linker)

Critical to digoxigenin 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. A_{280}) measurement. A scan however, provides greater information and assurance that the 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. If a commercial antibody sample contains any of these additives, please refer to the troubleshooting guide located in the Appendix to determine which materials may 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™ Digoxigenin	11 µg /1	room temp
1x Modification Buffer (pH 8.0)	1.5 ml /1	room temp
1x PBS (pH 7.2)	1.5 ml/ 1	room temp
DMF (anhydrous)	500 µl/1	room temp
Collection tubes	1.5 ml /4	room temp
Zeba™ Spin Columns	0.5 ml /2	room temp
Digoxigenin-IgG Control	100 µg /1	room temp
1M Tris·HCL (pH 8.9)	500 µl /1	room temp

g. Additional Materials Required But Not Provided

UV-VIS 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)
Standard 1.5 ml microfuge tubes, molecular grade water
Pipettes and tips for P-10, P-200, P-1000 (e.g. Rainin, Eppendorf or equivalent)

II. ChromaLink™ Digoxigenin 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 for digoxigenin labeling.

Sample (Solid Form)

Initial Sample: 100 µg/vial

Resuspend the sample in 100 µl 1X Modification Buffer (pH 8.0) to yield a 1 ± 0.1 mg/ml solution and proceed directly to the Sample Analysis section (II-b).

Initial Sample: > 120 µg/vial

Resuspend the sample in a sufficient volume of 1X Modification Buffer (pH 8.0) 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™ Digoxigenin 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 (minimum 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 a sample aliquot with 1X Modification Buffer (pH 8.0) to 1 mg/ml and 100 µl. 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 ug of total antibody is required (e.g. 100 µl @ 1mg/ml).

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 antibody 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).

b-1. UV-VIS Spectrophotometer

1. Program a spectrophotometer to scan from 220-400 nm. Follow the manufacturer's instructions for each 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. Record the A_{280} from the scan.
6. Recover the sample from the cuvette by placing it into a clean 1.5 ml microfuge tube.
7. Calculate the initial antibody concentration as illustrated in the example that follows.

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

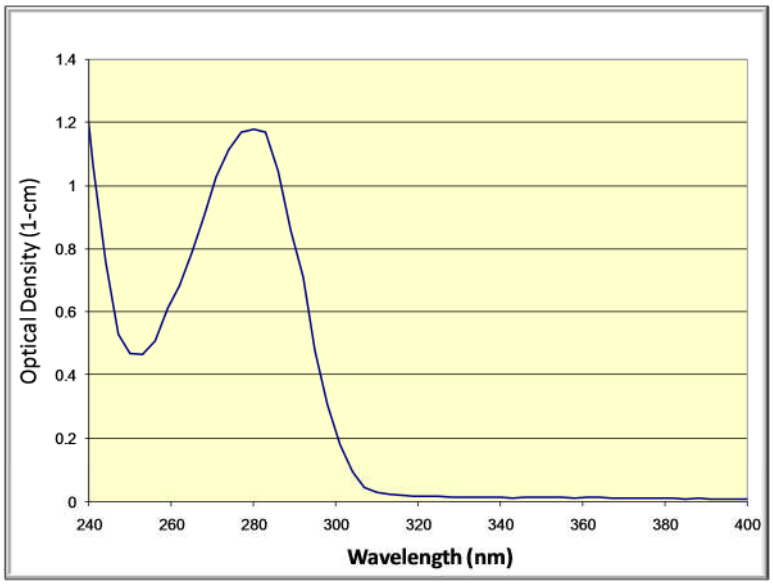


Figure 3. Scan of bovine IgG 100 µl @ 1 mg/ml in 1x Modification Buffer (100 mM sodium phosphate, 150 mM NaCl, pH 8.0) using a quartz semi-micro cuvette (50-100 µl).

Sample Calculation

Equation #1: [A_{280} /*E1% value] 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. Mass extinction coefficients (E1%) used to calculate antibody concentration**.** The E1% is the A_{280} of a 10 mg/ml solution using a 1-cm path length.

Sample: Bovine IgG 100 µl @ 1 mg/ml
A_{280} (from scan in Figure 3) = 1.18
Antibody E1% (Table 2) = 12.40

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

[1.18 / 12.40] x 10 mg/ml = 0.95 mg/ml

8. After confirming a sample's concentration, the sample is recovered and adjusted to 1 $\pm$ 0.1 mg/ml and 100 µl (if necessary) by addition 1x Modification Buffer. 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 µg. An accurate mass estimation is critical for maintaining the ratio of labeling reagent to antibody. Maintain sample volume as close as possible to 100 µl (e.g. $\pm$ 5 µl) for optimum 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 (section IV). A proper antibody spectrum is required to confirm an antibody's 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 µl drop of molecular grade water on the clean pedestal, click OK.
3. When the main menu appears, select the A_{280} menu option. **Note-** do not use the UV-VIS menu option on the NanoDrop™ to read the sample.
4. After the A_{280} menu appears, **click-off the 340 nm normalization option** using the mouse.
5. In the window labeled Sample Type, select 'Other protein E1%' option from the pull-down menu. Enter the appropriate E1% value **(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 µl 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 µl 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 µl 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 in the example below.

Example: Mouse IgG (100 µl @ 1 mg/ml) as illustrated in **Figure 4**.

