



ChromaLink™ Biotin Protein Labeling Kit

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I. Introduction

a. Product Description and Background

SoluLink's ChromaLink™ Biotin protein labeling kit has the necessary reagents for the traceable biotinylation of proteins. The kit provides sufficient materials to label, purify and quantify 5 protein reactions. ChromaLink Biotin incorporates UV-traceable biotin onto proteins containing lysine residues (amine groups) via a water-soluble-succinimidyl activated ester.

Biotin is a naturally occurring vitamin that binds with high affinity to avidin/streptavidin proteins ($K_d \sim 4 \times 10^{-14}$ M) and can readily be coupled to proteins without altering their biological activity. Labeled proteins can be detected using ELISA techniques/western blots or captured using immobilized avidin/streptavidin. Protocols provided with this kit can be used to successfully biotinylate proteins spanning a broad range of molecular weights (20-200 kD) and concentrations (0.25 to 10 mg/ml).

For example, quantities as small as 25 ug or as much as 1 milligram of an antibody can be biotinylated and the resultant incorporation determined (i.e. molar substitution ratio or MSR). Included in the kit are easy to use ChromaLink calculators located on the flash drive. These simple calculators are used to determine labeling reaction volumes and post-labeling calculations.

ChromaLink Biotin has been engineered to include many novel features. As illustrated in Figure 1, the molecule's structure contains a bis-aryl hydrazone chromophore **(a)**, linked by a PEG3 linker arm **(b)**, to biotin **(c)**. This reagent permits direct spectroscopic quantification of incorporated biotin. The extended PEG3 linker preserves biotin/streptavidin affinity and maintains protein solubility after modification while the water-soluble sulfo-succinimidyl ester functional group **(d)**, efficiently modifies lysines in aqueous buffers.

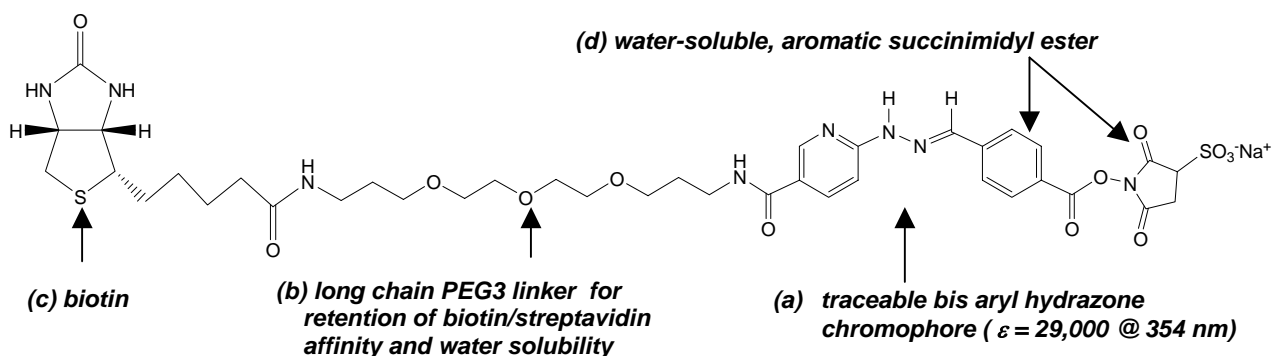


Figure 1. Molecular structure and formula of ChromaLink Biotin
(C₃₈H₄₉N₈NaO₁₃S₂) (M.W.- 912.96)

b. Benefits and Features

ChromaLink Biotin offers an easier solution to the quantitative biotinylation of proteins from as little as 25 ug of material. The instructions and protocols are easy to follow and are designed to make traceable biotinylation of proteins nearly foolproof. Labeling of proteins with ChromaLink Biotin eliminates the need to carry out cumbersome and time-consuming HABA assays often employed to quantify biotin incorporation. Instead, biotin incorporation is quantified by means of a simple spectrophotometric measurement at two wavelengths (A_{280} / A_{354}) or alternately at a single wavelength (A_{354}) in combination with a protein concentration (mg/ml) as determined by the BCATM protein assay.

Typical labeling results are illustrated by spectral overlay scans of four samples. As illustrated, Bovine IgG (100 ul @ 5 mg/ml) was labeled at 0, 5, 10, and 15 mole equivalents using ChromaLink Biotin. Spectral analysis illustrates how easy it is to visualize, confirm, and quantify biotin incorporation.

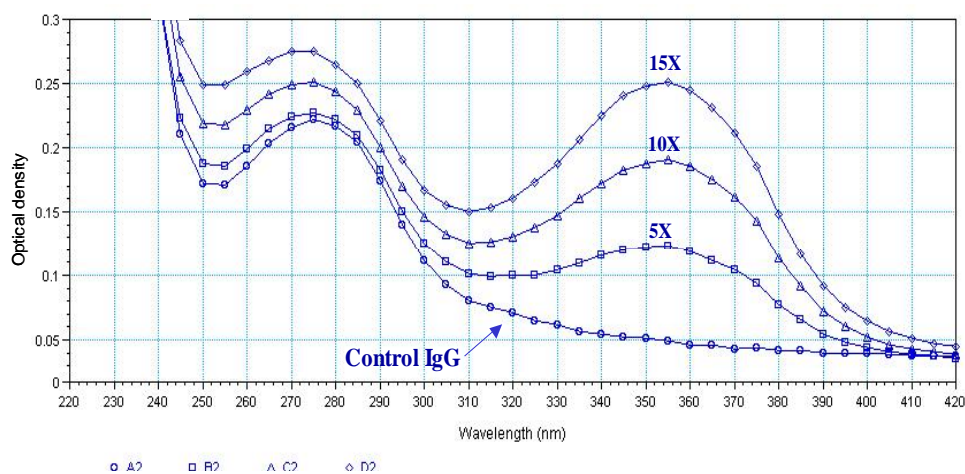


Figure 2. Superimposed spectra of bovine IgG biotinylated using ChromaLinkTM Biotin. Various biotin-to-protein mole equivalents (5X, 10X and 15X) were used. Note the traceable UV-signature @ 354nm indicating incorporation of biotin. All spectra were scanned on a Molecular Dynamics SpectraMax PlusTM UV-VIS plate reader (220-420 nm).

c. HABA vs. ChromaLink Biotin Spectrophotometric Assay

Comparison between the HABA (2-4'-hydroxyazobenzene-2-carboxylic acid) assay and the ChromaLink spectrophotometric assay reveals distinct differences between them. For example, the ChromaLink method will directly quantify incorporation non-destructively from as little as 25 ug of a biotinylated protein, whereas the HABA assay (destructively) requires up to 3 times that amount. Although there is a correlation between the two methods, they do differ (see Table 1).

Mole equivalents	Biotin/IgG	
	HABA	A ₃₅₄
5X	1.03	2.45
10X	1.60	4.71
15X	2.22	6.25

Table 1. Relationship between HABA assay results and the -Chromalink spectrophotometric assay results. All molar substitution ratios were measured using the same biotinylated bovine IgG at 5 mg/ml.

Molar substitution ratios (MSR) obtained using the ChromaLink spectrophotometric method are higher than those obtained using the HABA assay. The HABA dye displacement assay measures the number of biotins available for binding to streptavidin whereas the ChromaLink Biotin spectroscopic method measures absolute biotin incorporation by means of its internal UV-traceable hydrazone chromophore ($\epsilon = 29,000 @ 354 \text{ nm}$).

d. Procedure Overview

SoluLink's ChromaLink Biotin protein labeling procedure is depicted in Figure 3. The protein to be biotinylated is first exchanged into an optimized labeling buffer. After exchange, a small aliquot of the sample is used in a BCATM protein assay to determine protein concentration. The BCATM assay is used to establish precise stoichiometric proportions between the biotin labeling reagent and the protein. After labeling, excess reagent is removed using a ZebaTM spin column. Biotin incorporation is then quantified using one of two simple spectroscopic methods.

