



ChromaLink™ Biotin Protein Labeling Kit

User Manual Catalog # B-9007-105K)

Table of Contents

I. Introduction	3
<i>a. Product Description</i>	3
<i>b. Benefits and Features</i>	3
<i>c. Protocol Summary</i>	4
<i>d. Labeling Process Diagram</i>	4
<i>e. Materials Provided and Storage Conditions</i>	5
<i>f. Additional Materials Required but Not Provided</i>	5
II. ChromaLink™ Biotin Labeling Protocol	6
<i>a. Important Biotinylation Parameters to Consider Before Labeling</i>	6
<i>b. Sample Preparation</i>	7
<i>c. 1st Buffer Exchange</i>	8
<i>d. Determination of Protein Concentration (BCA Assay)</i>	9
<i>e. Biotinylation Procedure</i>	11
<i>f. 2nd Buffer Exchange</i>	12
<i>g. Determining Biotin Incorporation</i>	13
III. Examples and Applications	17
<i>a. Biotinylation of IgG</i>	17
<i>b. Streptavidin Binding to Biotinylated IgG</i>	17
<i>c. Traceable Capture of Biotinylated IgG using Streptavidin Beads</i>	18
<i>d. Nanodrop™ Spectra of Biotinylated IgG</i>	19
IV. Appendix	20
<i>a. Biotinylated IgG Control</i>	20
<i>b. IgG Control</i>	20
<i>c. HABA Assay vs. ChromaLink Biotin Spectrophotometric Assay</i>	21
<i>d. General Guide for Biotinylation of Proteins</i>	22
<i>e. Troubleshooting Guide</i>	23
<i>f. Instructions for Using a NanoDrop™ to Measure Biotin MSR</i>	24
<i>g. Kit Disclaimer</i>	24

I. Introduction

a. Product Description

The ChromaLink™ Biotin Protein Labeling kit has all the necessary reagents for the traceable biotinylation of any lysine-containing protein. This kit provides sufficient materials to biotinylate and purify up to 5 proteins in about 2 hours. The scale of each protein labeling reaction is chosen by the researcher and can span from as little as 25 ug to as much as 1 milligram of protein. **Please refer to kit disclaimer (pg 24).**

The ChromaLink™ Biotin labeling process is based on a patented UV-traceable linker called Sulfo-ChromaLink™ Biotin (**Figure 1**). This process is based on an ad-

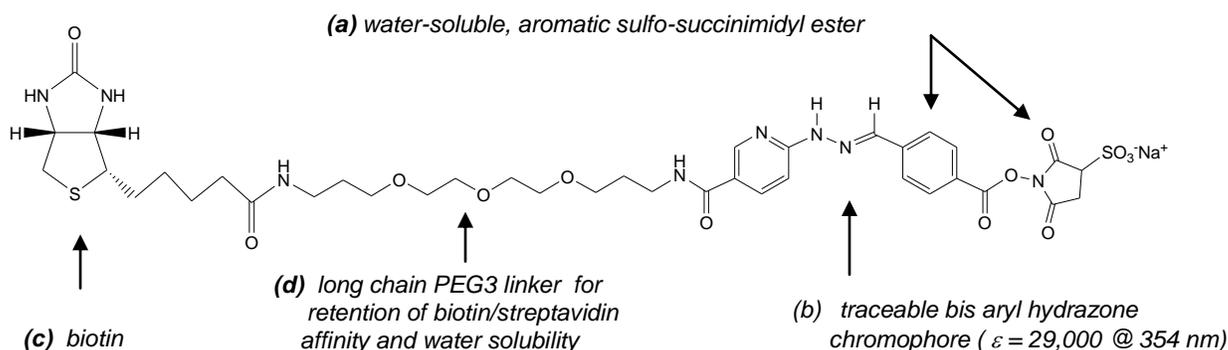


Figure 1. Molecular structure and formula of Sulfo-ChromaLink Biotin ($\text{C}_{38}\text{H}_{49}\text{N}_8\text{NaO}_{13}\text{S}_2$) (M.W. 912.96)

vanced protein biotinylation reagent containing an aromatic water soluble N-hydroxy-sulfosuccinimidyl ester functional group **(a)**, which efficiently modifies protein lysine residues in a phosphate buffer system. The linker possesses an embedded bis-aryl hydrazone structure **(b)**, which forms the compound's UV-traceable chromophore. The traceable nature of this unique chromophore enables the non-destructive quantification of biotin **(c)**, attached to the protein. These three diverse chemical elements are linked together through a long-chain PEG3 spacer **(d)**, which preserves streptavidin/biotin affinity while simultaneously maintaining antibody solubility.

b. Benefits and Features

The ChromaLink™ Biotin Protein Labeling kit offers an easier solution to the quantitative biotinylation of proteins starting from as little as 25 ug to as much as 1 milligram. Proteins ranging in molecular weights from 20-200kD are readily biotinylated using ChromaLink™ Biotin. The accompanying instructions and protocols are designed to make traceable biotinylation of proteins nearly foolproof. The use of ChromaLink™ Biotin eliminates the need to carry out cumbersome and indirect biotin binding assays such as the time consuming HABA or FluoroReporter™ (trademark of Invitrogen Corp.) assay used to determine the degree of biotin incorporation. Rather, after labeling the biotin molar substitution ratio is measured directly by a simple UV-spectrophotometric scan (220-420 nm) or

single point measurements at two wavelengths (A_{280} / A_{354}). Alternately, a single wavelength (A_{354}) can be employed in combination with a protein assay (e.g. BCA or Bradford) to quantify the molar substitution ratio (MSR) or number of biotins per protein molecule.

c. Protocol Summary

- Sample preparation (10 min.)
- 1st buffer exchange (10 min)
- Protein determination assay (BCA assay) (30 min)
- Biotinylation (90 min)
- 2nd buffer exchange (10 min)
- UV analysis and calculations (20 min)

d. Labeling Process Diagram

The ChromaLink™ Biotin protein labeling process is diagramed in **Figure 2**.