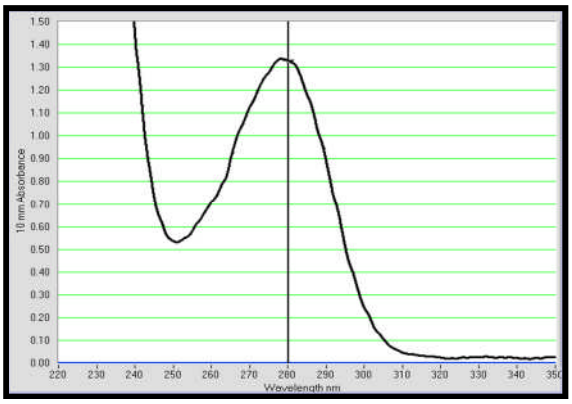


Figure 4. Mouse IgG 100 µl @ 1 mg/ml in 1x Modification Buffer (100 mM sodium phosphate, 150 mM NaCl, pH 8.0) scanned on a NanoDrop™ (220-350 nm).

Sample Calculation

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

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

Sample: Mouse IgG @ 1 mg/ml
A_{280} (from scan in Figure 3) = 1.34
Antibody E1% value (Table 2) = 14.00

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

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

9. After confirming a sample's concentration, the sample is recovered and adjusted to 1 ± 0.1 mg/ml and 100 µl (if necessary) by addition 1x Modification Buffer. 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 ug. The accuracy of this mass is critical to maintaining the ratio of labeling reagent to antibody. Maintain the sample volume as close as possible to 100 µl (e.g. ± 5 µl) for optimum results.

Important-if a sample 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 a sample's protein concentration.

C. First Buffer Exchange Procedure (10 min)

After adjusting a sample to 1 ± 0.1 mg/ml and 100 µl, buffer exchange the antibody using the following procedure.

1. Prepare the two spin columns provided by twisting off the bottom closures and loosening the red caps (do not remove caps).

2. Place each spin column into a separate 1.5 ml microcentrifuge collection tube (provided).

3. Place the spin columns opposite each other in the microcentrifuge and spin at 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 one cap with the letter **A** and the other spin column cap with the letter **B.**

7. Add 300 µl **1x Modification Buffer** (pH 8.0) 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 from 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 **B** spin column 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 spin column **A**.

12. Add 100 µl **1x PBS** buffer to spin column **B**. Recap the lid loosely. **Note**-this column will now serve as a balance tube.

13. Centrifuge the columns at **1500 x g for 2 minutes** to collect the eluate at the bottom of each collection tube.

14. Remove the spin column tube assembly **A** containing the buffer exchanged 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 300µl 1x PBS to the top of the resin bed to rehydrate the resin. Set the assembly aside for later use. **Important**-addition of 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 the bottom of collection tube **A** to confirm the amount of recovered antibody. **Note-** refer to previous Sample Analysis instructions (section II, b-1 or b-2) to calculate the recovered antibody concentration.

2. If the recovered antibody is at a concentration of **1 ± 0.2 mg/ml** and in a volume of **100 ± 5 µl**, proceed directly to the digoxigenin labeling procedure below (section 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 0.8 mg/ml and 100 ± 5 ul, do not attempt to label the sample. Refer to the troubleshooting guide found in the Appendix.

e. Digoxigenin Labeling Procedure (60 min)

1. Open a vial of ChromaLink™ Digoxigenin labeling reagent and add 5 µl DMF (provided) directly to the bottom of the vial. **Important**-do not add more than 5 µl DMF to the vial.

2. Using a P-10 pipette, carefully pipette the DMF solution up and down, rinsing the side wall (near the bottom) of the tube several times to completely dissolve the ChromaLink™ Digoxigenin reagent. The solution will appear clear but slightly yellow in color. **Note**-although Sulfo-ChromaLink™ Digoxigenin is water-soluble, a small amount of DMF greatly enhances labeling efficiency.

3. Add the entire volume (100 µl @ 1 mg/ml) of buffer exchanged antibody solution directly to DMF/ChromaLink™ Digoxigenin; mix by pipetting up and down several times.

4. Incubate for 60 minutes at room temperature.

5. After the reaction is complete, quench the reaction by addition of 10 µl 1M Tris (pH 8.9).

6. Centrifuge the quenched reaction mixture tube @ 1,500 x g for 30 seconds. After centrifugation, set the reaction mixture aside. **Note-**a mild centrifugation insures that any

insoluble linker hydrolysis byproducts (e.g. hydrolyzed ester) are gently spun to the bottom of the reaction tube prior to the second buffer exchange procedure.

7. Place the previously hydrated **B** spin column assembly containing 300 µl 1x PBS (section II, c-15) into the centrifuge. Loosely recap the lid and properly orient the assembly in the centrifuge.

8. Add 300 µl molecular grade water to the **A** spin column, and place the properly oriented assembly opposite **B** in the centrifuge. **Note-**The **A** assembly now serves as the balance tube.

9. Centrifuge at 1,500 x g for 1 minute. Discard the flow through from each spin column assembly.

10. Transfer the dry **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 digoxigenin labeling reaction (section II, e-6) to the center of the compacted **B** resin. Recap the column loosely.

2. Apply 100 µl of molecular grade water to the center of **A** spin column. Recap loosely. **Note**-the **A** column now serves as a 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 at the bottom of each collection tube.

