

## KPL APPLICATION NOTE

# SureLINK™ Chromophoric Biotin Labeling Kit for Labeling and Quantitation of Biotin in Protein Conjugates

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## INTRODUCTION

SureLINK™ Chromophoric Biotin Labeling Kits allow for the easy labeling of proteins with biotin as well as an accurate, precise, and inexpensive method of quantitating biotin in biotin-protein conjugates. Accurate measurement of this parameter is critical for the efficient design of reproducible immunoassays. The basis of the quantitation method is a biotinylation reagent that contains a chromophore in its linker region. Quantitation of biotin by absorbance of the biotin-protein conjugate at 354 nm is superior to the traditional quantitation of biotin using the HABA reagent. We prepared biotin-antibody conjugates using the SureLINK Chromophoric Biotin Labeling Kit, and then compared different quantitation methods. Absorbance at 354 nm was more reproducible than HABA-based quantitation, could be performed with lower concentrations of sample, and was nondestructive. The resulting biotin conjugates were used in ELISA, Western blot, and IHC applications<sup>1</sup>.

## BACKGROUND

The importance of biotin-protein conjugates and quantitation of biotin. The biotin-streptavidin bond is one of the strongest known natural interactions between a protein and ligand<sup>2</sup>. This interaction has been widely exploited in immunoassays and in molecular biology techniques. In immunoassays, a biotin-labeled antibody conjugate can be used in conjunction with streptavidin conjugated to reporter molecules to amplify the signal of the immunoassay. Many reagents are commercially available for conjugating biotin to antibodies and other proteins. However, it is difficult to precisely measure the number of biotins attached to the protein.

The traditional HABA method is inaccurate and consumes precious samples. Traditionally, researchers have used the reagent 4'-hydroxyazobenzene-2-carboxylic acid (HABA) to quantify the amount of biotin conjugated to a protein<sup>3</sup>. HABA binds weakly to avidin and the HABA-avidin complex absorbs at 500 nm. Biotin competes with HABA for binding to avidin, thereby lowering the absorbance at 500 nm. As we will demonstrate in this application note, HABA is only useful for concentrated proteins because the difference in absorbance due to biotin displacement of HABA from avidin is very small.

Incorporating a chromophore into the biotinylation reagent offers an alternative. SureLINK Chromophoric Biotin

Labeling reagent contains a chromophoric group (a bis-aryl hydrazone) in its linker section (Figure 1). In addition to the chromophoric group, SureLINK Chromophoric Biotin contains features that are only available in state-of-the-art labeling reagents: a long PEG spacer arm that provides solubility of biotin conjugates in aqueous solution and an aromatic NHS ester that allows for specific labeling of protein lysine side chain  $\epsilon$ -primary amines and protein N-terminal primary amines.

Biotin conjugates prepared with this reagent can be used in ELISA, Western blot, and IHC. In this application note, we describe conditions for using SureLINK Chromophoric Biotin Labeling Kits to produce biotin-antibody conjugates for use in ELISA, Western blot, and immunohistochemistry applications.

The Structure of SureLINK Chromophoric Biotin

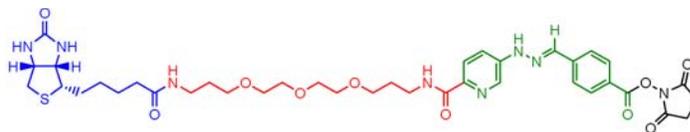


Figure 1: SureLINK Chromophoric Biotin has several features: 1. Biotin (blue); 2. A bis-aryl hydrazone chromophore that allows quantitation of biotin incorporation (green); 3. A PEG spacer that improves solubility of the biotin-protein conjugate (red); and 4. An aromatic N-hydroxysuccinimide (NHS) ester leaving group that reacts with primary amino groups (black).

## MATERIALS AND METHODS

**Reagents and supplies.** The SureLINK™ Chromophoric Biotin Labeling Kit (Catalog No. 86-00-01), HRP substrates, including ABTS (1-C) (Catalog No. 50-62-00), TrueBlue (Catalog No. 71-00-67), and LumiGLO® (Catalog No. 54-61-02) were from KPL. HRP-Streptavidin (Catalog No. 474-3000), wash solutions, blocking solutions, and IHC reagents were also from KPL. Slides with paraffin embedded human liver tissue (ProSci) were deparaffinized with Xylene Substitute (Shandon) for 15 minutes. SDS-PAGE protein gel and Western blotting reagents and supplies were from Bio-Rad Laboratories. HABA kits were purchased from Pierce (Supplier 1) and Anaspec Inc. (Supplier 2).