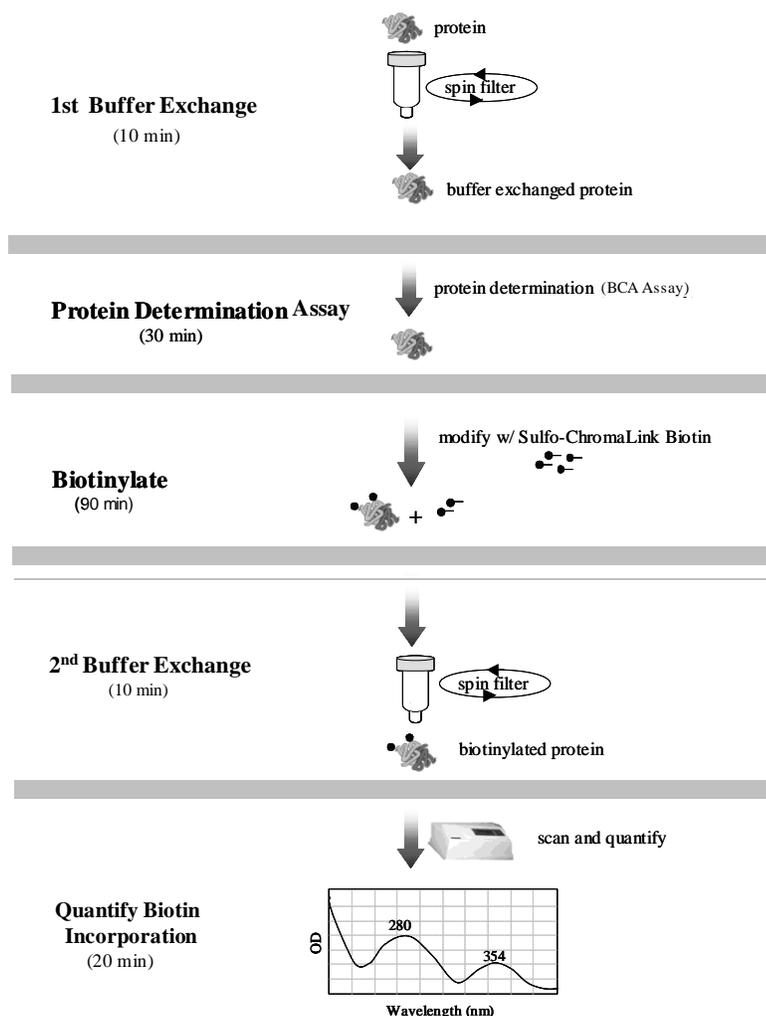


Figure 2. ChromaLink™ Biotin protein labeling process diagram.

e. Materials Provided and Storage Conditions

Component	Size/Quantity	Storage
Sulfo-ChromaLink™ Biotin	5 x 0.5 mg	room temp
10x Modification Buffer	2 x 1 ml	room temp
10x PBS	2 x 1 ml	room temp
*Zeba™ desalt spin columns	12	room temp
1.5 ml collection tube	24	room temp
1.5 ml storage tube	12	room temp
Bovine IgG	500 ug	lyophilized powder
Biotinylated Bovine IgG	500 ug	lyophilized powder
1 G Flash drive	1	room temp

*Zeba™ is a registered trademark of Pierce/Thermo Scientific

f. Additional Materials Required But Not Provided**Reagents and Consumables**

Molecular grade water

1.5 ml microfuge tubes

Standard polystyrene 96-well microplates (BCA protein assay)

BCA™ Protein Assay (Pierce/Thermo Scientific, #23223, 23224)

UV-VIS Instrumentation (any of the following)

UV-VIS Spectrophotometer containing a semi-micro cuvette holder

Nanodrop™ spectrophotometer (alternative instrument requiring no cuvette)

UV-VIS 96-well plate reader (requires UV-transparent microplates)

Other Required Instrumentation

Microcentrifuge (e.g. Eppendorf 5415D, IEC MicroMax or similar)

Microplates and Cuvette (if necessary)

UV-Transparent microplate (if using plate reader, 1/2 area plate, Cat. # 675801)

UV-Transparent microplate (if using plate reader, standard size, Cat. # 655801)

Quartz semi-micro-cuvette (50-100 µl) if using standard spectrophotometer)

Pipettes

Pipettes P-10, P-100, P-1000 (e.g. Rainin, Eppendorf or equivalent)

II. ChromaLink™ Biotin Labeling Protocol

a. Important Biotinylation Parameters to Consider Before Labeling

Protocols in this kit have been optimized to label a broad range of protein molecular weights (20–200kD) and concentrations (0.25 -10 mg/ml). Every biotin labeling reaction in this kit uses the same fixed volume of protein solution (100 µl) and either 10 or 20 mole-equivalents of ChromaLink Biotin reagent depending on the initial protein concentration. A calculator is provided with each kit (flash drive) that automatically calculates how to prepare the labeling reagent and how much reagent to add for a given molecular weight and concentration of protein. Reaction conditions have been chosen to incorporate multiple biotins into any lysine-containing protein regardless of starting molecular weight and/or initial protein concentration within the specified range summarized in **Table 1**. Generally, between 2 and 8 biotins are incorporated per protein molecule when used as directed.

Protein Concentration Range	Protein Mass Range	Molecular Weight Range	Reaction Volume
0.25 -10 mg/ml	25-1000 ug	20-200kD	100 ul

Table 1. Range of acceptable labeling conditions used in the ChromaLink™ Biotin protein labeling kit.

A properly calibrated UV-VIS scanning spectrophotometer or plate reader is required to quantify biotin incorporation. Any of the following (or similar) instruments can be used:

UV-VIS Scanning Spectrophotometer (e.g. Beckman or Perkin-Elmer)
NanoDrop™ ND-1000 Spectrophotometer (or similar)
UV-VIS Scanning Plate Reader (e.g. Molecular Devices SpectraMax)

Each instrument has its own particular set of requirements and limitations. For example, a conventional UV-VIS scanning spectrophotometer requires a quartz micro or semi-cuvette holder for measuring small volumes (50-100 µl). If using a UV-VIS plate reader, specialized UV-transparent polystyrene microplates will be required. If using a micro-volume UV-VIS spectrophotometer such as the Nanodrop™ ND-1000 spectrophotometer keep in mind that this instrument cannot be used to quantifying biotin incorporation at protein concentrations below 0.5 mg/ml. Please refer to the specific limitations and requirements of each instrument in section II-g of the manual. A word of caution, although the ChromaLink™ Biotin Protein Labeling Kit has been used to successfully label many different types and sizes of proteins, it still possible that biological function of some proteins may be compromised or lost during biotinylation. For further details see kit disclaimer (pg.24).

b. Sample Preparation (10 min)

Protein samples come in many different forms; solids or liquids, low or high concentration, micrograms to milligrams, and with or without various carriers and additives. Commercial proteins can be pre-dissolved in sample buffers that are incompatible with optimal labeling conditions (e.g. low ionic strength and/or pH) or that contain amine buffers such as Tris-HCl or glycine. **Note**-protein-based carriers are sometimes added to antibodies or other proteins to extend shelf-life. Protein carriers interfere with proper biotinylation of target proteins and must be removed before biotinylation can proceed. If a sample contains a carrier protein, a suitable method should be used to remove the carrier (gel filtration or affinity chromatography) before proceeding.