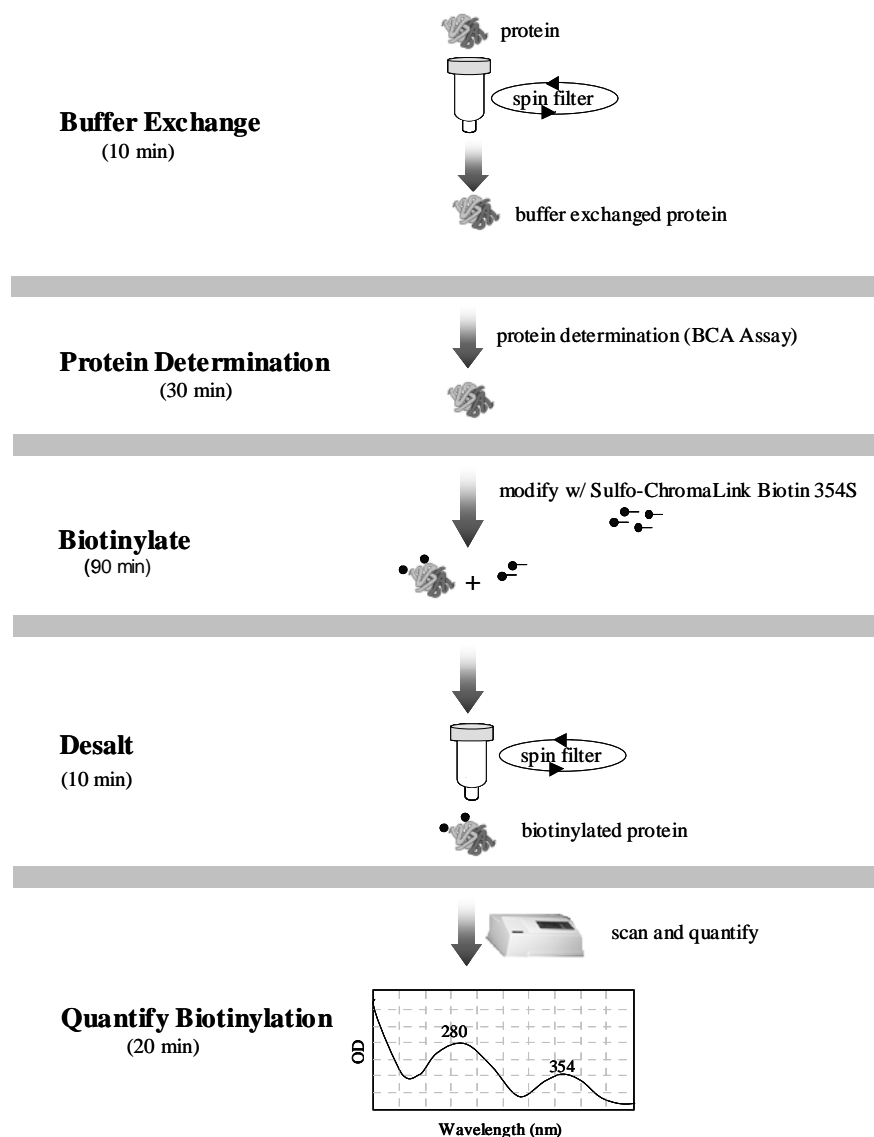


Figure 3. ChromaLink Biotin protein labeling procedure.

e. Materials Provided and Storage Conditions

Component	Size/Quantity	Storage Conditions
Sulfo-ChromaLink Biotin (solid)	5 x 0.5 mg	desiccated @ room temp
10x Modification Buffer	2 x 1 ml	room temp
10x PBS	2 x 1 ml	room temp
Zeba™ desalt spin columns	12	keep refrigerated 4-8 °C
1.5 ml collection tubes (no lids)	24	no special storage
1.5 ml storage tube	12	no special storage
Bovine IgG @ 1 mg/ml	0.5 ml	keep refrigerated 4-8 °C
Biotinylated IgG @ 1 mg/ml	0.5 ml	keep refrigerated 4-8 °C
128MB Flash drive	1	room temp

f. Additional Materials Required But Not Provided

Instruments

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

Water bath (40°C)

Reagents and Consumables

Albumin Standard, 2 mg/ml (Pierce Chemicals, #23209)

BCA™ Protein Assay Kit (Pierce Chemicals, #23225)

Molecular grade H₂O (e.g. Ambion # 9932 or similar)

15 ml disposable conical tubes (for BCA™ protein assay)

1.5 ml microfuge tubes

Standard polystyrene 96-well microplate

UV-VIS Instrumentation (any of the following)

UV-VIS Spectrophotometer and a semi-micro cuvette holder

Nanodrop™ spectrophotometer

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

Microplates and Cuvette

Greiner UV-Star™ 96 Well Microplate (half area plate, Cat. # 675801)

Greiner UV-Star™ 96 Well Microplate (standard UV plate, Cat. # 655801)

Quartz semi-micro-cuvette (100 ul volume)

Pipettes

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

g. Related Products

Product	Size/Qty	Cat. Number
Sulfo-ChromaLink Biotin 354S	5 x 1 mg	B1007-105
ChromaLink Biotin 354S	10 mg	B1001-110
ChromaLink Biotin 354M	5 x 1 mg	B1012-105

II. ChromaLink Biotin Protein Labeling Protocol

a. Important Biotinylation Parameters

SoluLink has optimized conditions to incorporate biotin onto proteins possessing maximum capture and detection efficiency using streptavidin. Protocols are provided that label a broad range of protein molecular weights (20–200kD) and concentrations (0.25 -10 mg/ml). Each labeling reaction uses a fixed protein volume (100 ul) and either 10 or 20 mole-equivalents of ChromaLink Biotin reagent (depending on protein concentration).

Reaction conditions used in this kit incorporate multiple biotins into any lysine-containing protein regardless of starting molecular weight and/or initial concentration within a specified range (see Table 2). Approximately ~ 3-6 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 2. Range of labeling parameters used in the ChromaLink Biotin protein labeling kit.

A buffer exchange is always used at the start of the procedure to transfer the protein into an optimized reaction buffer (1X Modification Buffer). This rapid buffer exchange guarantees reproducible biotinylation and must not be avoided or skipped. Zeba™ spin columns are provided for this purpose. These columns are rapid and easy to use while maintaining high protein recovery (>85%). The columns avoid sample dilution altogether and recover the same volume and mass that was applied to the column.

Molar substitution ratios (moles of biotin per mole of protein) obtainable with the ChromaLink assay can be measured using one of two spectroscopic methods ([Section II, g](#)). These two methods generally agree within ~5% but can differ slightly. Small protein volumes generated by the labeling protocol necessitate the use of a quartz micro-cuvette (100 ul) when measuring optical densities. Alternately, a UV-VIS plate reader can be used but this instrument requires a UV-transparent polystyrene microplate. Finally, if a micro-volume spectrophotometer such as a Nanodrop™ is employed to determine molar substitution ratios, no special plates or cuvettes are required. Refer to section II-g for additional information on determining quantitative molar substitution ratios with each instrument.

Protocols in this kit will modify proteins with an average MSR of ~3-6 biotins (if sufficient lysines are available) per protein molecule. We do not recommend higher levels of modification since over-modified proteins often precipitate. The relationship between molar substitution ratio, protein concentration (mg/ml), protein molecular weight (Daltons), and equivalents of ChromaLink Biotin in a reaction are summarized in [Appendix IV, section d, Table 6](#).

b. Biotinylation Protocol Summary

- Sample preparation (10 min.)
- Buffer exchange protein using Zeba™ spin column (10 min)
- Determine protein concentration (30 min)
- Biotinylate protein (90 min)
- Desalt reaction using Zeba™ spin column (10 min)
- Spectrophotometric analysis and calculations (20 min)

c. Sample Preparation (~10 min)

Protein samples come in many different forms; solid or liquid, low or high concentrations, micrograms or milligrams, with or without protein carriers. Often (if liquid) they come dissolved in buffers incompatible with optimal labeling conditions (e.g. Tris, varying ionic strength, and/or pH). To accommodate many of these sample differences, the protocol provided utilizes rapid Zeba™ spin columns to buffer exchange the protein without altering its concentration. Zeba™ columns have a maximum loading capacity of 130 ul, and for this reason, the labeling protocol limits the maximum protein labeling volume to 100 ul.

