WHITE PAPER

# UV-Traceable Reagent for Controlled Biotinylation of Antibodies

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## Summary

Biotin incorporation remains the most popular method of tagging antibodies for affinity capture and detection. In this report, we describe a UV-traceable biotinylation reagent that permits non-destructive quantification of biotin incorporation by means of a simple UV scan of the labeled antibody. The reagent was used to label a model antibody substrate (bovine IgG) to better understand the interaction between antibody concentration and reagent equivalents required to achieve a controlled biotin molar substitution ratio (MSR). In related experiments, an IL-2 specific detector antibody was biotinylated at different molar substitution ratios to assess how the degree of incorporation affects immunoassay sensitivity within a sandwich enzyme-linked immunosorbent assays (ELISAs) format. Bovine IgG labeling results indicate that within a given antibody concentration range the biotin molar substitution ratio doubles as reagent equivalents double. In related experiments, the data reveal that at a fixed antibody concentration biotin labeling efficiency increases in a predictable manner expressible as a percentage of the number of reagent equivalents used in the reaction. Finally, IL-2 sandwich ELISA results demonstrate a 2.7-fold increase in detection sensitivity as the MSR of a biotinylated detector antibodies increase from 0.70 through 8.4.

## Keywords

- Biotin
- UV-Traceable Biotinylation
- MSR
- Bovine IgG
- ELISA

## Introduction

ELISAs are a commonly used method of detecting and quantifying antigens (1, 2). In one ELISA format, matched pairs consisting of a capture and biotinylated detector antibody are used to quantify antigens (3, 4). The degree of biotin incorporation on a detector antibody has been shown to play a key role in immunoassay specificity (5, 6). Although the level of biotin incorporation on a detector antibody in this now widely used format is known to be important for assay performance, it often had been ignored or overlooked by researchers because rapid and direct methods of measuring biotin incorporation have only recently become available.

Over the years, 2 major indirect assays have prevailed as the standard for measuring biotin incorporation on antibodies and other proteins. The first of these assays, known as the HABA assay [2-(4-Hydroxyphenylazo)benzoic acid] was developed more than 40 years ago (7). In the HABA assay, incorporation is measured when biotin or a biotin-labeled molecule displaces the weaker binding avidin-bound HABA dye into the surrounding bulk solution, causing a reduction in the dye's absorbance at 500 nm. This reduction in signal is then quantified with an externally generated avidin/biotin calibration curve. In more recent years, a similar yet more sensitive indirect fluorescent assay has been developed by Johnson et. al (8) called the Invitrogen FluoReporter™ Assay. In this assay, biotin binding displaces an avidin-bound quencher dye back into the bulk solution, relieving the quenching of the covalently bound fluorophore. Neither of these indirect assays actually measure the number of biotin molecules on a protein's surface, rather they measure the number of biotin molecules capable of binding to avidin.

As a consequence, both assays tend to underestimate the absolute number of incorporated biotin molecules because any two molecules spaced closer than the binding footprint of avidin (or streptavidin) of 34 $nm^2$ (9) will bind to only one of them.

For this reason, improvements to these lengthy and complicated assays have included either an acid hydrolysis or protease digestion step to increase access of surface-bound biotin molecules (10–13). Although both of these indirect assays are still in use today, they are inconvenient, destructive in nature, time-consuming, and posing significant barriers to their routine implementation.

In this short report, we discuss a traceable biotin labeling reagent that addresses many of these shortcomings. This reagent can be used to measure biotin incorporation directly from a simple UV scan of the labeled antibody. The linker's intrinsic high molar extinction coefficient permits sensitive and direct measurement of biotin incorporation. Using this reagent, we labeled a model antibody substrate to explore the interplay between antibody concentration and reagent equivalents required to achieve a controlled biotin MSR. In related experiments, a rat anti-mouse IL-2 detector antibody was labeled at defined biotin in order to better understand the relationship between the degree of biotin substitution and relative assay sensitivity within the confines of a sandwich ELISA format.