To prepare a protein sample for biotinylation, proceed as directed below for your particular sample form.

Protein Sample (Solid Form)

If the protein to be biotinylated is in solid form (e.g. lyophilized) resuspend the protein as directed below:

Procedure: Solid Form (25 ug- 1 mg)

If the initial protein sample is a pre-weighed lyophilized solid containing from 25 ug to 1 mg of protein (free of any protein carrier) simply resuspend the sample in 100 μ l of 1x Modification Buffer and proceed directly to section II-c. **Important**-10x Modification Buffer is provided and must first be diluted with molecular grade water to a 1X dilution before using.

This kit is not designed to label a mass greater than 1 mg or a concentration greater than 10 mg/ml. Multiple labeling reactions must be performed if attempting to label protein quantities greater than 1 mg or volumes larger than 100 μ l.

Procedure: Bulk Solid Form (e.g. 5-100 mg)

If the initial protein sample is available in bulk form, weigh out approximately 5-10 mg on an analytical balance. Then add a volume of 1x Modification Buffer sufficient to bring the protein concentration into the appropriate range (0.25-10 mg/ml). Transfer a 100 μ l aliquot of the protein solution corresponding to a desired mass to a new 1.5 ml tube and proceed directly to the section II-c.

Protein Sample (Liquid Form)

If the initial protein sample is already in a liquid form (e.g. PBS or TBS) follow the instructions below to prepare the sample.

Procedure: Liquid Form (0.25 to 10 mg/ml)

If the protein sample is already dissolved in a buffered solution at a concentration ranging between 0.25 mg/ml and 10 mg/ml, simply transfer 100 μ l into a new 1.5 ml tube and proceed directly to section II-c. **Note**-do not attempt to label protein samples below 0.25 mg/ml or volumes significantly larger or smaller than 100 \pm 10 μ l.

µl. If the protein to be labeled is outside this concentration range and volume either concentrate or dilute into appropriate range before proceeding.

c. First Buffer Exchange Procedure (10 min)

This kit provides Zeba™ spin columns to buffer exchange proteins without altering their initial concentration. These columns have a maximum loading capacity of 130 µl and thus limit the labeling protocol to a maximum sample volume of 100 µl. For each protein to be labeled two Zeba™ spin columns (**Figure 3**) are always prepared (equilibrated) **in tandem using two different buffers**. A total of 12 spin columns are provided with each kit, sufficient for five biotinylation reactions plus one control biotinylation reaction.



Figure 3. Zeba™ buffer exchange spin column.

1. For each protein sample to be buffer exchanged, prepare 1.5 ml of 1x Modification buffer **and** 1.5 ml of 1x PBS buffer from 10x concentrate. **Note-** use molecular grade water to prepare 1x PBS and 1x Modification Buffer from the concentrate.
2. Twist off the bottom closures of two spin columns and loosen the red caps (do not remove caps).
3. Place each spin column into a separate 1.5 ml microcentrifuge collection tube (provided).
4. 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.
5. Remove the columns from the centrifuge and discard the solutions from the bottom of the collection tubes. **Note-**do not discard the collection tube.
6. Using a marker pen, place a mark on the side of each spin column where the compacted resin is slanted upward.
7. 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**.
8. 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, do not disturb the resin bed with the pipette tip.
9. 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.
10. Repeat steps 8 and 9 two additional times, discarding the flow-through buffer each time.

11. 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.
12. Carry out the 1st buffer exchange on the protein sample by loading the entire 100 μ l of the sample to the top of spin column **A**.
13. Add 100 μ l 1x PBS buffer to the top of the equilibrated spin column **B**. Recap the lid loosely. **Note**-**B** column serves as a balance tube.
14. Centrifuge the columns @ 1500 x g for 2 minutes to collect the eluate at the bottom of each collection tube.
15. Remove spin column assembly **A** containing the protein sample to be biotinylated from the centrifuge and transfer to a clean storage tube (provided). Label the tube appropriately.
16. Remove spin column assembly **B** from the centrifuge and discard the bottom eluate. Add 300 μ l 1x PBS to the top of resin bed **B** to rehydrate the resin, cap and **set aside until near the end** of the biotinylation reaction. **Important**-this 300 μ l of PBS is needed to keep the resin hydrated.

d. Determination of Protein Concentration (BCA™ Assay) (30 min)

After buffer exchange, a small aliquot (2-10 μ l) of the protein sample is used to measure protein concentration. SoluLink highly recommends the BCA™ Protein Assay (Pierce, Cat. #23225, BCA is a registered trademark ThermoScientific/Pierce) for this purpose. Other protein assays can also be used (e.g. Bradford assay).

If the protein concentration is 1 mg/ml or less, a 10 μ l aliquot is generally used in the BCA assay. If the protein concentration is 2-10 mg/ml, a 2 μ l aliquot is generally used in the BCA assay. **Note**- although BCA™ reagents are not provided with this kit, a standard microplate BCA™ protein assay protocol is provided for reference.

BCA™ Microplate Procedure

Required Materials (sufficient for ~25 protein assays)

BCA Reagent A- 5 ml	96-well polystyrene plate
BCA Reagent B-100 μ l	40° C water bath
BSA standard: 2 mg/ml	1X PBS (10 ml)
Molecular grade water	P-2, P100 pipettes

1. Prepare a working solution of BCA reagent just prior to use by adding 5 ml of BCA Reagent A to a clean 15 ml conical tube followed by addition of 100 μ l of BCA Reagent B. Mix the two solutions until a clear green solution forms. **Note**- Prepare the BCA working reagent fresh daily.
2. For each protein to be measured, place the indicated volume of 1x PBS (see **Table 2**) into a microplate well and add the appropriate aliquot of the protein sample to the PBS (see table below). The final volume of each sample in the plate must 20 μ l. Record the dilution factor.

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

Table 2. Preparation of protein samples for BCA assay.