Protein carriers are sometimes added to antibody preparations and these will prevent proper labeling of the target protein. Protein samples may first require purification using affinity chromatography (or other methods) to remove stabilizing carrier proteins. Carriers must be removed before proceeding. To prepare a particular sample for biotinylation, refer to the following instructions:

Initial Protein Sample (Solid Form)

If the initial protein to be labeled is in solid form (e.g. lyophilized) it must first be resuspended in an appropriate volume of 1x Modification Buffer. The protocol limits the amount of protein that can be biotinylated within a single reaction. This range spans from a minimum of 25 ug to a maximum of 1 mg. All labeling reactions require a protein volume of 100 ul corresponding to protein concentrations that can span from 0.25 mg/ml to 10 mg/ml.

Procedure: Solid Form (25 ug- 1mg)

If the initial sample is a pre-weighed solid containing from 25 ug to 1 mg of protein (free of any other protein carrier) simply resuspend the sample in 100 ul of 1x Modification Buffer.

Important-10x Modification Buffer is provided as a 10X concentrate and must be diluted with molecular grade water to 1X before using.

Important- Do not attempt to label a mass greater than 1 mg or a concentration greater than 10 mg/ml. Multiple labeling reactions must be performed if labeling quantities larger than 1 mg.

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

If the protein to be labeled is available in larger bulk form, weigh out approximately 5-10 mg using an analytical balance. Add a volume of 1x Modification Buffer sufficient to bring the final protein concentration anywhere in a range from 0.25-10 mg/ml. Subsequently, transfer a 100 ul aliquot corresponding to the desired mass to be labeled into a new 1.5 ml tube.

Once a protein sample has been resuspended in 100 ul 1x Modification Buffer at the appropriate concentration range (0.25 mg/ml to 10 mg/ml), proceed directly to the biotinylation procedure ([section II, d](#)).

Initial Protein Sample (Liquid Form)

If the initial protein to be labeled is in liquid form (e.g. PBS or TBS) it must be in a concentration range from 0.25 mg/ml to 10 mg/ml. All labeling reactions require a protein volume of 100 ul, thus limiting the amount of protein that can be labeled in each reaction from a minimum of 25 ug to a maximum of 1 mg.

Procedure: Liquid Form (25 ug - 1 mg)

If the protein to be labeled is already dissolved in buffered solution at a concentration that ranges between 0.25 mg/ml and 10 mg/ml, simply transfer 100 ul into a new 1.5 ml tube and proceed to the biotinylation procedure ([section II, d](#)).

Note- Do not attempt to label protein samples below 0.25 mg/ml. Do not attempt to label protein volumes larger or smaller than 100 ul (volume accommodated by Zeba™ spin column). Always maintain the final protein concentration between 0.25 mg/ml and 10 mg/ml in a reaction volume of 100 ul. If more than 1 mg of protein is to be labeled, multiple reactions must be performed.

d. Protein Biotinylation Procedure (~90 min)

Note- 10x PBS and 10x Modification Buffer are provided as 10X concentrates and must be diluted to 1X with molecular grade water before using.

- 1) Buffer exchange the desired protein into 1x Modification Buffer using a Zeba™ spin column as outlined in [section \(II-e\)](#). The protein concentration must be between 0.25 mg/mL to 10 mg/mL in a volume of 100 ul.
- 2) After buffer exchange, use an aliquot of the protein to determine the protein concentration using the BCA assay as outlined in [section \(II-f\)](#).
- 3) Using the ChromaLink Biotin protein labeling calculator provided on the flash drive (Folder #1), input the name, protein concentration, and M.W. of the protein to be labeled.

Important-The calculator automatically calculates the volume of 1x Modification Buffer required to dissolve ChromaLink Biotin (0.5 mg solid) and create a working solution. The calculator also determines the volume of this working solution required in the labeling reaction. A sample calculator is provided below (Figure 4) for illustration purposes.

ChromaLink Biotin Protein Labeling Calculator	
Name of Protein	IgG
Molecular Weight (<i>Daltons</i>)	150,000
Protein Concentration (<i>mg/ml</i>)	0.50
(<i>mg</i>) of Protein being biotinylated	0.050
Volume of Protein being labeled (<i>ul</i>)	100
Mass of solid ChromaLink Biotin (<i>mg</i>)	0.50
Volume 1X Modification Buffer used to resuspend ChromaLink Biotin (<i>uL</i>)	1000
Concentration of ChromaLink Biotin Working Solution (<i>mg/ml</i>)	0.5
Equivalents of ChromaLink Biotin added	20
Volume of ChromaLink Biotin working solution added to protein (<i>ul</i>)	12.2

Figure 4. Sample ChromaLink™ Biotin protein labeling calculator located on the flash drive.

- 4) Prepare a ChromaLink Biotin working solution by adding the indicated volume of 1x Modification Buffer (from the calculator output) to a vial of solid reagent. Immediately mix the solution **vigorously** for 2-4 minutes using a vortex mixer to resuspend the solid. Spin the tube briefly to collect the liquid at the bottom of the tube if necessary. The final solution will appear clear and yellow in color.

Important- Insure the working solution contains no visible solids before using. **Once the working solution is prepared, do not let it stand. Use it immediately.** Do not attempt to store or reuse the working solution.

Important- Calculator outputs depend on initial reaction conditions (M.W., and protein concentration). The volume of 1x Modification Buffer added to solid (0.5 mg) ChromaLink Biotin varies depending on initial reaction parameters. The concentration of the reagent varies from reaction to reaction depending on the protein being labeled. Always use the calculator to determine the volume of 1x Modification Buffer needed to prepare the working solution.

- 5) Immediately add the indicated volume of freshly prepared working solution (from the calculator output) to 100 ul of protein solution and mix well.
- 6) Allow the reaction to proceed for exactly 90 minutes at room temperature.

Important- Do not allow the reaction to proceed longer than 90 minutes.

- 7) Fifteen minutes before the end of the reaction period, equilibrate a new Zeba™ spin column in **1x PBS** buffer as outlined in [section II-e](#). This column will be used to remove excess labeling reagent from the reaction.

e. Zeba™ Spin Column Buffer Exchange/Desalt Procedure (~ 10 min)

Twelve spin columns are provided in the kit (Figure 5). Five columns are used for buffer exchange (prior to biotinylation) and five to desalt and remove excess biotinylation reagent after the protein is labeled. Two additional columns are provided for running control reactions (Section IV-a) if desired.



Figure 5. Zeba™ desalt spin column used to rapidly exchange or desalt samples. Zeba™ columns have a maximum loading capacity of 130 ul.

Buffer Exchange/Desalt Protocol

Note- 10x PBS and 10x Modification Buffer are provided as 10X concentrates and must be diluted to 1X with molecular grade water before using. Prepare 1 ml of 1x Modification Buffer and 1 ml of 1x PBS for each spin column.

- 1) For each protein sample to be buffer exchanged or desalted, prepare a Zeba™ desalt spin column by twisting off the bottom closure and loosening the top red cap (do not remove the cap).
- 2) Place the spin column into a 1.5 ml microcentrifuge collection tube (provided).
- 3) Centrifuge the spin column @ 1,500 x g for 1 minute to remove storage solution. Remove the column from the centrifuge and discard the storage solution. Do not discard the collection tube since it will be reused.
- 4) Using a marker pen, place a mark on the side of the spin column where the compacted resin is slanted upward.

Note- this mark is used to orient the spin column in all subsequent centrifugation steps with the mark aiming outward and away from the center of the rotor.

- 5) Equilibrate the spin column using **1x Modification buffer (pH 7.4)** to exchange the protein into this optimized labeling buffer prior to biotinylation.

Note- **1x PBS buffer (pH 7.2)** is used to equilibrate the column if preparing the column for desalting excess reagent from the protein after completion of the biotinylation reaction.

- 6) Add 300 ul of the appropriate equilibration buffer to the top of the resin bed.
- 7) Place the column back into the reused collection tube and centrifuge at 1,500 x g for 1 minute to remove the buffer. After centrifugation the column matrix will appear white in color and dry.
- 8) Repeat steps 6 and 7 two additional times, discarding the flow-through buffer each time.
- 9) The column is now equilibrated with 1x buffer and ready for sample loading.