**Preparation of Biotin-Antibody Conjugates.** Goat polyclonal anti-mouse IgG (KPL Catalog No. 18-06-10) was biotinylated using the SureLINK™ Chromophoric Biotin Labeling Kit. Prior to conjugation, the antibody was rehydrated in the 1X Modification Buffer provided. The conjugation reactions were initiated by the addition of 0.2 mg (0.2 mL or 0.4 mL) of antibody to 10 to 60-fold molar excess of chro-

mophoric biotin. The conjugation reactions were incubated for two hours at room temperature with gentle agitation. Following the conjugation reactions, the excess biotin was removed using the 5,000 MWCO Spin Filters included in the kit.

**Comparison of Biotin Quantitation Methods.** Mouse monoclonal anti-goat IgG (OEM Concepts Inc.), was labeled with a 20-fold molar excess of SureLINK Chromophoric Biotin at an antibody concentration of 1 mg/mL. After a 2 hour conjugation at room temperature with gentle agitation, the conjugate was purified using a 50,000 MWCO VivaSpin 6 centrifugal concentrator from Sartorius<sup>1</sup>. The purified biotinylated antibody was serially diluted two-fold, yielding eight samples of the conjugates at various concentrations. The ratio of biotin to antibody was calculated in each of the samples using both the chromophoric and HABA methods. Triplicate measurements were made using both methods. HABA assay kits from two different suppliers were tested.

**ELISA Assays.** Microplate wells were coated using 250 ng of mouse IgG. Plates were blocked with 1X BSA (KPL Catalog No. 50-61-00), washed with 2% sucrose, and stored desiccated at room temperature. Biotinylated goat anti-mouse IgG conjugates described above were added to each well in two-fold serial dilutions, and then incubated for 30 minutes at room temperature. The wells were washed and incubated with HRP-Streptavidin. After washing, ABTS Substrate was added and incubated for 15 minutes. The reaction was stopped and absorbance read at 405 nm.

**Western Blot Assay.** Mouse IgG was run on SDS-PAGE and blotted onto a 0.45 µm nitrocellulose membrane. The membrane was blocked and then incubated for 1 hour with 12.5 ng/mL of goat anti-mouse antibody conjugated with SureLINK Chromophoric Biotin. The membranes were washed and then incubated with HRP-Streptavidin. Detection was performed using chemiluminescent LumiGLO Substrate and photographic film.

**Immunohistochemistry.** Standard procedures were used for immunohistochemistry experiments<sup>5</sup>. Paraffin-embedded human liver tissue slides were used along with a mouse monoclonal anti-human epithe-

lial membrane antigen (EMA) antibody (DakoCytomation). Biotinylated Goat Anti-Mouse IgG conjugates (5 µg/mL, prepared as described above), were used in place of the biotinylated antibody in the immunohistochemistry kit (Catalog #71-00-18). The slides were incubated with TrueBlue Peroxidase Substrate for 10 minutes, followed by Contrast Red for counterstaining as described in product technical information<sup>5</sup>. Images were visualized using a compound light microscope.

## RESULTS AND DISCUSSION

SureLINK Chromophoric Biotinylation Kit was used to conjugate a monoclonal antibody with biotin, as described in Materials and Methods. A 20-fold molar excess of biotin to antibody was used. This yields a conjugate with a molar substitution ratio (MSR) of 5-10 biotins per antibody molecule. Two commercially available HABA assay kits were used to quantitate the MSR of biotin substitution. The HABA assay was performed at a range of protein concentrations in order to assess the utility of the method.

**Chromophoric biotin quantitation is more reliable than the HABA assay.** The results of biotin quantitation in the conjugate using the two HABA kits are shown in Figure 2. For comparison, the results of quantitation using absorbance of the SureLINK chromophore at 354 nm are shown as well as error bars for triplicate measurements of each data point. Figure 2 demonstrates that the HABA assay is highly variable and inaccurate at protein concentrations below 0.4 mg/mL.