4. Transfer the digoxigenin-labeled antibody from the bottom of **B** collection tube to a new 1.5 ml microfuge tube. Proceed to determine digoxigenin incorporation as directed in the following section.

g. Determining Digoxigenin Incorporation (10 min)

Digoxigenin incorporation is determined by scanning the final labeled antibody sample on either a UV-VIS or NanoDrop™ spectrophotometer. Proceed as directed 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.
3. Discard the blank solution.
4. Transfer the digoxigenin-antibody sample to the cuvette and scan.

5. Record the A_{280} and A_{354} from the spectrum.
6. Recover the digoxigenin labeled sample from the cuvette by transferring the solution back to a clean 1.5 ml microfuge tube. Label the sample and store refrigerated at 4°C.
7. Input the A_{280} and A_{354} and the corresponding E1% value into the **ChromaLink Digoxigenin Protein MSR Calculator** to automatically calculate the digoxigenin molar substitution ratio (MSR). **Note-**typical MSRs values range from 2-8 digoxigenin per antibody molecule. The amount of digoxigenin-labeled antibody (mass) recovered can range from 50 to 100 µg depending on the exact starting amount being labeled.

***g2*. 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, click 'OK'.
3. When the main menu appears, select the A_{280} menu option. Note- do not use the UV-VIS menu option on the NanoDrop™ to read the sample.
4. After the A_{280} menu appears, click-off the 340 nm normalization option using the mouse.
5. In the window labeled Sample Type, select 'Other protein E1%' option from the pull-down menu. Enter the appropriate E1% value (see Table 2) corresponding to your particular antibody sample type.
6. Blank the NanoDrop™ spectrophotometer by placing a 2 µl drop of 1x PBS and on the pedestal and using the mouse click 'Blank'.
7. 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.
8. Transfer a 2 µl volume of the digoxigenin-labeled antibody solution @ 1 mg/ml to the pedestal, and click 'Measure'. Wait until the spectrum (220-350 nm) appears in the window.
9. Record the absorbance @ 280 nm from the λ absorbance window.
10. Record the absorbance @ 354 nm by typing the numeric value '354' into the λ window. Refrigerate the labeled sample.
11. Input the A_{280} and A_{354} and the corresponding E1% value into the **ChromaLink Digoxigenin Protein MSR Calculator** to automatically calculate the digoxigenin molar substation ratio (MSR). **Note-**typical MSRs range from 2-8 digoxigenin per antibody. The amount of digoxigenin-labeled antibody (mass) recovered can range from 50 to 100 µg depending on the exact mass and type of antibody labeled.

III. ChromaLink Digoxigenin Labeling of Antibodies: Some Examples

a. One-Shot Digoxigenin Labeling of Rabbit Anti-BSA IgG

An aliquot of a commercial rabbit anti-BSA IgG (7 mg/ml liquid) was adjusted to 1.0 mg/ml and 100µl using 1x Modification Buffer (100 mM sodium phosphate, 150 mM NaCl, pH 8.0). An aliquot (2µl) was scanned using a NanoDrop™ spectrophotometer **(Figure 5).** The shape of the spectrum appears ideal with a flat baseline near 350 nm. The initial concentration was estimated to be 1.04 mg/ml (E1% value of 13.50). After the 1st buffer exchange the sample was rescanned to yield a recovery of 105 µl @ 0.89 mg/ml solution **(Figure 6).** The buffer exchanged sample was then labeled with ChromaLink Digoxigenin reagent for 60 minutes, quenched and excess reagent removed with the second buffer exchange procedure (1x PBS). The labeled sample was then rescanned (220-350 nm) as shown in **Figure 7**. The calculator-estimated digoxigenin molar substitution ratio was 3.7 with 95µg of recovered antibody.