10) Place the spin column into a new 1.5 ml collection tube, remove the red cap and slowly apply 100 µl of protein solution to the center of the compacted resin bed.

Note- Avoid contact with the sides of the column when loading samples.

11) Centrifuge at 1,500 x g for 2 minutes to collect the protein sample. Approximately 100 ul of protein sample will be recovered in the collection tube.

12) Transfer the protein solution to a clean storage tube (provided). Cap and label appropriately.

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

After a protein is buffer exchanged and/or desalted, an aliquot of the protein sample (2-10 ul) is used to measure protein concentration. SoluLink highly recommends the BCA™ Protein Assay (Pierce, Cat. #23225) for this purpose because of its high sensitivity, accuracy and speed.

If a protein's expected concentration is 1 mg/ml or less, a 10 ul aliquot is used in the BCA assay. If a protein's expected concentration is 2-10 mg/ml, a 2 ul aliquot is used in the BCA assay.

Although BCA™ reagents are not provided with this kit, a comprehensive microplate BCA™ protein assay protocol is provided in the following section. Any commercial UV-VIS microplate reader or spectrophotometer can be used for conducting the BCA assay.

Note- BCA™ is a registered trademark of Pierce Chemical, Rockford, Illinois

BCA™ Microplate Procedure

Materials (sufficient for ~25 protein assays)

BCA Reagent A	5 ml	96-well polystyrene plate
BCA Reagent B	100 ul	40° C water bath
BSA standard: 2 mg/ml		10X PBS (10 ml)
Molecular grade water		

BCA™ Protein Determination Assay (96-Well Protocol)

1) In a 1.5 ml microfuge tube prepare 1 ml of 1 x PBS by diluting 100 ul of 10 x PBS buffer (provided) with 900 ul of molecular grade water.

- 2) 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. Prepare the BCA working reagent fresh daily.
- 3) For each protein determination, place the required volume of 1x PBS into a microplate well and mix an aliquot of the protein sample with the PBS in the plate (see table below). The final volume of each sample in the plate will be 20 μ l. Record the dilution factor.

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

- 4) Prepare a BCA protein standard curve by making a 2-fold serial dilution from 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 50 μ l 1X PBS and 50 μ l 2mg/ml BSA standard to a well (1mg/ml)
 Well #2- Add 50 μ l 1X PBS and 50 μ l from the 1st well to a 2nd well (0.5mg/ml)
 Well #3- Add 50 μ l 1X PBS and 50 μ l from the 2nd well to a 3rd well (0.25mg/ml)
 Well #4- Add 50 μ l 1X PBS and 50 μ l from the 3rd well to a 4th well (0.125mg/ml)
 Well #5- Add 50 μ l 1X PBS and 50 μ l from the 4th well to a 5nd well (0.0625mg/ml)
 Well #6 -Add 50 μ l 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. bovine IgG standard, Pierce Cat. #23212) instead of a 2 mg/ml albumin standard.

- 5) Transfer 20 μ l aliquots from each of the 2-fold serially diluted albumin standards to six empty microplate wells, preferably adjacent to wells containing 20 μ l of the protein sample to be assayed (from step 3 above).
- 6) Transfer and mix 150 μ l freshly prepared BCA reagent to each well containing 20 μ l of each dilution standard and sample being assayed.
- 7) 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.

- 8) Remove the plate from the water bath, dry the bottom of the plate and read the wells in a suitable plate reader (e.g. Molecular Devices) @ 562 nm. A typical BCA assay result is depicted in Figure 6.

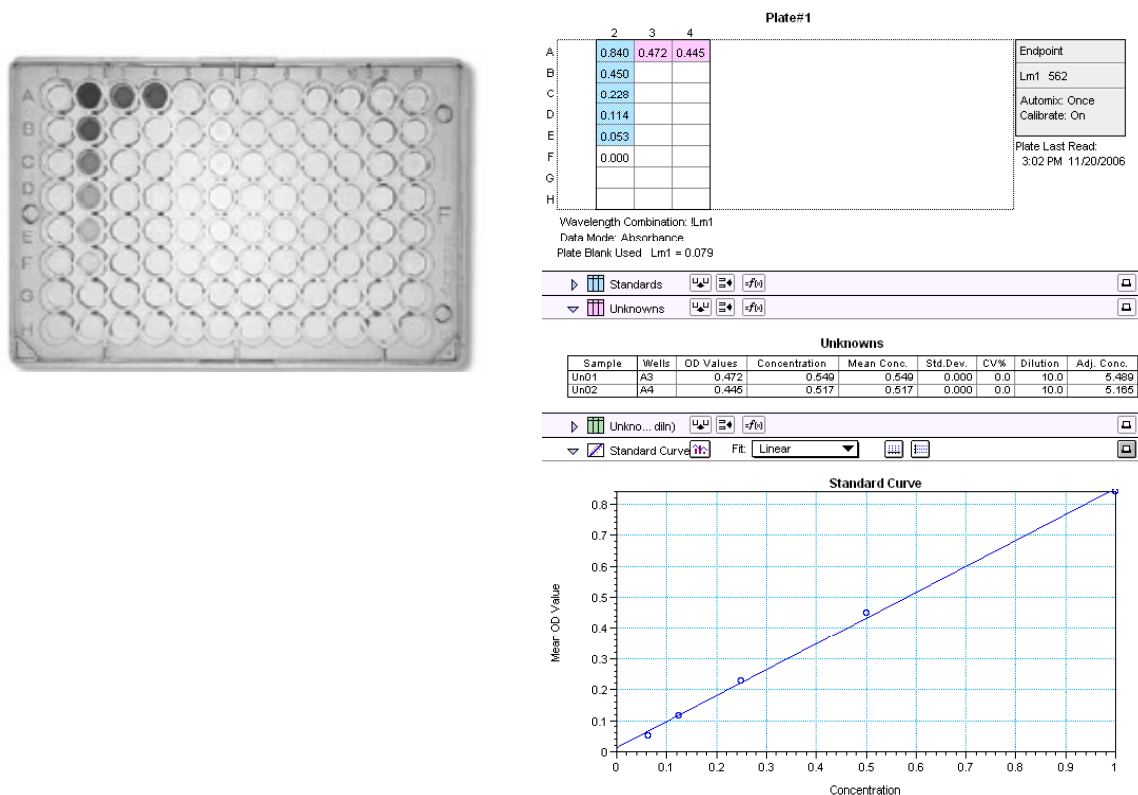


Figure 6. BCA protein microplate assay result. On the left is a plate containing a dilution series of BSA standards (A2-F2) along with two protein samples (A3, A4). On the right is an output from a Molecular Devices UV-VIS microplate reader illustrating BCA assay result.

g. Spectrophotometric Analysis and Calculations (~ 20 min)

Biotin incorporation is quantified by determining the molar substitution ratio (MSR) of the biotinylated protein sample. This ratio can be determined using one of two spectroscopic methods:

- **Method #1: Protein Extinction Coefficient Method ('A' Value Method)**
- **Method #2: Protein Concentration Method (BCA Method)**

After biotinylation, sample volumes will range from 100-130 ul. Since samples accommodated by this kit can span a broad range of protein concentrations (40-fold) some samples may require dilution prior to taking

optical density measurements. In addition, since optical density measurements are often taken using different instruments, some precautions are warranted. For example, when using a conventional spectrophotometer the sample holder should be able to accommodate a 1-cm quartz microcuvette (100 ul capacity). Alternately, if using a UV-VIS plate reader, a UV-transparent 96-well microplate (both standard or 'half-area' plate) may be required.

Newer optical instruments capable of measuring micro-volumes can also be used to determine the MSR. For example, a Nanodrop™ spectrophotometer may be used to measure optical densities. However, in our experience, this instrument is not sensitive enough to detect and measure the MSR of dilute protein samples (e.g. ≤ 0.5 mg/ml). The Nanodrop™ spectrophotometer should only be used to measure the MSR of samples having a protein concentration equal to or greater than 1 mg/ml. Each optical instrument has its own advantages and disadvantages. Use the table below to determine the compatibility of your instrument with a given sample concentration.