3. Prepare a BCA protein standard curve by making a 2-fold serial dilution of a 2 mg/ml albumin standard (e.g. Pierce Chemical, Product Number 23209) or other protein standard into individual wells of a microplate as follows:

Well #1- Add 50ul 1X PBS and 50ul 2mg/ml BSA standard to a well (1mg/ml)
 Well #2- Add 50ul 1X PBS and 50ul from the 1st well to a 2nd well (0.5mg/ml)
 Well #3- Add 50ul 1X PBS and 50ul from the 2nd well to a 3rd well (0.25mg/ml)
 Well #4- Add 50ul 1X PBS and 50ul from the 3rd well to a 4th well (0.125mg/ml)
 Well #5- Add 50ul 1X PBS and 50ul from the 4th well to a 5nd well (0.0625mg/ml)
 Well #6 -Add 50ul 1X PBS to the 6th well (Buffer blank).

Note- For more accurate results, a BCA standard curve can be made using a protein similar to the one being assayed (e.g. if using antibodies use a bovine IgG standard, Pierce Cat. # 23212).

4. Transfer 20 µl aliquots from each of the 2-fold serially diluted albumin standards to six empty microplate wells, preferably adjacent to wells containing 20 µl of the protein sample to be assayed (from step 2 above).
5. Add 150 µl freshly prepared BCA reagent to each well containing 20 µl of each dilution standard and sample to be assayed; mix well.
6. Seal the wells using clear adhesive film or scotch tape and incubate the plate at 37-40°C in a water bath for 15-20 minutes.
7. Remove the plate from the water bath, dry the bottom of the plate and read the plate in a suitable reader (e.g. Molecular Devices) at 562 nm. A typical BCA assay result is depicted in **Figure 4**.

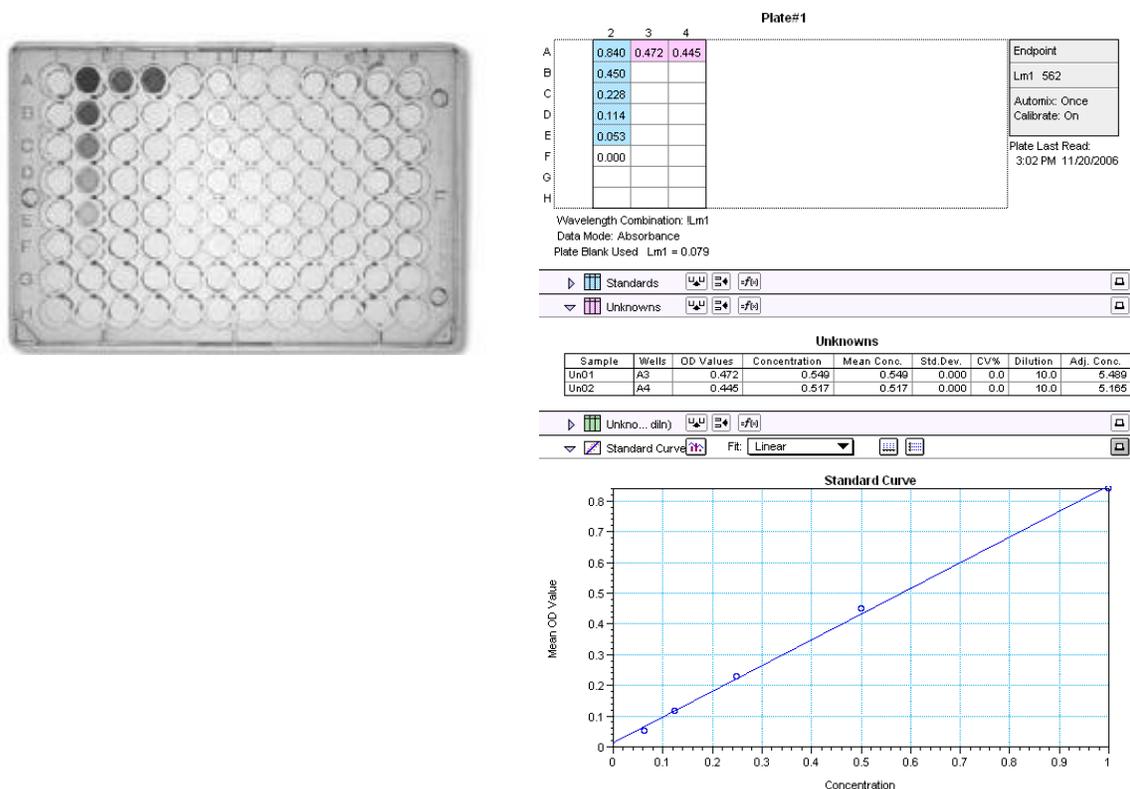


Figure 4. BCA protein microplate assay result. On the left is a plate containing a dilution series of BSA standards (wells A2-F2) along with two protein samples (A3, A4). On the right is the plate output from a Molecular Devices UV-VIS microplate reader illustrating BCA assay result.

e. Biotinylation Procedure (90 min)

1. After confirming (BCA assay) the buffer exchanged protein concentration (90-100 μ l) is in the proper range (i.e. 0.25 -10 mg/ml), the sample is ready to be biotinylated.
2. Prepare a Sulfo-ChromaLink Biotin working solution as follows:

Use the ChromaLink Biotin protein labeling calculator located on the flash drive and input the name of the protein, molecular weight, and BCA concentration (mg/ml) for your sample as illustrated in **Figure 5**. From the calculator output (1st red cell) add the indicated volume of 1x Modification Buffer to resuspend the Sulfo-ChromaLink Biotin reagent (0.5 mg).

Name of Protein	IgG	← User inputs
Molecular Weight (Daltons)	150,000	
Protein Concentration (mg/ml)	1.00	
(mg) of Protein being biotinylated	0.100	
Volume of Protein being labeled (ul)	100	← Calculator outputs
Mass of solid ChromaLink Biotin (mg)	0.50	
Volume 1X Modification Buffer used to resuspend ChromaLink Biotin (ul)	500	
Concentration of ChromaLink Biotin Working Solution (mg/ml)	1.0	
Equivalents of ChromaLink Biotin added	40	
Volume of ChromaLink Biotin working solution added to protein (ul)	24.3	↙

Figure 5. Sample ChromaLink™ Biotin protein labeling calculator located on the flash drive.