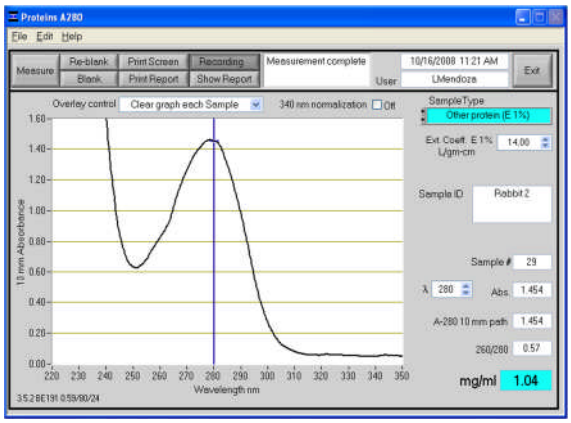


Figure 5. Antibody before buffer exchange

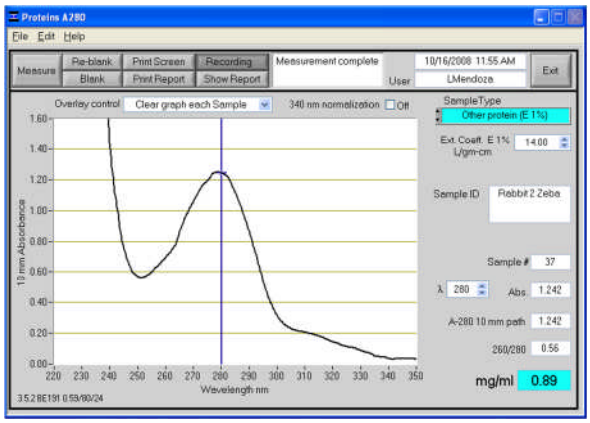


Figure 6. Antibody after buffer exchange

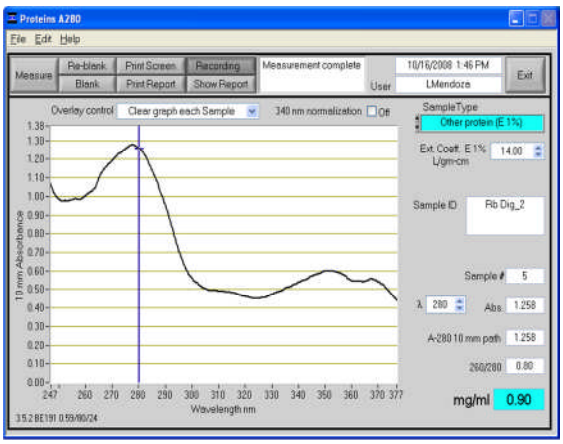


Figure 7. Antibody after digoxigenin-labeling

b. One-Shot Digoxigenin Labeling of Donkey Anti-Mouse IgG

An aliquot of a commercial donkey anti-mouse IgG (1.3 mg/ml liquid) was adjusted to 1.0 mg/ml and 100µl using 1x Modification Buffer (pH 8.0). An aliquot (2 µl) of the sample was scanned on a NanoDrop™ spectrophotometer **(Figure 8).** The shape of the spectrum appeared nearly ideal with a flat baseline near 350 nm. The initial concentration was determined to be 0.94 mg/ml (E1% value of 15.00). After the 1st spin column, the sample was rescanned to yield a sample concentration of 0.90 mg/ml solution **(Figure 9).** The sample was then labeled with ChromaLink Digoxigenin reagent for 60 minutes, quenched and excess reagent removed with a second buffer exchange spin column (1x PBS). The labeled sample was then rescanned (220-350 nm) as shown in **Figure 10.** The calculator estimated digoxigenin molar substitution ratio was 6.80 with 65.9µg of recovered antibody.

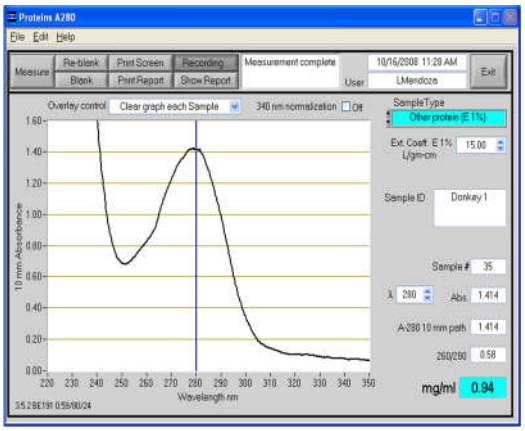


Figure 8. Antibody before buffer exchange

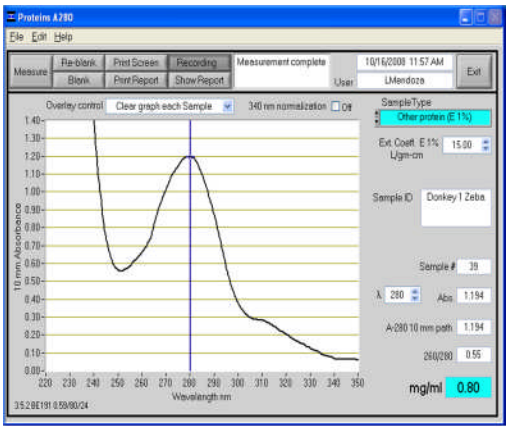


Figure 9. Antibody after buffer exchange

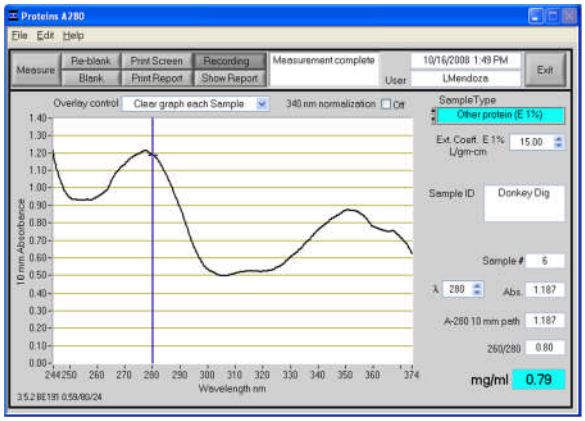


Figure 10. Antibody after digoxigenin labeling

c. One-Shot Digoxigenin Labeling of Chicken IgY

A commercial chicken IgY @ 10mg/ml in PBS was diluted to 100 µl and 1 mg/ml using 1x Modification Buffer. The IgY was scanned using a NanoDrop™ spectrophotometer to confirm an initial protein concentration of 0.99 mg/ml **(Figure 11).** After the 1st spin column the concentration was determined to be 0.95 mg/ml (E1% value of 12.76) as shown in **Figure 12.** The sample was then labeled with ChromaLink Digoxigenin reagent for 60 minutes, quenched and excess reagent removed with a second spin column. The labeled sample was then rescanned (220-350 nm) **Figure 13**. The final digoxigenin-labeled chicken IgY sample had a calculated molar substitution ratio of 6.0 with 85µg of recovered antibody.