Instrument	Protein Concentration	Quartz Microcuvette (100 ul)	Quartz Cuvette (1 ml)
UV-VIS Spectrophotometer	0.25 mg/ml 0.50 mg/ml 1-10 mg/ml	√ √ √*	N/R N/R N/R
Instrument	Protein Concentration	UV-Microplate (Standard well)	UV-Microplate (Half-area well)
UV-VIS Plate Reader	0.25 mg/ml 0.50 mg/ml 1-10 mg/ml	N/R N/R √*	√ √ √*
Instrument	Protein Concentration	Cuvette or Well	1.5 ul volume
Nanodrop™ Spectrophotometer	0.25 mg/ml 0.50 mg/ml 1-10 mg/ml	none none none	N/R N/R √

N/R = not recommended

√ = compatible

√* = compatible but protein sample may require dilution prior to measurement

Finally, before diluting the biotinylated protein sample it is always wise to measure the optical density of the undiluted sample to insure sufficient protein was recovered during the labeling procedure. Follow the instructions

below if you wish to manually calculate the molar substitution ratio of your biotin-labeled protein sample otherwise use the MSR calculators provided on the flash drive (Folders #2 or #3).

Method #1: Protein Extinction Coefficient Method (i.e. 'A' Value)

Use this method to calculate the biotin molar substitution ratio (MSR) if the 'A' value of the protein is known. The 'A' value is the optical density @ 280 nm of a 1 mg/ml protein solution. Some commonly published 'A' values are listed in Table 5.

Protein Name	Protein A280 Value (protein extinction coefficient or 'A' Value)
Human IgG	1.36
Mouse IgG	1.40
Rabbit IgG	1.35
Bovine IgG	1.24
Human Secretory IgA	1.26
Human IgM	1.18
Human IgE	1.53
Rabbit Fab	1.50
Rabbit Fc	1.20
Human J chain	0.68
Bovine Serum Albumin	0.67
Trypsin	1.60
Chymotrypsin	2.02
Ovalbumin	0.79
α -amylase	2.42

Table 4. Published 'A' values for several common proteins. The 'A' value is the optical density of a 1 mg/ml solution at 280 nm using a 1-cm path length.

Note- A Nanodrop™ spectrophotometer can read concentrated protein samples and thus will not require dilution prior to making measurements. However, some protein samples may be too dilute to measure using this instrument.

Protocol

To use this method, measure the A_{280} and A_{354} of the biotinylated protein on a blanked spectrophotometer or microplate. Input the measured A_{280} and A_{354} values along with the known 'A' value of the protein into the Biotin MSR calculator (Method #1) located on the flash drive (Folder #2). Calculations can also be performed manually with the equations that follow:

Method #1: Protein Extinction Coefficient Method

Determine the A_{280} and A_{354} of the modified protein and use the equations below.

1) Determine corrected absorbance (A_{C280})

$$A_{C280} = A_{280} - (A_{354} * 0.23) \quad \text{eq. 1}$$

2) Determine moles protein

$$\left[\frac{(A_{C280} / \text{protein 'A' value}) * (\text{volume})}{1000} \right] / \text{protein MW} = \text{moles protein} \quad \text{eq. 2}$$

For example: protein 'A' value for human IgG is 1.36 (see table 4).

3) Determine moles biotin

$$\left(\frac{A_{354}}{29000} \right) * \left(\frac{\text{volume}}{1000} \right) = \text{moles biotin} \quad \text{eq. 3}$$

4) Biotin/protein MSR determination

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

NOTE: volume in ml and protein in mg/ml

Method #2: Protein Concentration Method (i.e. BCA Method)

If the 'A' value of the protein is not known, then the molar substitution ratio can be determined by measuring the optical density of the biotinylated protein at a single wavelength (i.e. A_{354}) followed by a BCATM assay to determine the protein concentration.

Important- Use a standard spectrophotometer and quartz micro-cuvette (100 ul capacity) or a NanodropTM spectrophotometer when using this method. Do not use a plate reader. A UV-VIS plate reader is not recommended when using the BCA method (Method #2) since the path length conversions (required for calculations) is often unknown in microplates.

Protocol

To use this method, measure the A_{354} of the biotinylated protein on a blanked spectrophotometer or NanodropTM. Determine the A_{354} of the sample, then remove an aliquot (~ 2.5 ug) and perform a BCA assay to determine the sample's concentration. Input values obtained into the Biotin MSR calculator (Method #2) located on the flash drive. Calculations can also be performed manually with the equations that follow:

1) Determine moles protein:

$$\left[\frac{(\text{protein concentration}) * (\text{volume})}{1000} \right] / \text{protein MW} = \text{moles protein} \quad \text{eq. 1}$$

2) Determine moles biotin present (volume in ml):

$$\left(\frac{A_{354}}{29000} \right) * \left(\frac{\text{volume}}{1000} \right) = \text{moles biotin} \quad \text{eq. 2}$$

3) Biotin/protein MSR determination

$$MSR = \frac{\text{eq.2}}{\text{eq.1}} \quad \text{eq.3}$$

NOTE: volume in ml and protein in mg/ml

III. Examples and Applications

a. Biotinylation of IgG

Bovine IgG was biotinylated as described in the kit. Five different concentrations (0.5, 1, 2.5, 5, and 10 mg/mL) of bovine IgG were reacted using 10 equivalents of ChromaLink Biotin. Reactions were desalted and scanned on a Molecular Dynamics UV-VIS microplate reader. All molar substitution ratios were calculated using optical density measurements @ A_{280} and A_{354} (quartz micro-cuvette) with an 'A' value of 1.24. Some samples were diluted prior to scanning.

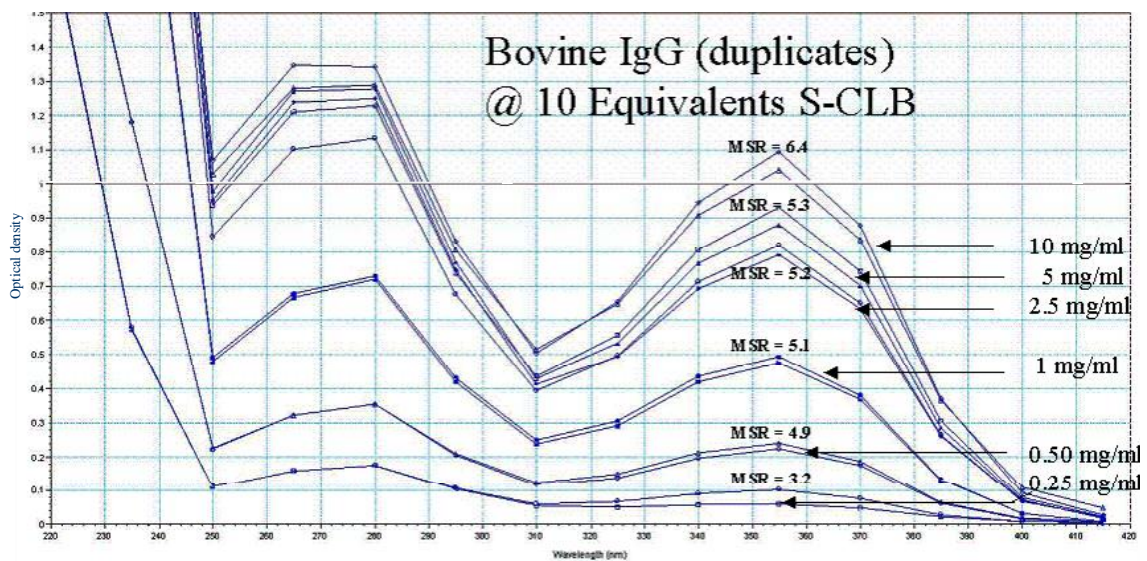


Figure 7. Overlaid spectra of biotinylated bovine IgG reactions taken on a Molecular Dynamics UV-VIS plate reader. Duplicate reactions at five different initial IgG concentrations are represented.

b. Micro-scale Biotinylation of IgG

Bovine IgG was biotinylated using the protocol described in the ChromaLink Biotin protein labeling Kit at 0, 5, and 10, and 20 equivalents of ChromaLink Biotin. Each reaction contained twenty-five micrograms of bovine IgG (duplicates) at the indicated equivalents. Reactions were desalted using Zeba™ spin columns and reaction volumes were scanned in 'half area' microplates on a Molecular Dynamics UV-VIS microplate reader. All molar substitution ratios were calculated using optical density measurements @ A_{280} and A_{354} (quartz micro-cuvette) with an 'A' value of 1.24.