Variability in the Quantitation of Biotin in a Biotin-Antibody Conjugate with HABA Assay Kits and Absorbance at 354 nm

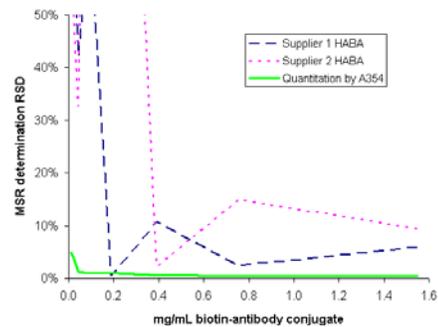


Figure 3: See the legend to Figure 2 for a description of the experiment. Figure 3 plots the RSD (CV) of the MSR determination of biotin for the two HABA kits and the A<sub>354</sub> measurement for triplicate measurements of the conjugate at each concentration.

To examine this variability from a different perspective, the relative standard deviation (RSD or coefficient of variation (CV)) between triplicate measurements for each HABA kit and 354 nm absorbance at different protein concentrations is plotted in Figure 3. The variability of the HABA assays is greater than the variability of the absorbance measurement at 354 nm at all protein concentrations, but particularly below 0.4 mg/mL.

To understand the variability in the HABA assay, it is instructive to examine the raw data summarized in Table 1. The

Quantitation of Biotin in a Biotin-Antibody Conjugate with HABA Assay Kits and Absorbance at 354 nm

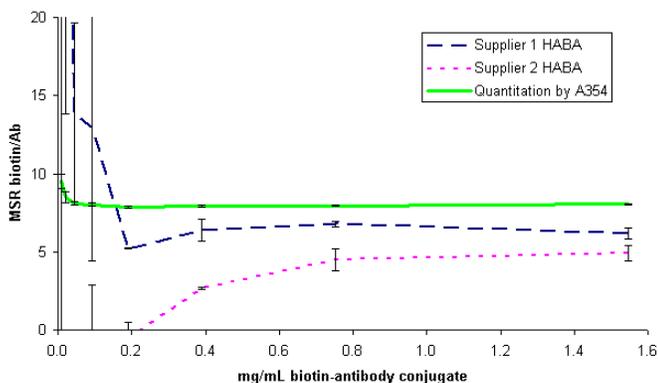


Figure 2: A biotin conjugate of a mouse anti-goat monoclonal antibody was prepared using the SureLINK Chromophoric Biotinylation Kit, as described in the text. The conjugate was diluted to the following concentrations: 0.01, 0.02, 0.04, 0.09, 0.19, 0.39, 0.75, and 1.55 mg/mL, and tested with two HABA assays and by measuring absorbance at 354 nm in triplicate. The MSR (molar substitution ratio) of biotin was determined for each sample.

change in absorbance due to addition of the biotinylated antibody is very small relative to the baseline  $A_{500}$ . Changes in absorbance of this magnitude are very difficult to measure reproducibly. In contrast, at 0.4 mg/mL, the biotinylated antibody has an absorbance of 0.140 at 354 nm when prepared with SureLINK Chromophoric Biotinylation Kit. This absorbance is well within the linear range of the spectrophotometer.

Raw absorbance data for HABA quantitation of biotin for a biotin-antibody conjugate

	$A_{500}$ for the HABA-avidin complex, no biotinylated Ab added	$A_{500}$ for the HABA-avidin complex, biotinylated Ab added	$A_{500}$ due to displacement of HABA by the biotinylated Ab
Supplier 1 HABA	0.520	0.494	0.026
Supplier 2 HABA	0.360	0.348	0.012

Table 1: Raw absorbance data at 500 nm (average of triplicates) for analysis of the MSR of biotin in the conjugate at 0.39 mg/mL.

Chromophoric biotin allows for a broader linear range of biotin quantitation as compared to HABA. As Table 2 indicates, the linear range for HABA is 8- to 16-fold and the linear range for the absorbance of chromophoric biotin is 40-fold. The sensitivity of biotin quantitation chromophoric biotin absorbance is 5- to 10-fold greater than the sensitivity of the HABA method.