## Materials and Methods

### Biotinylation of Antibodies

All antibodies in this report were biotinylated using Vector Laboratories Sulfo-ChromaLINK® Biotin reagent. Briefly, solid antibodies were resuspended in phosphate buffered saline (100 mm sodium phosphate, 150 mm NaCl, pH 7.4) followed by buffer exchange into the same buffer using Thermo Scientific™ Zeba™ desalting spin columns. Purified antibody concentrations were quantified by their intrinsic $A_{280}$ absorbance as measured on a Thermo Scientific NanoDrop™ Spectrophotometer. Bovine IgG stock solutions (0.5, 1.0, and 2.5 mg/ml) were prepared from a more concentrated stock (13.9 mg/ml) in phosphate buffered saline. Sulfo-ChromaLINK Biotin labeling reagent, an amorphous solid [>95% pure as confirmed by $^1H$ NMR, reverse phase HPLC 95%, and mass spectrometry (MWt 912.96)] was prepared by dissolving known quantities into 100 mm sodium phosphate, 150 mm NaCl at pH 7.4. Model bovine IgG substrate solutions (100µl) were biotinylated in triplicate by the addition of equal volumes (5 µl) of Sulfo-ChromaLINK Biotin reagent corresponding to 5-, 10- or 20-fold mole equivalents for 2 hours at room temperature. Similarly, rat anti-mouse IL-2 specific detector antibody (100 µl at 1 mg/ml) (BioLegend) was biotinylated by incubation of the antibody with 0-, 2-, 4-, 10-, and 28-fold excess of Sulfo-ChromaLINK Biotin. After labeling, excess reagent was desalted using Zeba spin columns equilibrated in PBS. Aliquots from the purified labeling reactions (2 µl) were scanned on a NanoDrop Spectrophotometer (220–420 nm) and the resulting $A_{280}$ and $A_{354}$ values were used to calculate biotin MSRs.

### IL-2 Sandwich ELISA

Initially, IL-2 specific capture and detection monoclonal antibodies were titrated against each other in a preliminary checkerboard optimization process. Sandwich ELISAs were performed by coating a 96-well plate (100 µl per well) with rat anti-mouse IL-2 capture antibody (1.25 µg/ml) for 30 minutes at 37°C. Unbound capture antibody was washed away with 10 mM sodium phosphate, 250 mM NaCl, 0.05% Tween® 20, pH 7.2 (3X) followed by careful blotting of plates between washes using paper towels. Capture antibody-coated wells were then blocked for 2 hours at room temperature using 200 µl blocking buffer (10 mm sodium phosphate, 250 mm NaCl, 1% BSA, 0.05% Tween 20, pH 7.2). All ELISA capture plates were sealed and stored at 4°C until used. Lyophilized carrier-free recombinant IL-2 antigen 3 (BioLegend) was resuspended in PBS at 100 ng/ml and 2-fold serially diluted across the plate (100 ng/ml to 19.5 ng/ml) using replicates. After incubating for 1 hour at room temperature, unbound antigen was washed away. Biotinylated IL-2 detector antibody (100 µl at 0.5 µg/ml) was then incubated for 1 hour at room temperature followed by additional washes. All wells were then incubated for 1 hour with horseradish peroxidase-streptavidin at 1.5 µg/ml. Immune complexes were detected by addition of 100 µl tetramethylbenzidine substrate (Thermo Fisher Scientific). Substrate development was stopped after 15 minutes using 1m $H_2SO_4$. ELISA signals were read at 450 nm on a Molecular Devices SpectraMax™ Plus™ 384 plate reader and the data plotted using the instrument's 4-parameter curve- fitting software routine.