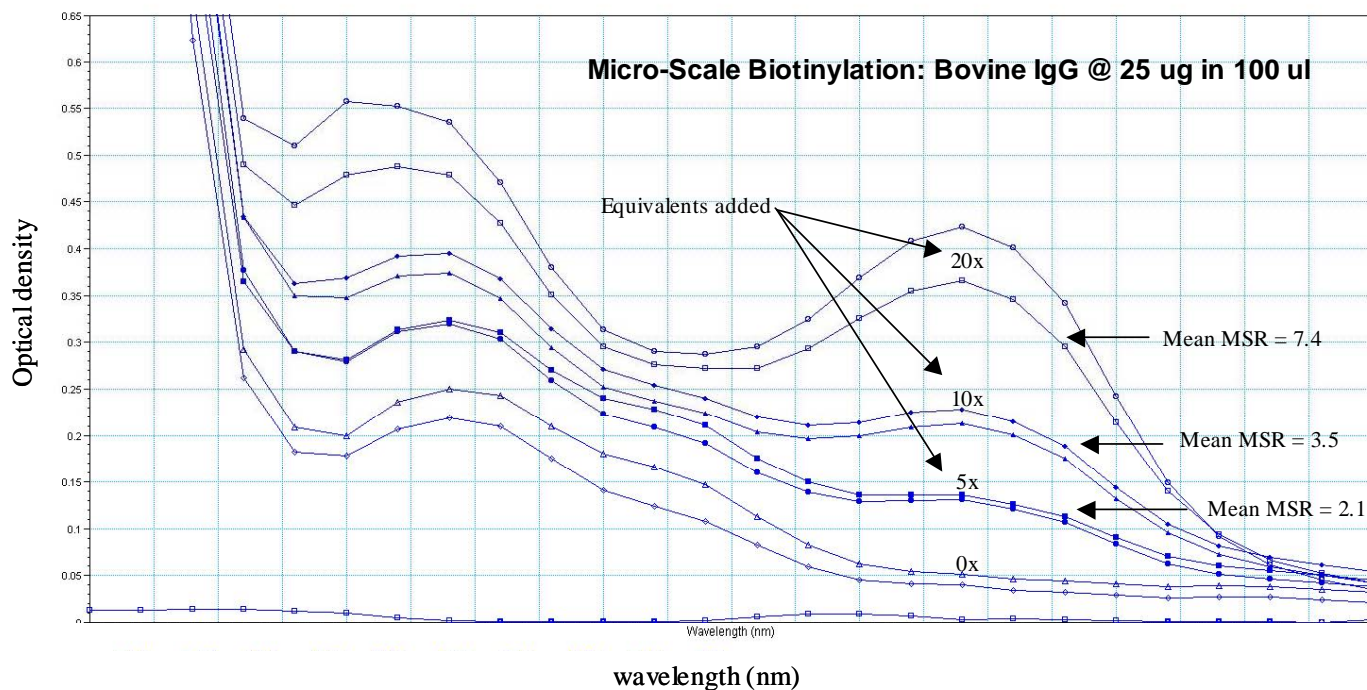
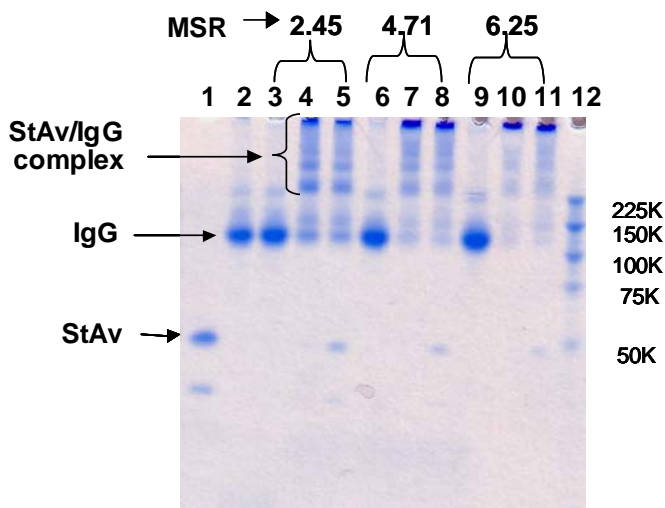


Figure 8. Overlaid spectra generated by scanning desalted reactions on a Molecular Dynamics UV-VIS plate reader. Micro-scale biotinylation reactions conducted using 25 ug of IgG (100 ul volume) at the indicated equivalents (duplicates).

c. Streptavidin Binding to Biotinylated IgG

Bovine IgG at 5 mg/ml was biotinylated at 5, 10, and 15 mole equivalents. Molar substitution ratios were determined for each reaction by A_{280} and A_{354} absorbance readings. To demonstrate the streptavidin-binding efficiency of ChromaLink Biotin, biotinylated bovine IgG was treated with 1 and 2 mole equivalents of streptavidin and binding was confirmed by PAGE gel-shift analysis (Figure 9). These results demonstrate increasing streptavidin binding efficiency with increasing 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	IgG-(CL biotin) 2.45	0
4	IgG-(CL biotin) 2.45	1
5	IgG-(CL biotin) 2.45	2
6	IgG-(CL biotin) 4.71	0
7	IgG-(CL biotin) 4.71	1
8	IgG-(CL biotin) 4.71	2
9	IgG-(CL biotin) 6.25	0
10	IgG-(CL biotin) 6.25	1
11	IgG-(CL biotin) 6.25	2
12	MW markers	N/A

Figure 9. Bovine IgG was reacted with 2.5X, 5X and 10X ChromaLink™ Biotin at 5 mg/mL. Protein concentrations were determined and molar substitution ratios calculated. Aliquots of biotinylated IgGs were then incubated with 1X and 2X equivalents of streptavidin and binding confirmed using PAGE gel-shift analysis (Coomassie blue staining).

d. Traceable Capture of Biotinylated IgG w/ Streptavidin Beads

Capture of a biotinylated antibody can be quantitatively monitored using ChromaLink Biotin. In this experiment, bovine IgG was biotinylated using 10 equivalents ChromaLink Biotin and desalted as described in the manual. Fixed aliquots (100 ul) containing 90 ug of biotinylated antibody (BCA) were incubated with either 125, 250, or 500 ug of NanoLink™ Magnetic Streptavidin particles for 30 minutes on a platform shaker to keep the beads suspended. Reactions were placed on a magnet to remove solid phase, and clarified supernatants recovered and scanned (220-420 nm). Resulting spectra clearly demonstrate increasing IgG depletion from the supernatant (binding) @ A_{354} and A_{280} with increasing solid phase. Quantitative depletion of 90 ug of biotinylated IgG was achieved in 30 minutes using 500 ug of NanoLink™ Magnetic Streptavidin particles.