Quantitation of biotin incorporation by the absorbance of chromophoric biotin does not consume sample. The HABA method relies on a biotin-labeled sample competing with HABA for binary with avidin. Thus, the assay consumes the biotin conjugate sample. Table 2 lists the amount of sample consumed for a single replicate for a biotin conjugate in the HABA assay. This amount varies with the MSR of the biotin conjugate. For biotin conjugates with 1-5 biotin molecules per antibody, the HABA assay requires 15-60  $\mu\text{g}$  conjugate per assay replicate. In contrast, because the absorbance of the

Linear range and sample consumption for quantitation of biotin by HABA or by absorbance of SureLINK Chromophoric Biotin at 354 nm.

	Linear Range	Sample consumption for conjugate with 1 biotin/antibody, single replicate	Sample consumption for conjugate with 5 biotin/antibody, single replicate
HABA Assay	20 - 160 $\mu\text{M}$ biotin	60 $\mu\text{g}$	12.5 $\mu\text{g}$
Absorbance of Chromophoric Biotin at 354 nm	1.75 - 70 $\mu\text{M}$ biotin	None	None

Table 2: Sample consumption is calculated assuming a single replicate for HABA requires 20  $\mu\text{L}$  of sample and a protein with a molecular weight of 150,000 Daltons. Sample consumption for the HABA assay is determined at the lower limit of the linear range. The extinction coefficient for the SureLINK Chromophoric Biotin reagent is 29,000  $\text{M}^{-1} \text{cm}^{-1}$  at 354 nm. The linear range of the quantitation at 354 nm is defined as the amount of chromophoric biotin that will give a reading between 0.05 and 2.0 absorbance units.

SureLINK biotin conjugate can be measured directly in a spectrophotometer, no conjugate is consumed.

**Use of SureLINK™ Chromophoric Biotin conjugates in ELISA.** SureLINK Biotin Conjugate can be used in a variety of ELISA formats. The use of these conjugates in an indirect ELISA is illustrated in Figure 4. Antigen is coated onto a plate and then probed with a biotinylated antibody. Following plate washing, the biotinylated antibody/ antigen complex is probed with HRP-streptavidin. Figure 4 shows that the sensitivity of the ELISA is improved by preparing biotin-antibody conjugates with a higher amount of SureLINK Chromophoric biotin.

**Use of SureLINK™ Chromophoric Biotin conjugates in**

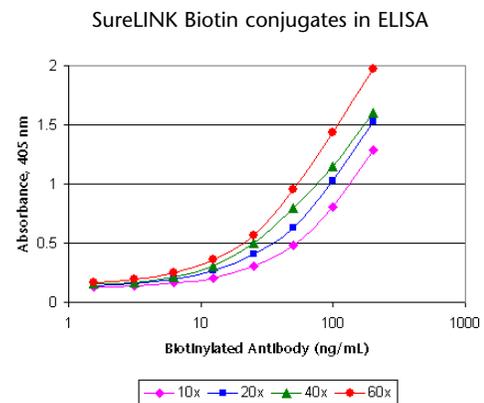


Figure 4. Detection of IgG in ELISA with varying levels of biotinylated antibody. SureLINK Chromophoric Biotin conjugates were conjugated at 1 mg/mL Ab with 10, 20, 40, or 60-fold excess biotin added. HRP-SA conjugate was used for detection in conjunction with ABTS Substrate.

**Western blots.** Western blots were performed with SureLINK biotin conjugates prepared using SureLINK™ biotin and an anti-IgG polyclonal antibody (Figure 5). Varying amounts of IgG antigen were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Figure 5 shows that preparing biotin-antibody conjugates with a wide range of SureLINK Chromophoric biotin (10- to 60-fold molar excess over antibody) resulted in conjugates with similar performance in a Western blot assay.

**Use of SureLINK™ Chromophoric Biotin conjugates in immunohistochemistry.** Biotinylated anti-IgG antibody conjugates were prepared for use in an immunohistochemistry application (described in Materials and Methods). IHC results with a biotin-antibody conjugate prepared with SureLINK chromophoric biotin are shown in Figure 6. The preparation of biotin-antibody conjugates with a wide range of SureLINK Chromophoric biotin (10- to 60-fold molar excess over antibody) resulted in conjugates with similar performance in IHC assays.