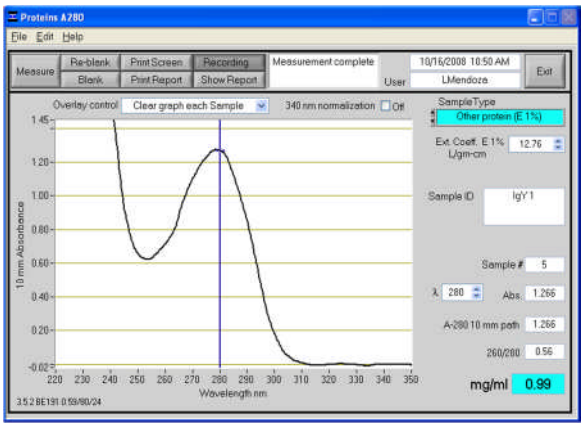


Figure 11. Antibody before buffer exchange

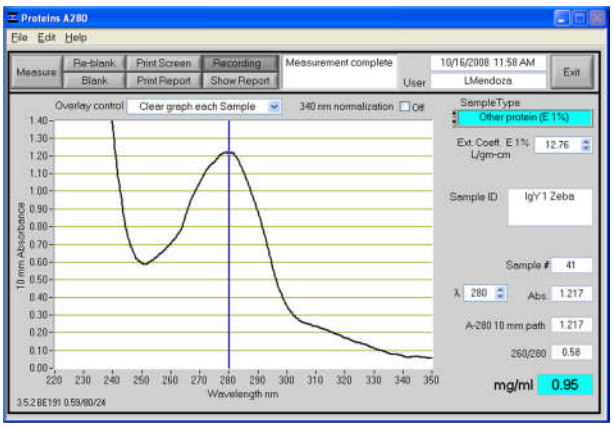


Figure 12. Antibody after 1st buffer exchange

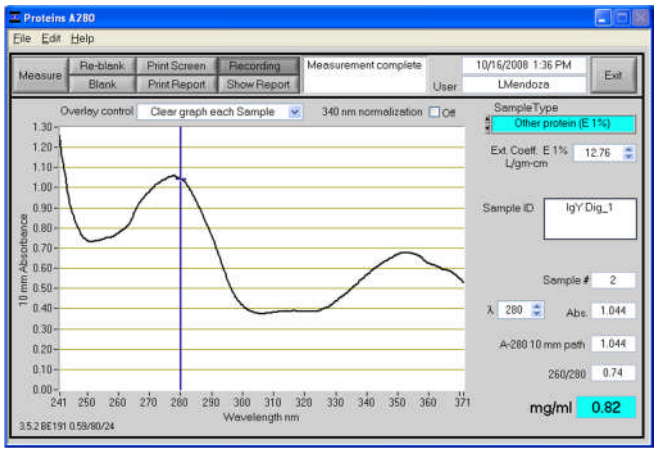


Figure 13. Antibody after digoxigenin labeling.

IV. Appendix

a. Digoxigenin IgG Control

The ChromaLink™ Digoxigenin One-Shot Antibody Labeling Kit comes complete with a digoxigenin-labeled antibody control. This control consists of a lyophilized digoxigenin-labeled bovine IgG at a precisely known digoxigenin molar substitution ratio (refer to the lot specific certificate of analysis). This control is used to validate the accuracy of a given spectrophotometer 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 PBS (220-400 nm). Discard the blank buffer solution from the cuvette. **Note-**if using a NanoDrop™ follow the procedure on page 9.

2. Resuspend the control (lyophilized Dig-IgG, 50 µg) using 100 µl molecular grade water to a final concentration of 0.5 mg/ml ± 0.1 mg/ml and close the lid.

3. Centrifuge the control very briefly at low speed (30 seconds at 1500xg) to pellet any insoluble salts that may be present.

4. Remove an aliquot and scan the digoxigenin labeled antibody.

5. Record the A_{280} and A_{354} values generated from the spectrum.

6. Input these values along with the E1% (12.40) and M.W. (150 kD) into the **ChromaLink Digoxigenin Protein MSR Calculator** to determine the digoxigenin molar substitution ratio.

7. Confirm the value obtained with the lot specific MSR found on the product data sheet.

b. Troubleshooting Guide

This section of the manual is intended to be used as a technical troubleshooting guide. Many 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 A_{280} altering additives. These spectral aberrations are often revealed when scanning a sample but not from a single point, A_{280} measurement. Distortions to an antibody's intrinsic spectrum or other spectral aberrations such as baseline offset errors will 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 Digoxigenin).

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)
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 examples of reference spectra for troubleshooting purposes. These are intended to aid the user with proper measurement of protein concentration. Each section includes a sample spectrum and possible corrective action.

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 14.** 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 A_{280} 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 $\pm$ 0.1 mg/ml).