### UV Spectra and Biotin MSRs

All UV spectra were obtained from a calibrated NanoDrop ND-1000 spectrophotometer employing a 1 mm path length. Data were collected by scanning 2 µl aliquots using the instrument's Protein $A_{280}$ menu (340 nm normalization feature turned off). The $A_{280}$ and $A_{354}$ from each sample's absorption spectrum were then used to calculate the biotin molar substitution ratio as follows:

1. $A_{c280} = A_{280} - (A_{354} \times 0.23)$
2. Moles of antibody = V (ml) × [($A_{c280}$) / (E1%)] × [(10 mg/ml) / 1000 mg/ml) / MWt IgG]
3. Moles of biotin = ($A_{354}$ / $\varepsilon_{354}$) × [V (ml) / 1000 (ml/L)]
4. Biotin MSR = moles of biotin/moles of antibody

Equation 1 is used to measure the corrected $A_{280}$ of the labeled antibody sample. It employs a correction factor that subtracts the $A_{280}$ contribution originating from the labeling reagent itself.

This correction factor was originally determined by scanning an ethanolamine quenched Sulfo-ChromaLINK Biotin solution (354 nm, 1 mm path length) in phosphate buffer and expressing the $A_{280}$ correction factor as a fraction of the quenched reagent's absorbance at 354 nm. Equation 2 is used to quantify the moles of antibody from the labeled antibody's known sample volume (V) in milliliters, the corrected $A_{280}$ as well as the antibody's mass extinction coefficient (E1%) and its molecular weight in Daltons. Equation 3 is used to quantify the moles of antibody-incorporated biotin using the reagent's quenched molar extinction coefficient ($\varepsilon_{354}$ = 29,000 $M^{-1}$ $cm^{1}$), its sample's volume (ml), and its $A_{354}$. Equation 4 then combines the results of equation 2 and 3 to obtain the biotin MSR of the labeled antibody.

## Results and Discussion

The chemical structure of the biotin labeling reagent (Sulfo-ChromaLINK Biotin) used in this report is illustrated in Figure 1, Panel A. As seen in the figure, this compound possesses a water-soluble N-hydroxysulfosuccinimidyl ester that acylates antibody lysine residues under mild aqueous buffer conditions (100 mm sodium phosphate, 150 mm NaCl, pH 7.4). The UV-traceable portion of the linker consists of an embedded bis-aryl hydrazone chromophore attached through a PEG3 spacer to the biotin moiety. The chromophore's high molar extinction coefficient ($\varepsilon_{354}$ = 29,000 $m^{-1}$ $cm^{1}$) originates from the extended conjugation of the bis-aryl hydrazone. In Figure 1, Panel B illustrates the unquenched solution phase UV-absorption spectrum of the labeling reagent whereas Panel C is the spectrum of a biotin-labeled antibody.

Using this traceable biotin reagent we labeled a model antibody substrate (bovine IgG) at three different protein concentrations to better understand the interplay between initial antibody concentration and the number of reagent mole equivalents required to achieve a given biotin MSR. Aliquots of bovine IgG at 3 different concentrations (0.5, 1.0, and 2.5 mg/ml) were labeled using 5-, 10-, and 20-fold mole excess reagent in triplicates. After desalting all 27 reactions using Zeba spin columns to remove excess reagent, the UV spectrum (220–420 nm) of each sample was acquired.