### Western blot assay using SureLINK Biotin-Antibody conjugates

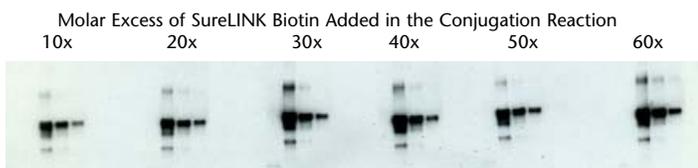


Figure 5. Western blots were prepared with various amounts of IgG and probed with the SureLINK Biotin conjugates and HRP-SA. LumiGLO® was used for chemiluminescent detection of HRP activity. The three lanes in each set were loaded with 250, 50, and 10 ng IgG, respectively.

## CONCLUSIONS

The SureLINK Chromophoric Biotin Labeling kits offer a superior method for labeling and quantitation of biotin in protein conjugates. The SureLINK chromophoric biotin reacts with primary amino groups on immunoglobulin or other proteins to produce high-performance biotin conjugates that can be used in a variety of immunoassays including, but not limited to, ELISA, Western blotting and IHC. The performance of biotin conjugates prepared with the SureLINK Chromophoric Biotin varied by application. Conjugates prepared using a 60-fold molar excess of biotin performed optimally in ELISA, whereas conjugates prepared with a 10-fold molar excess of biotin performed well in Western blot and IHC applications.

The unique chromophoric group enables direct quantitation of the biotin incorporated into a biotin-protein conjugate through a spectrophotometric reading at 354 nm. It overcomes many of the limitations of the traditional HABA method of biotin quantitation. SureLINK Chromophoric Biotin allows for accurate quantitation of biotin incorporation at protein concentrations below 0.05 mg/mL. In contrast, we demonstrated that HABA assay kits are highly non-reproducible and cannot be effectively used with protein concentrations below 0.4 mg/mL. In addition, the measurement of biotin incorporation using chromophoric biotin can be performed over a broader linear range

### Immunohistochemistry assay using biotin-antibody conjugates prepared with the SureLINK Chromophoric Biotinylation Kit

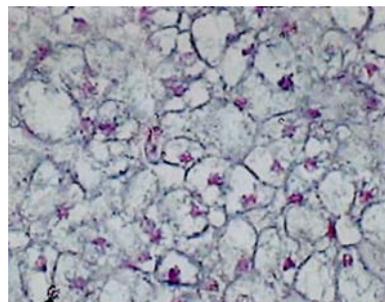


Figure 6. Detection of epithelial membrane antigen in human liver tissue by immunohistochemistry using SureLINK™ Chromophoric Biotin conjugates. 5 mg/mL of conjugate was added to each slide. Slides were stained/counterstained with TrueBlue/Contrast Red, resulting in a blue/purple and pink stain of the antigen and nuclei, respectively.

of protein concentrations as compared to HABA. Finally, quantitation of biotin incorporated into a conjugate using SureLINK Chromophoric Biotin does not consume the conjugate sample, making it an economic alternative to the HABA method.

## REFERENCES

- <sup>1</sup>Abbreviations—HABA, 4'-hydroxyazobenzene-2-carboxylic acid; HRP, horseradish peroxidase; SA, streptavidin; Ab, antibody; mAb, monoclonal antibody; pAb, polyclonal antibody; ELISA, Enzyme-linked immunosorbent assay; IHC, immunohistochemistry; MWCO, Molecular Weight Cutoff; MSR, Molar Substitution Ratio.
- <sup>2</sup>Hermanson, G.T. (1996) Bioconjugate Techniques, Academic Press, San Diego, CA
- <sup>3</sup>Green, N.M. (1965) Biochem J., 89, 609-620.
- <sup>4</sup>In this particular experiment, the larger MWCO spin filter was used for more rapid purification.
- <sup>5</sup>See Technical Information for KPL's HistoMark Biotin-Streptavidin Peroxidase Kit, Mouse Primary Antibody, Catalog #71-00-18.

## Product Information

Description	Size	Catalog No.
SureLINK Chromophoric Biotin Labeling Kit	5 x 1.0 mg	86-00-01
SureLINK Chromophoric Biotin Labeling Kit	2 x 0.5 mg	86-00-02
SureLINK Chromophoric Biotin	10 mg	86-00-03



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