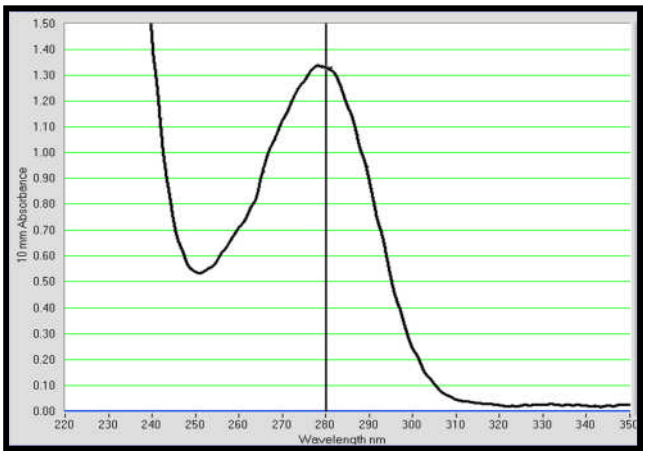


Figure 14. UV-spectrum (220-350 nm) of a highly purified mouse IgG antibody @ 1 mg/ml without any additives or preservatives. Note the uniform shape and flat baseline @ 350 nm.

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

Table 4. Expected A_{280} 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 at 0.05 or 0.1% in a sample of bovine IgG at 0.9 mg/ml is illustrated in **Figure 15**. 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 this 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 15.** 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. Passage through the 1st spin columns will remove all traces of azide.

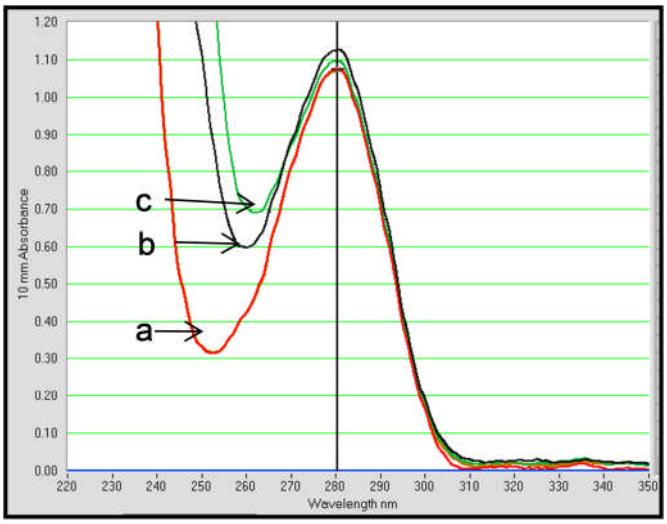


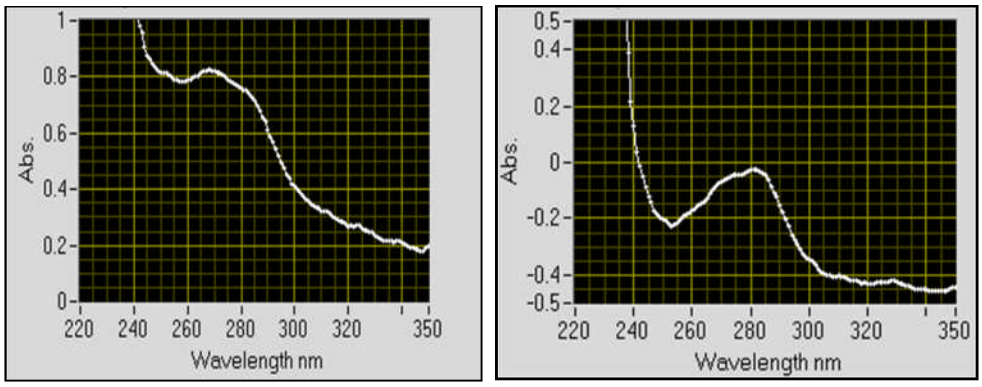
Figure 15. Superimposed spectra of purified bovine IgG @ 0.90 mg/ml with a) no sodium azide, b) spiked with 0.05% sodium azide, or c) spiked with 0.1% sodium azide.

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 accurate estimates of initial protein concentration. Two examples are illustrated in **Figure 16.**

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 $\pm$ 0.1 mg/ml, proceed to the digoxigenin labeling 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.



Panel A **Panel B**

Figure 16. 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 17** 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 A_{280}. When using a NanoDrop™, offsets may also occur due to trapped air bubbles on the pedestal.

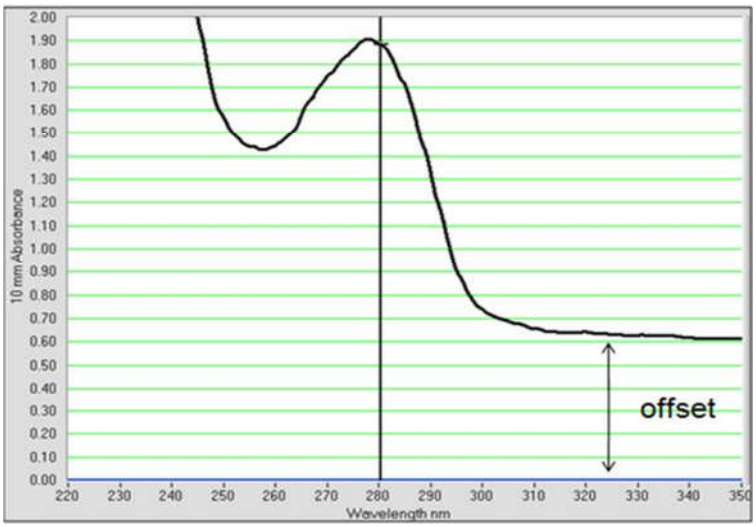


Figure 17. Baseline offset in a commercial rabbit polyclonal antibody preparation (5 mg/ml) diluted to 1 mg/ml in 1x Modification Buffer. Based on the A_{280} value the estimated concentration was 1.40 mg/ml. The actual protein concentration after desalting the sample on a spin column to remove the buffer blank offset was 1 mg/ml.