- Mix the working solution thoroughly by pipetting up and down repeatedly (20-30 times) or until no visible solids remain. The solution should appear clear and yellowish in color. **Important-** insure the working solution is fully dissolved before use.
- Add the indicated volume of Sulfo-ChromaLink Biotin working solution from the calculator output (2nd red cell) to the protein solution (90-100µl) and pipette up and down to mix.
- Allow the reaction to incubate for 90 minutes at room temperature. **Note-** as the reaction proceeds; some cloudiness (usually after 15-30 minutes) is sometimes observed from more concentrated protein samples due to partial hydrolysis of the concentrated ester.
- Shortly before the end of the labeling reaction (~5 min), transfer the still hydrated PBS equilibrated **B** spin column (section II-c, step16) into the centrifuge. Place the **A** column containing 300 µl of molecular grade water opposite the **B** column into the centrifuge as a balance tube.
- Spin the columns at 1,500 x g for 1 minute; discard the flow-through buffer from each collection tube.
- Transfer the **B** spin column to a new, clean collection tube and immediately proceed to the second buffer exchange procedure.

f. Second Buffer Exchange Procedure (10 min)

- Using a pipette, transfer the contents of the completed biotinylation reaction to the top of the **B** resin. Properly orient the spin column back in the centrifuge as before.
- Add 100µl molecular grade water to the resin in column **A**; properly orient it opposite the **B** column assembly in the centrifuge.
- Spin at **1,500 x g for 2 minutes** to collect the buffer exchanged biotinylated protein sample.

4. Transfer the eluate from the bottom of **B** collection tube to a new storage tube (provided). The biotin molar substitution ratio of the labeled protein is now be determined.

g. Determining Biotin Incorporation (20 min)

Degree of biotin incorporation is determined by scanning the biotinylated protein sample using a **UV-VIS spectrophotometer**, **UV-VIS plate reader**, or **NanoDrop™**. Refer to **Table 3** for each instrument's specific requirements and limitations. For example, when scanning a sample with a conventional UV-VIS spectrophotometer the sample holder needs to accommodate a micro- or semi-micro cuvette (50-100 μ l capacity). As indicated by the table, more concentrated protein samples (2-10 mg/ml) will require dilution prior to scanning. If using a UV-VIS plate to scan, you will require specialized UV-transparent 96-well microplates (standard or 'half-area' plates) and more concentrated protein samples (2-10 mg/ml) will also require dilution before scanning. When using the **NanoDrop™ spectrophotometer**, some protein samples will be too dilute for accurate UV ratio measurements due to the short path length of this instrument.

Instrument	Protein Concentration	Quartz Semi-Micro Cuvette (50-100 μ l)	Quartz Cuvette (1 mL capacity)
UV-VIS Spectrophotometer	<0.5 mg/ml 1.0 mg/ml 2-10 mg/ml	√ √ √*	N/R N/R N/R
Instrument	Protein Concentration	UV-Microplate (Standard well)	UV-Microplate (Half-area well)
UV-VIS Plate Reader	<0.5 mg/ml 1.0 mg/ml 2-10 mg/ml	N/R √ √*	√ √ √*
Instrument	Protein Concentration	Cuvette or Well	1.5 μ l volume
NanoDrop™ Spectrophotometer	<0.50 mg/ml 1.0 mg/ml 2-10 mg/ml	none none none	N/R √ √

Table 3. Instrument-specific sample requirements.

N/R = not recommended, √ = compatible, √* = compatible but sample will require dilution

As a general rule of thumb for all instruments, the optimal protein concentration for scanning a sample is 1 mg/ml.

Determine the protein biotin molar substitution ratio using either of the following methods:

- E1% Method
- BCA Method

The E1% method is used when the protein's E1% value is known. The E1% or mass extinction coefficient (L/gm-cm) is the A_{280} value of a 1% solution (10 mg/ml) as measured in a 1-cm path length. A list of known E1% values (published) is provided in **Table 4** for reference.

Protein	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
Human IgA	12.60
Human IgM	11.80
Bovine Serum Albumin	6.70
Trypsin	16.00
Chymotrypsin	20.20
Ovalbumin	7.90
alpha-amylase	24.20

Table 4. Known E1% values for commonly biotinylated proteins.

If the E1% of a protein is not available or is unknown, then the BCA method can be used to determine the biotin molar substitution ratio (MSR) of the biotinylated protein.

E1% Method

1. Program a conventional spectrophotometer or plate reader to scan from 220-420 nm.
2. Blank the spectrophotometer using 1x PBS.
3. Scan ~100 μ l of the biotinylated protein sample from 220 nm to 420 nm.
4. Record the A_{280} and A_{354} values from the scan.

- Calculate the biotin molar substitution ratio using the E1% Biotin MSR Calculator located on the flash drive by inserting the following input data:

name of biotinylated protein, protein sample volume scanned (μl), A_{280} and A_{354} , E1% value, molecular weight of the protein (daltons)

- Record the calculator output (biotins/protein molecule or MSR).

If you do not wish to use the E1% calculator to automatically calculate the MSR, the following equations can be used for manual calculations:

- Determine corrected absorbance (A_{280})

$$\text{Corrected } A_{280} = A_{280} - (A_{354} * 0.23) \quad \text{eq. 1}$$

- Determine moles protein

$$\left[\frac{(\text{Corrected } A_{280} / \text{E1\% value}) * (\text{volume})}{10} \right] / \text{protein M.W.} = \text{moles protein} \quad \text{eq. 2}$$

NOTE: volume in ml and protein E1% in mg/ml

- Determine moles biotin

$$\left(\frac{A_{354}}{29,000} \right) * \left(\frac{\text{volume}}{1,000} \right) = \text{moles biotin} \quad \text{eq. 3}$$

NOTE: volume in ml

- Biotins/protein MSR determination

$$\text{MSR} = \frac{\text{eq. 3}}{\text{eq. 2}} \quad \text{eq. 4}$$

BCA Method

1. Blank a spectrophotometer @ 354 nm using 1x PBS.
2. Measure the A_{354} of the biotinylated protein sample and record the value.
3. Measure the biotinylated protein concentration using BCA protein.
4. Calculate the biotin molar substitution ratio using the BCA Biotin MSR Calculator located on the flash drive by inserting the following input data:

name of biotinylated protein, biotinylated protein sample volume recovered (μ l), molecular weight of protein (daltons), A_{354} , BCA concentration (mg/ml),

5. Record the calculator output (biotins/protein molecule or MSR).

If you do not wish to use the BCA method calculator to automatically calculate the MSR, the following equations can be used for manual calculations:

- 1) Determine moles protein:

$$\left[\frac{(\text{protein concentration}) * (\text{volume})}{1000} \right] / \text{protein M.W.} = \text{moles protein}$$

eq. 1

NOTE: volume in ml and BCA protein concentration in mg/ml

- 2) Determine moles biotin present

$$\left(\frac{A_{354}}{29,000} \right) * \left(\frac{\text{volume}}{1,000} \right) = \text{moles biotin}$$

eq. 2

NOTE: volume in ml

- 3) Biotin/protein MSR determination

$$\text{MSR} = \frac{\text{eq. 3}}{\text{eq. 2}}$$

eq.3

III. Examples and Applications

a. Biotinylation of IgG

Bovine IgG was biotinylated at six different protein concentrations (0.25, 0.5, 1, 2.5, 5, and 10 mg/mL) and a fixed number of Sulfo-ChromaLink Biotin (S-CLB) equivalents (10X). Reactions were buffer-exchanged (PBS) and scanned (220-420 nm) on a Molecular Dynamics UV-VIS microplate reader (**Figure 6**) in UV-transparent 96-well microplates. All molar substitution ratios were calculated using optical density measurements @ A_{280} and A_{354} with a quartz micro-cuvette using an E1% value of 12.4. More concentrated samples were diluted prior to scanning. Actual results may vary somewhat depending on the type and source of antibody used.

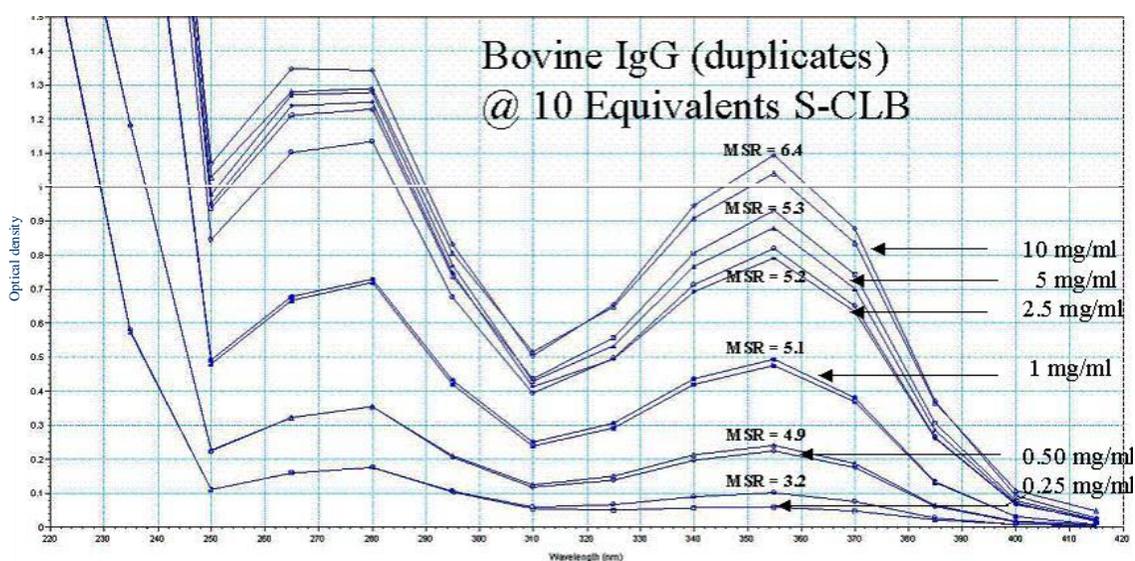
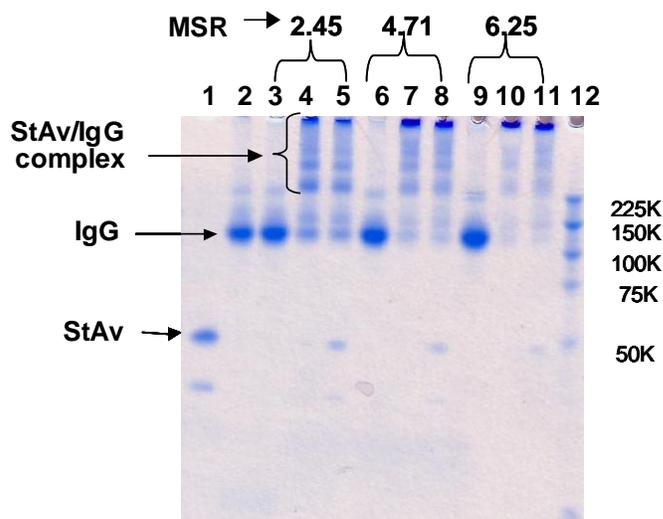


Figure 6. Spectral overlays of biotinylated bovine IgG using a Molecular Dynamics UV-VIS plate reader. Duplicate reactions at six different initial IgG concentrations are represented.

b. Streptavidin Binding to Biotinylated IgG

Bovine IgG at 5 mg/ml was biotinylated at 5, 10, and 15 mole equivalents using ChromaLink Biotin. Molar substitution ratios were determined for each reaction using A_{280} and A_{354} method. To demonstrate the streptavidin-binding efficiency of ChromaLink Biotin, biotinylated bovine IgG was incubated with 1 and 2 mole equivalents of streptavidin and binding confirmed by SDS-PAGE gel-shift analysis (**Figure 7**). These results demonstrate increasing streptavidin binding efficiency with an increase in biotin modification. The lack of complete binding at lower biotins/IgG levels is likely due to inaccessibility of some biotins by streptavidin.



Lane	Protein	StAv Equiv added
1	Streptavidin	N/A
2	Bovine IgG	N/A
3	Biotinylated IgG MSR = 2.45	0
4	Biotinylated IgG MSR = 2.45	1
5	Biotinylated IgG MSR = 2.45	2
6	Biotinylated IgG MSR = 4.71	0
7	Biotinylated IgG MSR = 4.71	1
8	Biotinylated IgG MSR = 4.71	2
9	Biotinylated IgG MSR = 6.25	0
10	Biotinylated IgG MSR = 6.25	1
11	Biotinylated IgG MSR = 6.25	2
12	Protein M.W. markers	N/A

Figure 7. Bovine IgG at 5 mg/ml was reacted with 5X, 10X and 15X ChromaLink™ Biotin and molar substitution ratios determined. Aliquots of biotinylated IgG at the indicated MSR were then incubated with 1X or 2X equivalents of streptavidin and binding confirmed using PAGE gel-shift analysis (Coomassie blue staining).

c. Traceable Capture of Biotinylated IgG Using Streptavidin Beads

Bovine IgG was biotinylated at an MSR of 3.2 biotins/IgG using ChromaLink Biotin. The biotinylated IgG (500 ug) was then incubated in binding buffer with 1 mg of NanoLink Streptavidin Magnetic beads (blocked w/casein) for 30 minutes. Sample supernatants were scanned from 220-420 in order to quantify binding of biotinylated IgG (**Figure 8**). Controls consist of no bead controls as well as (500 ug) unbiotinylated IgG. Approximately half or 250 ug biotinylated IgG bound to 1 mg of beads in 30 minutes.