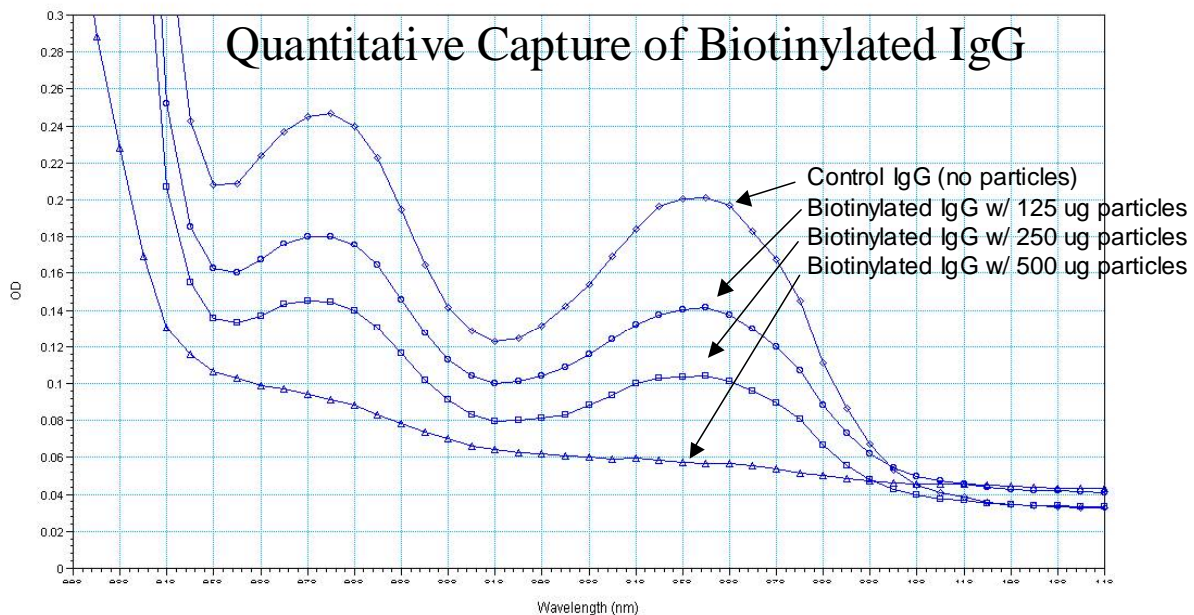


Figure 10. UV scans illustrating traceable capture of ChromaLink Biotin labeled IgG (90 ug per well with an MSR of 6.0) incubated with increasing amounts of streptavidin-coated magnetic particles (NanoLink™ Magnetic Streptavidin @ 125, 250, and 500 ug of particles). Five hundred micrograms of streptavidin-coated particles are required to quantitatively bind and deplete the supernatant of 90 ug traceable biotinylated IgG in 30 minutes. Unbiotinylated IgG controls showed no appreciable depletion (binding) when incubated with NanoLink Magnetic Streptavidin beads (data not shown).

e. Nanodrop™ Spectra of Biotinylated IgG

Overlaid UV spectra of biotinylated IgG scanned using a Nanodrop™ spectrophotometer. 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). Samples were reacted with 10 equivalents of ChromaLink Biotin reagent (see protocol in manual). Desalted reactions (1.5 ul aliquots) were then scanned from 220-420 nm on a Nanodrop™ spectrophotometer. Note low absorbance signals for samples below 1 mg/ml using the Nanodrop™.

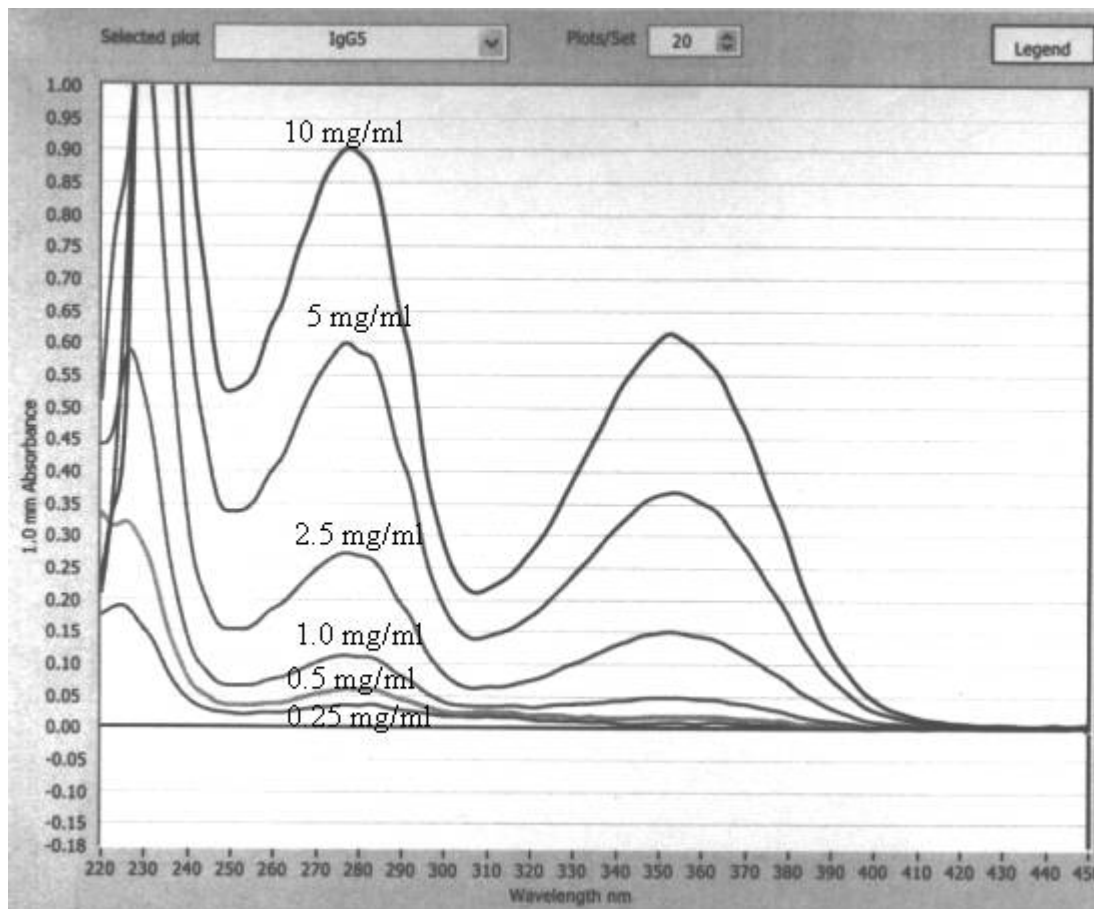


Figure 11. Overlaid spectra (220-450 nm) of biotinylated IgG samples using a Nanodrop™ spectrophotometer.

IV. Appendix

a. Positive Control Reaction

To establish if the kit is working properly, a control protein consisting of bovine IgG at 1 mg/ml \pm 0.05 mg/ml is provided (refer to product data sheet for lot specific concentration). This control protein is desalted and ready for direct labeling with ChromaLink Biotin. To use this control, proceed as follows:

- 1) Transfer 100 μ l of the unbiotinylated control IgG @ 1mg/ml to a clean 1.5 ml tube.
- 2) Prepare a 0.5 mg/ml working stock of ChromaLink Biotin in 1x Modification Buffer as directed in [Section II, d-3](#).
- 3) Add 12.2 μ l of ChromaLink Biotin working solution to the control IgG. Mix well.
- 4) Allow the reaction to proceed for 90 minutes.
- 5) Desalt the reaction using a Zeba™ spin column equilibrated in 1x PBS as directed in [Section II, e-2](#)
- 6) Measure the molar substitution ratio of the sample as directed in [Section II, g](#).
- 7) A properly working kit should yield a biotin molar substitution ratio between 3-6 biotins per IgG molecule.

b. Biotinylated Positive Control

A biotinylated IgG control @ 1 mg/ml \pm 0.05 mg/ml is also provided. This protein is biotinylated using ChromaLink Biotin at a molar substitution ratio that may range between 2.0 and 6.0. (Refer to the product data sheet for lot specific MSR). This control can be used to confirm the accuracy of either MSR method. To use the control proceed as follows:

- 1) Blank the spectrophotometer using a quartz micro-cuvette (100 μ l nominal capacity) with 1x PBS at 280 nm and 354 nm. Discard the blank solution.

Note-some spectrophotometers can be blanked at two wavelengths simultaneously otherwise independent blanks will be required at each wavelength where necessary.