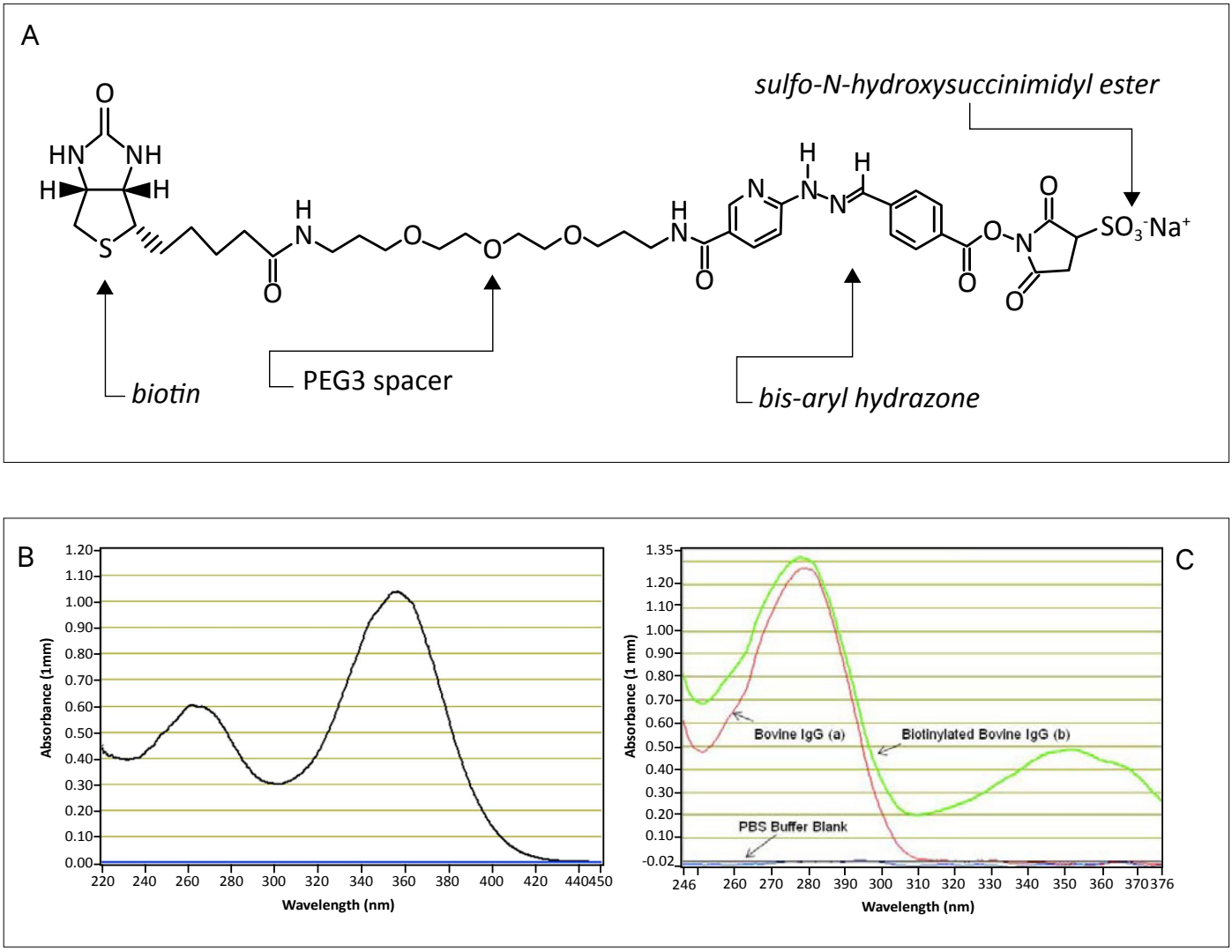


***Figure 2*** *The chemical structure of Sulfo-ChromaLink Biotin (Panel A) and the solution phase absorption spectra of the linker before (Panel B) and after (Panel C) incorporation. Panel A summarizes the different functional moieties comprising the labeling reagent. Panel B is the solution phase absorption spectrum (NanoDrop, 1 mm path length) of a 350 µm stock solution made from pure (unquenched) reagent dissolved in 100 mm sodium phosphate, 150 mm NaCl, pH 7.4. Panel C is the overlaid absorption spectra of buffer blank (PBS), unlabeled bovine IgG at 1 mg/ml, and Sulfo-ChromaLink Biotin-labeled bovine IgG at 1 mg/ml (MSR of 2.57).*