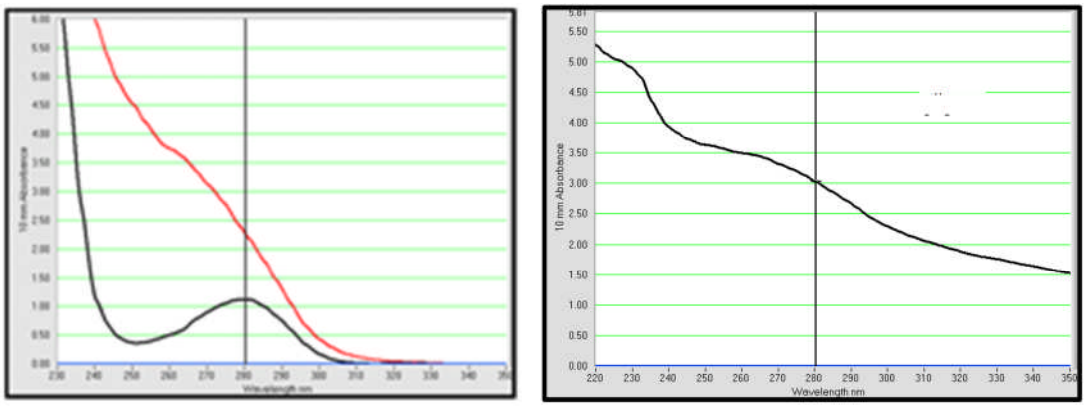
Corrective Action: For samples containing large baseline offset errors, we recommend passing the sample through the 1st spin column and rescanning to obtain a properly blanked sample spectrum. Since the spin column exchanges the antibody into a known buffer solution (1x Modification Buffer) it normally removes any offset error originating from the buffer blank. After confirming sample concentration proceed to the digoxigenin labeling procedure (II-e). If after buffer exchange, it is determined that a sample contains significantly more or less than 1 mg/ml then 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 µl before proceeding.

Example #5: Bovine IgG containing thimerosal preservative

A bovine IgG sample was spiked with thimerosal preservative as illustrated in **Figure 18** (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 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 the 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 $\pm$ 0.1 mg/ml, proceed to the digoxigenin labeling 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 µl 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.



Panel A **Panel B**

Figure 18. 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 19** 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.).

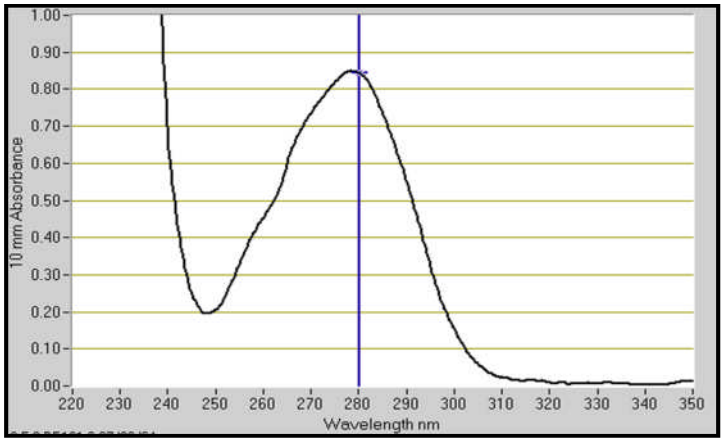


Figure 19. A commercial bovine IgG sample containing significantly less antibody (68 µg) than expected from the original package label.

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 ug of buffer exchanged antibody using this procedure.

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

The ChromaLink Digoxigenin 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 Digoxigenin 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 Digoxigenin 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 significantly alter 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 completely 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 digoxigenin 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 where 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 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 digoxigenin labeling reaction	-improper mixing of reaction components -improper operation of the spectrophotometer -presences of amine contaminants -improper storage of the ChromaLink Digoxigenin reagent may have caused it to hydrolyze	-make sure to mix the antibody ChromaLink Digoxigenin reaction mixture completely -use the digoxigenin-labeled IgG positive control provided to validate that the spectro-photometer is operating properly -remove all amine contaminants such as glycine before labeling -store and keep ChromaLink Digoxigenin in dessicated pouch at room temp at all times
Molar substitution ration was out of recommended range (2-8 digoxigenin/antibody)	-initial antibody concentration used was too low or too high. -antibody precipitated due to over-modification of available lysine residues	-make sure to properly determine 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 to insure that low amounts of antibody were not used
Low antibody recovery and/or sample precipitation	-antibody may have been aggregated before it was labeled -incorrect antibody concentration -antibody was over-modified	-do not use aggregated samples -follow the recommended protocol

	-on rare occasions some antibodies precipitate on modification of any lysine residues	-attempt to label a different antibody

c. Relationship between Molar Substitution Ratio & ELISA Sensitivity

The ChromaLink™ Digoxigenin One-Shot antibody labeling kit was optimized to incorporate between 2 and 8 digoxigenin per antibody molecule. Various levels of digoxigenin incorporation were evaluated for their effects on direct ELISA assay signals.