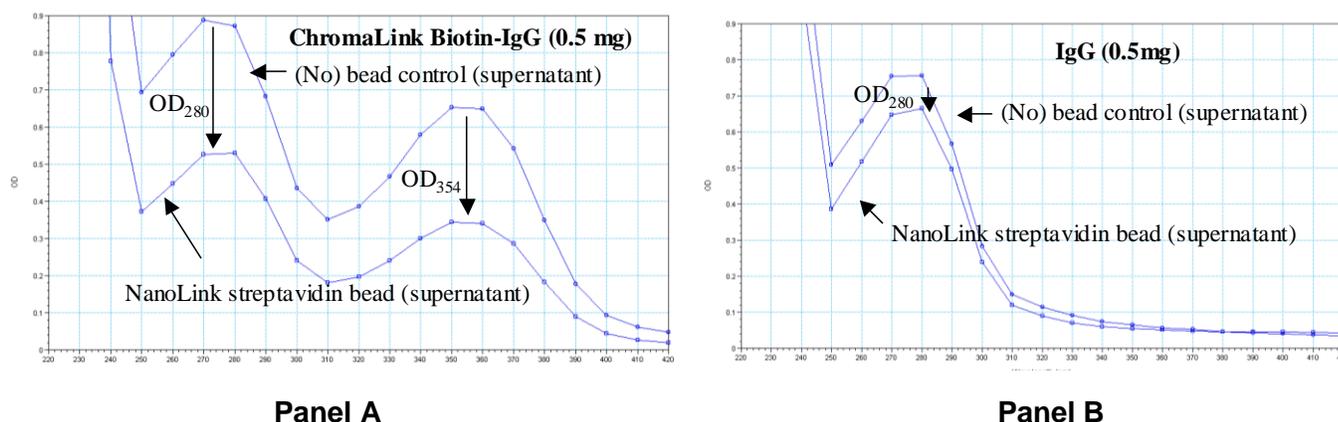


Figure 8 Capture of ChromaLink Biotin-IgG as measured by reduction of supernatant A_{280} versus a negative control (unbiotinylated IgG). Each binding reaction (150 μ l) consisted of 1 mg of casein-blocked NanoLink™ Streptavidin magnetic beads incubated for 30 minutes with either 500 μ g biotin-IgG (Panel A) or 500 μ g unbiotinylated IgG (Panel B). Note the lack of A_{354} in the unbiotinylated control. Reduction in the A_{354} (Panel A) can be used to calculate moles biotinylated-IgG specifically captured by the streptavidin beads.

d. NanoDrop™ Spectra of Biotinylated IgG

Overlaid UV spectra (220-450 nm) of various biotinylation reactions (1.5 μ l) scanned on a NanoDrop™ spectrophotometer (**Figure 9**). Biotinylation reactions were set up containing a series of different antibody concentrations (0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml). All samples were reacted using either 10 or 20X Sulfo-ChromaLink Biotin.

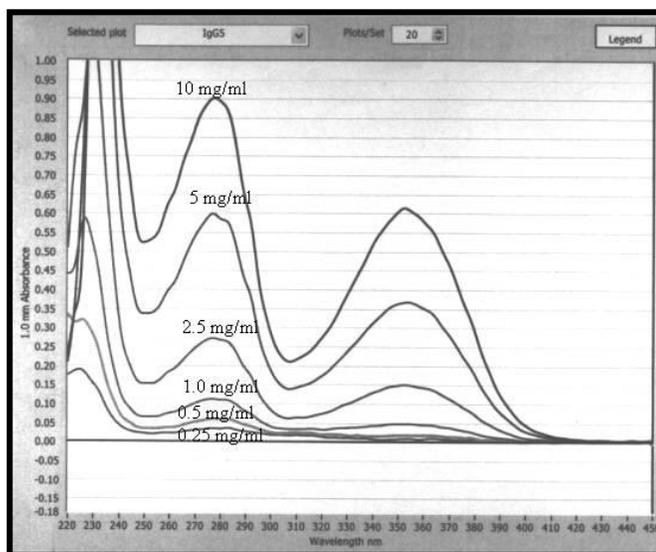


Figure 9. Overlaid spectra (220-450 nm) of biotinylated IgG samples scanned on a Nanodrop™ spectrophotometer.

IV. Appendix

a. *Biotinylated IgG Control*

The ChromaLink™ Biotin Protein Labeling Kit comes complete with a lyophilized, pre-biotinylated bovine IgG (500ug) control. This control can be used to validate the reliability of a given spectrophotometer or 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 with 1x PBS (220-420 nm). Discard the blank buffer solution from the cuvette.

Note-if using a NanoDrop™ follow the instructions on page 24.

2. Thoroughly resuspend the biotinylated antibody control (lyophilized solid, 500 µg) using 500 µl molecular grade water @ 1 mg/ml.
3. Briefly spin the tube @ 1500xg for 15 seconds.
4. Transfer 100 µl (or 2 µl with the NanoDrop™) and scan the biotinylated antibody from 220-420 nm.
5. Record the A_{280} and A_{354} values generated from the scan.
6. Enter the name of biotinylated protein, protein sample volume scanned (µl), A_{280} and A_{354} , E1% value, molecular weight of the protein into the E1% 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 located on the flash drive.

b. *IgG Control (unbiotinylated)*

The ChromaLink™ Biotin Protein Labeling Kit also comes with an unlabeled bovine IgG (500ug) control ready for biotinylation. To use the control; simply resuspend the lyophilized bovine IgG (500 ug) in 500 µl molecular grade water to obtain a 1mg/ml solution. Biotinylate the control as follows:

- 1) Transfer 100 µl of the (unbiotinylated) control IgG @ 1mg/ml to a clean 1.5 ml tube.
- 2) Prepare a 0.5 mg/ml working stock of Sulfo-ChromaLink Biotin by resuspension of the reagent in 1000 µl 1x Modification Buffer as directed in Section II, e-2.
- 3) Add 24.3 µl of ChromaLink Biotin working solution to 100 ul of the control IgG. Mix well.