The resulting $A_{280}$ and $A_{354}$ absorbance values along with measured sample volumes were then used to calculate biotin MSRs. A bar graph summarizing these biotin incorporation data is illustrated in Figure 2 below. As seen from the graphical data, at lower fixed antibody concentrations (0.5 and 1 mg/ml) the biotin MSR approximately doubles as reagent equivalents double. For example at 0.5 mg/ml IgG, we observe a 2.3-fold and 1.9-fold increase in biotin MSR as we double the number of equivalents from 5- to 10-fold and again from 10- to 20-fold, respectively. At 1 mg/ml IgG, the corresponding increases are 2.1- and 1.90-fold. While at 2.5 mg/ml the increase is 2.0- and 1.56-fold, respectively. The observed drop in 2-fold proportionality at 2.5 mg/mL and 20-equivalents implies label saturation of the antibody. This saturation phenomenon has also been observed by Smith (11) when attempting to label other types of antibodies using biotin NHS esters. We surmise, as have others (6), that label saturation occurs because of reduced antibody solubility (aggregation/precipitation) at the highest molar substitution ratios.

The experimental results in Figure 2 also reveal another interesting relationship between antibody concentration and molar substitution ratio. Namely, that biotin labeling efficiency (at a fixed excess of reagent equivalents) is predictable across different antibody concentrations. That is, labeling efficiency is expressible as a simple percentage of the number of input equivalents used to modify the antibody. For example, at a fixed 5-fold mole excess of Sulfo-ChromaLINK Biotin, the labeling efficiency averages 29 ± 2.3%

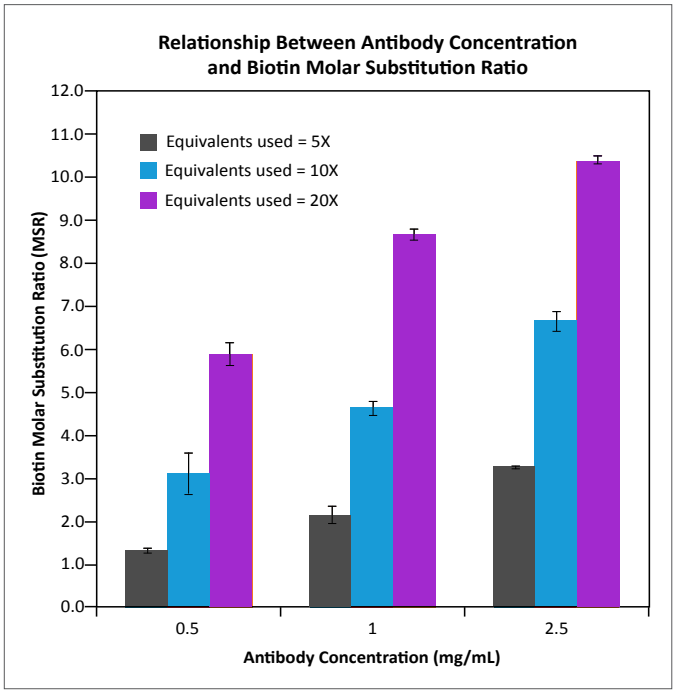


***Figure 2*** *Plot summarizing Sulfo-ChromaLINK Biotin labeling results with bovine IgG as the model substrate. Three different IgG concentrations labeled at 5-, 10-, and 20- equivalents are represented in triplicates. Error bars represent ± 1 standard deviation.*

the number of input equivalents at all three concentrations tested. At a fixed 10-fold mole excess the labeling efficiency increases to 42 ± 1.7% at all three IgG concentrations, while at 20 mole equivalents the labeling efficiency rises to 66 ± 0.4% at 0.5 and 1.0 mg/ml but declines to 52% at 2.5 mg/ml as label saturation is reached. The observed decline at 2.5 mg/ml is consistent with label saturation causing aggregation and precipitation of the antibody at highest substitution ratios.

Using this predictable relationship, we biotinylated a rat anti-mouse IL-2 antibody (1 mg/ml) at various mole equivalents to generate a defined set of biotin MSRs with the same antibody. Overlaid absorption spectra of this IL-2 detector antibody are illustrated in Figure 3. This detector antibody labeled at three substitution levels (MSR 0.7, 3.7, and 8.38) was then evaluated for relative assay sensitivity in an IL-specific sandwich ELISA.