Direct ELISA

A bovine IgG antibody was labeled with digoxigenin using ChromaLink Digoxigenin across a series of different molar substitution ratios. Each digoxigenin labeled antibody was then immobilized using a standard ELISA procedure in a 96-well plate in a 2-fold serial dilution (0.5 - 5,000 ng/ml). After immobilization (4 hr at RT), the wells were blocked with 1% casein/PBS and subsequently washed. The immobilized antigen was then incubated with an anti-digoxigenin HRP conjugate at 150 mμ/ml for 60 minutes. After washing, TMB substrate (3,3',5,5'- tetramethylbenzidine) was added for 30 minutes before being stopped with 1M H_2SO_4. Signals were measured on a Molecular Devices SpectraMax Plus plate reader at 650 nm. Direct ELISA dose response curves were plotted as illustrated in **Figure 20**.

Results: Signal/noise increased approximately 2.6-fold (linear portion of the curve) as the MSR increased from 2.4 to 8.0. Background controls were constant across the various MSRs (data not shown)

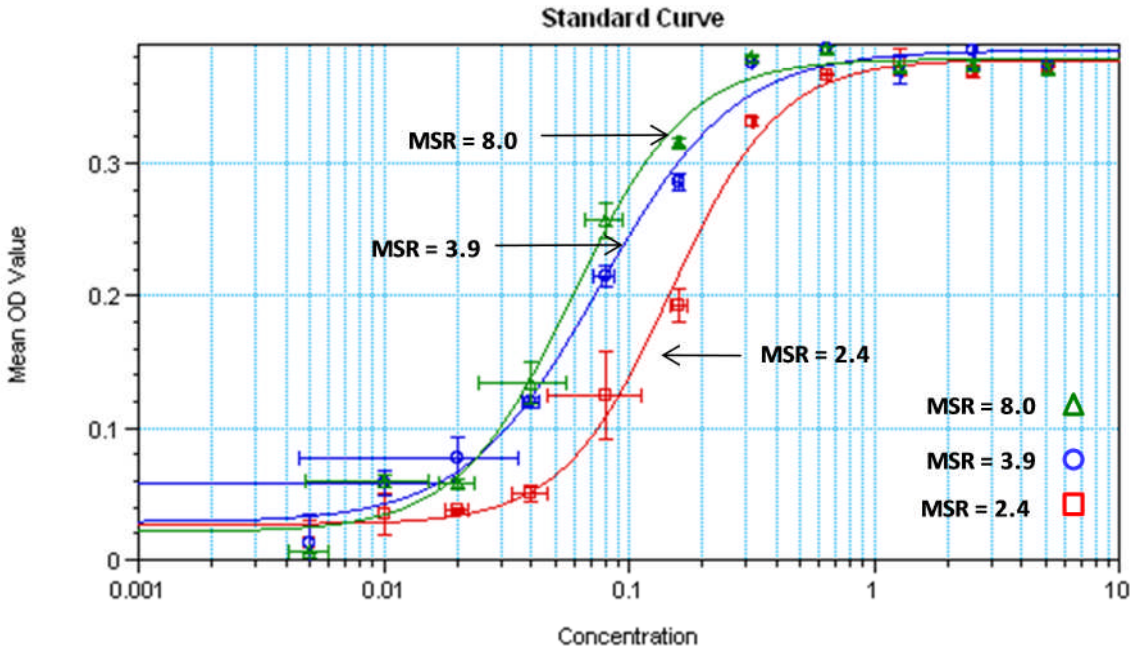


Figure 20. Direct ELISA response curves illustrating the relationship between digoxigenin molar substitution ratio and direct ELISA signals at 650 nm.

d. Spin Column Antibody Recovery Yields

Table 5 summarizes typical antibody recovery yields from Zeba™ spin columns used in the 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 on a Zeba™ column as described in the One-Shot procedure. Protein concentrations were measured using a NanoDrop™ spectrophotometer (220-350 nm). Recovery yields average 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. Zeba spin column protein recovery yields.

e. References

1. Non-radioactive labeling of RNA transcripts in vitro with the hapten digoxigenin; hybridization and ELISA-based detection. H J Höltke, C Kessler. Nucleic Acids Res. 1990 Oct 11; 18 (19):58**.** Nucleic Acids Res. 1990 Oct 11:18 (19):5843-51

2. Two-color, rolling-circle amplification on antibody microarrays for sensitive, multiplexed serum-protein measurements. Heping Zhou,Kerri Bouwman, Mark Schotanus, Cornelius Verweij, Jorge A Marrero, Deborah Dillon, Jose Costa, Paul Lizardi, and Brian B Haab. Genome Biology. 2004 5(4):R28 Mar 30

f. Kit Disclaimer

Although Solulink has extensively optimized the ChromaLink™ Digoxigenin One-Shot Antibody labeling kit and used it to label dozens of different antibodies successfully, it is nonetheless still possible that antibody binding affinity may be compromised or even lost during the digoxigenin labeling 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 labeling. Additionally, Solulink has observed that some antibodies possess critical lysine residues (not necessarily at the binding site) that are absolutely critical for maintaining antibody stability/solubility. Once modified, these critical residues cause complete loss of antibody recovery due to precipitation. This phenomenon is sometimes observed even at very low digoxigenin substitution levels and is not related to over-modification, namely, a high digoxigenin molar substation ratio (MSR).

These situations are beyond the control of this kit and any other NHS-ester based labeling kit used to label 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 cases. We feel our customers should be made aware that the digoxigenin labeling process used in this kit can in some instances compromise the biological function of the antibody being modified.