- 4) Allow the reaction to proceed for 90 minutes.
- 5) Buffer exchange the reaction using a Zeba™ spin column equilibrated in 1x PBS as directed in the protocol.
- 6) Measure the molar substitution ratio of the sample as previously directed.
- 7) A properly working kit will yield a biotin molar substitution ratio between 2-8 biotins per IgG molecule.

c. HABA vs. ChromaLink Biotin Spectrophotometric Assay

Comparisons between the HABA assay (2-4'-hydroxyazobenzene-2-carboxylic acid dye-binding assay) and the ChromaLink™ spectrophotometric assay (CSA) are summarized in **Table 5**. 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 one-third the value obtained using ChromaLink.

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

Table 5. Comparison of HABA and ChromaLink™ assays for determination of biotin molar substitution ratios (MSRs). Bovine IgG samples were biotinylated at different reaction equivalents and the results tabulated.

As illustrated in the table, the HABA dye-binding assay generally underestimates the true biotin molar substitution ratio because HABA measures the number of moles of biotin available for binding to streptavidin and not the absolute number of biotin molecules attached to an antibody surface. For example, two biotin molecules in proximity to each other are likely to bind to one streptavidin molecule. Another major difference includes the non-destructive nature of ChromaLink versus HABA. For example, HABA consumes and destroys a minimum of 75 micrograms of a biotinylated antibody per assay whereas ChromaLink™ requires as little as 25 µg biotinylated sample, all fully recoverable. Finally, the HABA assay requires an external spectrophotometric calibration curve whereas the ChromaLink™ method requires no such external standard calibration.

d. General Guide for Biotinylation of Proteins

Table 6 summarizes the expected degree of biotinylation for several different proteins at a given protein concentration and mole-equivalents of Sulfo-ChromaLink Biotin. Generally, as protein concentration increases the MSR also increases. Lower molecular weight proteins tend to have fewer available lysines so smaller proteins tend to have lower MSRs under a given set of conditions.

Bovine IgG (150 kD)	Equivalents		
	5x	10x	20x
mg/ml	MSR	MSR	MSR
5	2.5	4.6	7.8
2.5	2.3	4.1	7.3
1	1.4	3.5	5.7
0.5	1.1	2.2	3.9
0.25	1.2	1.8	2.1

BSA (67 kD)	Equivalents		
	5x	10x	20x
mg/ml	MSR	MSR	MSR
5	2.3	3.9	5.4
2.5	2.8	3.6	5.8
1	1.6	3.3	5.2
0.5	1.6	2.3	4.8
0.25	1.0	2.0	2.7

CEWA (44 kD)	Equivalents		
	5x	10x	20x
mg/ml	MSR	MSR	MSR
5	4.9	7.7	9.3
2.5	3.5	5.9	8.4
1	1.9	3.5	4.1
0.5	1.2	1.9	3.0
0.25	0.8	1.1	1.9

β-Lactoglobulin (18.3 kD)	Equivalents		
	5x	10x	20x
mg/ml	MSR	MSR	MSR
5	2.7	3.3	3.4
2.5	2.6	3.2	2.9
1	1.8	3.3	3.0
0.5	1.2	2.7	3.0
0.25	1.2	2.6	2.2

Table 6. Guideline for biotinylation of various proteins.

e. Troubleshooting Guide

Problem	Possible Cause	Recommended Action
Poor biotin modification of protein	-initial protein concentration is outside the optimal range for this protocol	-recheck initial protein concentration -concentrate or dilute the protein into the required range (e.g. 0. 25-10 mg/ml)
	-low number of lysines or sterically hindered lysine residues	-check the primary sequence of the protein being modified using NCBI Blast to insure lysine groups are available for modification
	-a large excess of amine contaminants may be present in the protein sample, e.g. Tris or glycine buffers could be contaminating the protein sample	-buffer exchange the protein sample. Some samples are so overly contaminated that two buffer exchanges may be required before proceeding
Complete failure of biotin labeling reaction	-improper mixing of reaction components -improper functioning of spectrophotometer	-use the control IgG (unbiotinylated) sample provided and biotinylate according to the recommended protocol -UV lamp output low -recalibrate spectrophotometer
Molar substitution ratio are out of detectable range	-protein concentrations are out of recommended range. -precipitation of biotin modified proteins may occur due to over-modification of available lysines and a drastic change in the isoelectric properties of the modified protein.	-concentrate or dilute protein samples into recommend range -after the biotinylation reaction is complete, addition of 1M Tris (pH 9.0) can sometimes be used to resuspend the biotinylated protein -reduce number of equivalents used to label protein
Precipitation of protein	- over-modification of the protein -critical lysine residues necessary for solubility have been modified	-check the concentration of Sulfo-ChromaLink Biotin working stock used to label the protein -attempt to label for shorter periods of time

f. Instructions for Using a NanoDrop™ to Measure Biotin MSR

1. Turn on the NanoDrop™ spectrophotometer and click on the NanoDrop™ icon to launch the software.
2. Place a 2 µl drop of molecular grade water on the clean pedestal, click 'OK'.
3. When the main menu appears, select the A280 menu option. **Note-** do not use the UV-VIS menu option on the NanoDrop™ to read 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 protein sample.
6. Blank the NanoDrop™ spectrophotometer by placing a 2 µl drop of 1x PBS 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 protein 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.
9. Record the absorbance @ 280 nm from the λ absorbance window.
10. Record the absorbance @ 354 nm after typing the numeric value 354 into the λ window.
11. 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).

g. Kit Disclaimer

Although SoluLink has extensively optimized the ChromaLink™ Biotin Protein Labeling kit and used it to successfully label dozens of different proteins, it is nonetheless possible that the biological activity of some proteins may be compromised or even lost during the biotinylation process.

Although somewhat rare in frequency, this phenomenon may occur because the protein in question may possess one or more critical lysine amino acid residues directly at the active site (AS) and whose function for biological activity is compromised during biotinylation. Additionally, SoluLink has observed certain instances where some proteins may possess critical lysine residues (not necessarily at the active site) that are absolutely crucial for maintenance of protein stability and/or solubility. The modification of these few critical residues may cause partial or complete precipitation/aggregation of the protein in question. This phenomenon has sometimes even been observed even at very low biotin substitution levels.

These instances are beyond the control of this and any other NHS-ester based labeling kit used to biotinylate proteins in a non site-selective (stochastic) manner. Use of this labeling kit does not come with any implied guarantee or warranty for either loss of protein due to precipitation/aggregation or loss of biological activity/function. We feel our customers should be made aware that biotinylation may sometimes compromise the biological function/activity of the protein being modified.