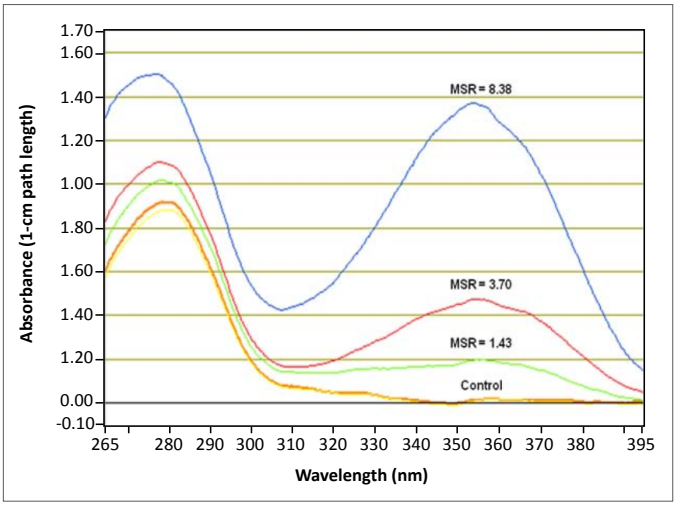


***Figure 3*** *Absorption spectra (265–395 nm) of the same biotinylated anti-mouse IL-2 monoclonal antibody labeled at three different molar substitution ratios. The series was generated with mouse anti-IL-2 IgG at 1 mg/ml using 0-, 4-, 10-, and 28-mole-equivalents of Sulfo-ChromaLINK Biotin labeling reagent.*

The resulting dose-response curves are illustrated in Figure 4. The data reveal a gradual increase in IL-2 detection sensitivity as biotin molar substitution ratio increases through the series. The leftward shift in the inflection point of the response curves reveals a 2.7-fold increase in IL-2 detection sensitivity as the biotin MSR progress from 0.7 to 8.4. In summary, we demonstrate the utility of using a UV-traceable biotin labeling reagent to achieve controlled and quantifiable biotin incorporation with antibodies. The reagent's traceable nature allows rapid and non-destructive measurement of biotin incorporation, greatly simplifying immunoassay standardization. These experiments reveal that antibody biotin labeling efficiency is predictable and highly reproducible under well-defined reaction conditions. Lastly, IL-2 sandwich ELISA results confirm that biotin MSRs do affect relative assay sensitivity as demonstrated in the specific ELISA format chosen for this study.

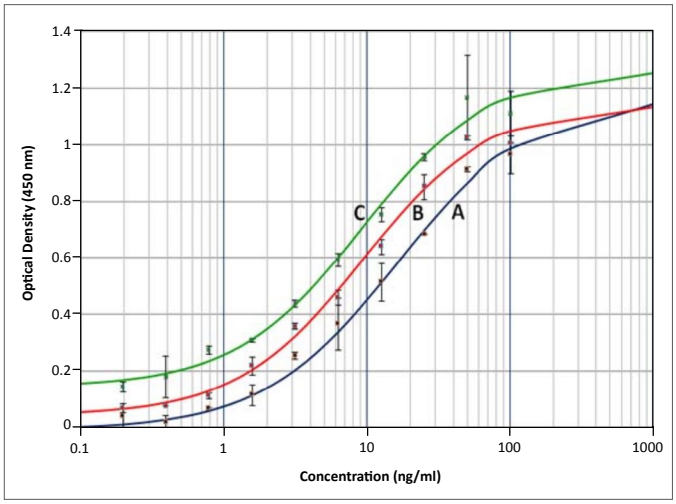


***Figure 4*** *This panel illustrates the IL-2 dose-response curves corresponding to molar substitution ratios of 0.7 (A), 3.7 (B), and 8.38 (C). Four-parameter ELISA curves plot the average of replicates with error bars representing +1 standard deviation.*


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