- 2) Transfer 100 μ l of biotinylated IgG to an empty micro-cuvette and measure the optical density @ 280 nm and 354 nm. Input the values into the ChromaLink Biotin MSR calculator (see flash drive) to determine the MSR. The biotinylated control should give the same MSR as indicated in the product data sheet located on the flash drive.

c. Troubleshooting Guide

Problem	Possible Cause	Recommended Action
Poor biotin modification of protein	-initial protein concentration is outside the optimal range for this protocol	-Recheck the 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 with the protein sample, e.g. Tris or glycine buffer could be contaminating the protein sample	-Adequately desalt the protein sample. Some samples are so overly contaminated that two desalting steps may be required.
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 recommend-ed protocol -properly calibrate spectro-photometer
Molar substitution readings 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 by adjusting the pH above the pI of the protein -reducing the number of equivalents can sometimes prevent precipitation but it will also reduce the MSR
Precipitation of protein on modification	- over-modification of the protein	-Recheck the concentration of SCLB working stock used to label the protein

d. Comprehensive Molar Substitution Table

		Bovine IgG (150 kD)		
		10 Equivalents	5 Equivalents	2.5 Equivalents
Bovine IgG				
mg/ml		Biotin MSR	Biotin MSR	Biotin MSR
10		6.8	3.7	1.8
5		6.5	3.2	2.0
2.5		5.1	3.5	2.1
1		4.8	2.3	1.6
0.5		4.2	2.5	1.4
0.25		2.8	1.8	1.3

		Bovine Serum Albumin (67 kD)		
		10 Equivalents	5 Equivalents	2.5 Equivalents
BSA (67 kD)				
mg/ml		Biotin MSR	Biotin MSR	Biotin MSR
10		6.2	4.4	2.4
5		5.8	4.0	2.4
2.5		5.4	3.4	2.4
1		5.6	2.4	2.0
0.5		4.4	2.5	1.3
0.25		4.0	2.6	1.3

		Chicken Egg Albumin (44 kDa)		
		10 Equivalents	5 Equivalents	2.5 Equivalents
Chicken Egg Albumin (44 kDa)				
mg/ml		MSR	MSR	MSR
10		3.3	3.3	2.3
5		4.6	3.8	2.5
2.5		5.1	3.6	2.5
1		4.2	2.3	1.8
0.5		4.2	1.8	1.7
0.25		3.8	1.5	1.3

		beta-lactoglobulin (18.3 kD)		
		10 Equivalents	5 Equivalents	2.5 Equivalents
beta-lactoglobulin (18.3 kD)				
mg/ml		MSR	MSR	MSR
10		1.0	1.5	1.7
5		2.3	2.8	2.2
2.5		2.6	2.4	1.9
1		2.2	1.7	1.6
0.5		2.1	1.4	1.1
0.25		1.9	1.2	0.8

Table 6. Relationship between molar substitution ratios (MSR), protein concentration, protein molecular weight, and reaction stoichiometry for various proteins. Data were generated using ChromaLink Biotin protein labeling kit and a Nanodrop™ spectrophotometer (Method #1).

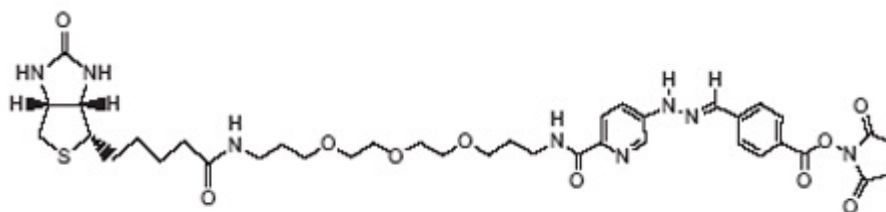
f. Additional Biotinylation Reagents

ChromaLink Biotin 354S

$C_{38}H_{50}N_8O_{10}S$

MW 810.92

yellow solid



Application: Original ChromaLink Biotin 354S traceable reagent. Non-sulfonated NHS-ester analog of ChromaLink Biotin 354S. Can be used to quantify biotin incorporation by means of direct spectroscopic methods.

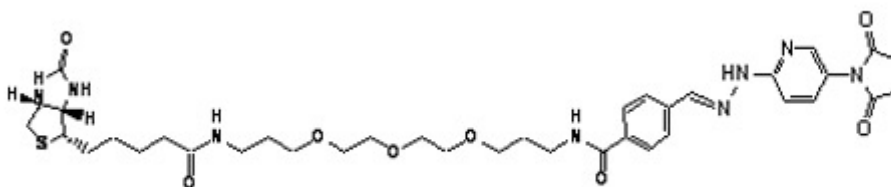
B1001-100	5 X 1.0 mg	\$165
B1001-110	10 mg	\$195

ChromaLink Biotin 354M

$C_{37}H_{49}N_8O_8S$

MW 764.89

yellow solid



Application: Maleimide-activated ChromaLink Biotin 354 reagent used for traceable biotinylation of thiol containing proteins. Can be used to quantify biotin incorporation by means of direct spectroscopic methods.

B1012-105

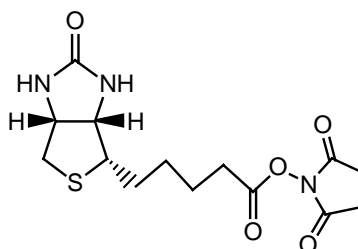
5 X 1.0 mg

\$200

Biotin succinimidyl ester $C_{14}H_{19}N_3O_5S$

MW 341.38

white solid

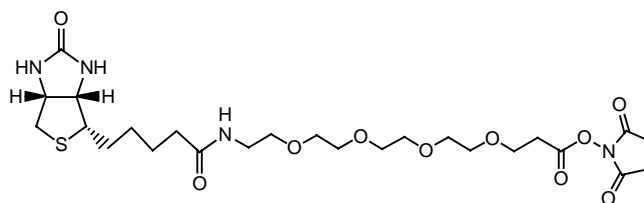
**Application:** Incorporation of biotin on amine-reactive biomolecules.**B1005-120** 10 X 2.0 mg \$ 195**B1005-100** 50 mg \$ 295**B1005-110K Kit** 5 X 2.0 mg \$ 225

Kit Components	5 X 2.0 mg
DMF (anhydrous)	1.0 mL
10XModification Buffer	1.5 mL
diafiltration apparatus 5K MWCO	4

Biotin/PEG4/succinimidyl ester $C_{25}H_{40}N_4O_{10}S$

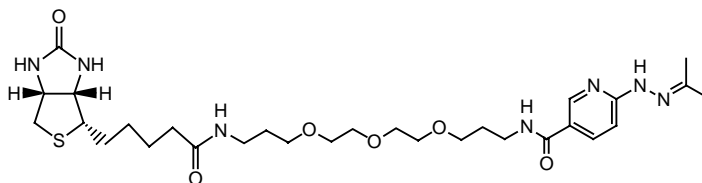
MW 588.67

white solid

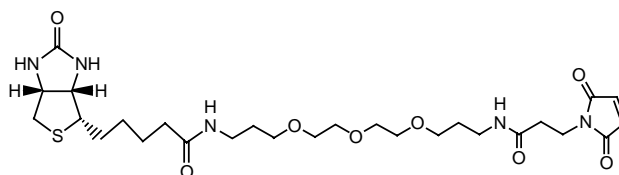
**Application:** Incorporation of biotin with a PEG4 linker on amino-containing biomolecules.**B1002-120** 10 X 2.0 mg \$ 195**B1002-025** 25 mg \$ 295**B1002-110K Kit** X 2.0 mgs \$ 225

Kit Components	5 X 2.0 mg
DMF (anhydrous)	1.0 mL
10XModification Buffer	1.5 mL
diafiltration apparatus	4

5K MWCO

Biotin/PEG4/hydrazino nicotinamide acetone hydrazone**C₂₉H₄₇N₇O₆S****MW 621.79****yellow solid****Application:** Incorporation of biotin with a PEG4 linker on carbonyl containing biomolecules.**B1003-105 5 X 1.0 mg \$ 195****B1003-110K Kit 5 X 2.0 mg \$ 225**

Kit Components	5 X 2.0 mg
DMF (anhydrous)	1.0 mL
10XModification Buffer	1.5 mL
diafiltration apparatus 5K MWCO	4

Biotin/PEG4/maleimide**C₂₇H₄₃N₅O₈S****MW 597.73****white solid****Application:** Incorporation of biotin with a PEG4 linker on sulfhydryl-containing biomolecules.**B1004-120 10 X 2.0 mg \$ 195****B1004-050 50 mg \$ 295****B1004-110K Kit 5 X 2 mgs \$ 225**

Kit Components	5 X 2.0 mg
DMF (anhydrous)	1.0 mL
10XModification Buffer	1.5 mL
diafiltration apparatus 5K MWCO	4

