

Micro-Volume Determination of Labeling Efficiency Using Spectral Scanning Analysis in a Standard Microplate Reader

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A variety of methods and reagents have been developed to efficiently label biomolecules. These labels greatly improve assay sensitivity in conjunction with modern analytical techniques and instrumentation. Many of these labeling methods involve covalent modifications of proteins or nucleic acids resulting in the addition of a fluorophore or a functional moiety via a flexible linker. The use of labeled reagents in a variety of experimental methods such as immunofluorescence, TR-FRET, and ELISA has been shown to provide substantial improvements in assay sensitivity while allowing sample and reagent conservation in a high-throughput context. Here we describe the analysis of protein-antibody conjugates labeled with biotin or digoxigenin using micro-volume spectral-scanning analysis in a microplate reader.

Introduction

Spectrophotometric analysis has been widely used in the biological sciences for decades for detection and quantification of a variety of substances. Commonly used methods for analysis of biological samples rely on absorption, fluorescence and luminescence detection. Furthermore, these methods rely on a relatively narrow range of bandwidths spanning the UV-visible to near infrared spectral region with wavelengths within the ~200-999 nm range. Many molecules have intrinsic properties that allow direct analysis using spectrophotometric methods. However, it is not uncommon to encounter complex experimental conditions that result in signal interference in the spectral region required for direct analysis of the target analyte resulting in poor signal-to-noise. Thus, a variety of labeling chemistries have been developed that can aid in both minimizing background interference while simultaneously increasing assay sensitivity and dynamic range.

One common analytical method is sandwich immunoassays which involve capture of a target analyte by antibodies immobilized on a solid support, such as a microplate well surface; as is the case with a typical bridging type immunoassay. Following capture, analyte detection is achieved using a labeled antibody to a different epitope on the analyte. Common labels are digoxigenin or biotin.

Custom labeling of a specialized protein that may not be commercially available in the desired form may be necessary during assay development. Efficient labeling and quantification of the labeled product is critical to insure the success of downstream applications. Several commercially available products have been developed to simplify labeling efficiency. Here we describe methods for labeling of antibodies, and subsequent quantification of product yield and labeling efficiency, required for validation of an immunogenicity assay using a model system for detection of anti-drug antibodies (ADA), which may be present in serum samples, against a biological drug. The ADA detection assay required labeling of the biologic of interest with either a digoxigenin or biotin moiety. Reagents available from Solulink (San Diego, CA) contain features that aid in both labeling and quantification of labeled product by incorporation of both common linker chemistry and a UV-traceable chromophore for calculating the number of labels per molecule (Figure 1). The molecular substitution ratio (MSR), the ratio of labels per molecule of antibody can then be used as a qualification measure once validated empirically during assay development.

Key Words:

Protein Labeling Efficiency

Biotin

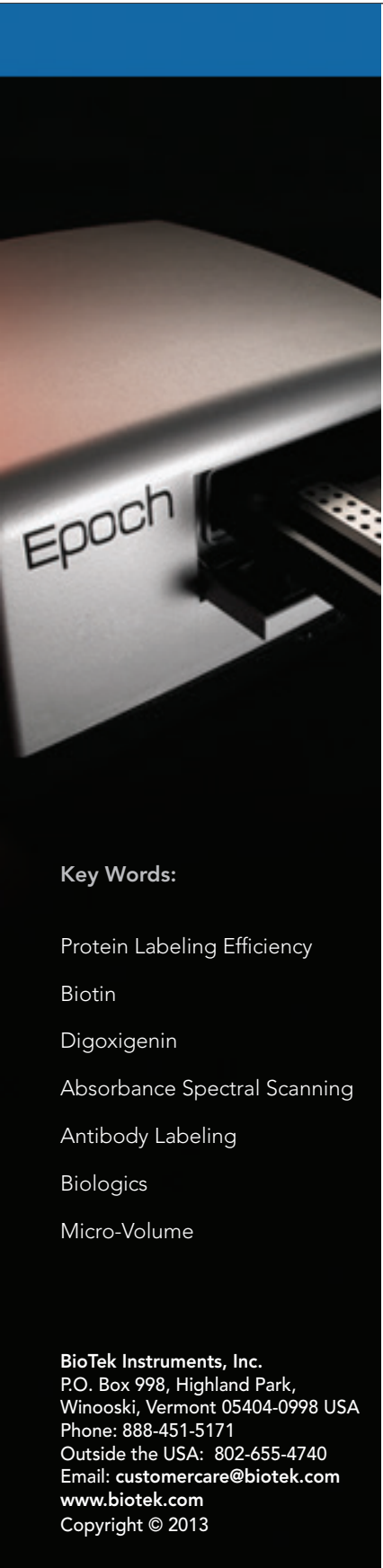
Digoxigenin

Absorbance Spectral Scanning

Antibody Labeling

Biologics

Micro-Volume



MSR ratios from 2-8 are commonly achievable. Measurement of the absorption of UV light at a wavelength of 280 nm by the labeled antibody and absorption of light at a wavelength of 354 nm by the traceable bis-aryl hydrazone chromophore of the labeling reagent are

used to calculate the MSR. Measurement data can be gathered by performing a spectral scan on as little as 2 μ L of sample using the Take3™ Micro-Volume Plate and a standard absorbance capable microplate reader.

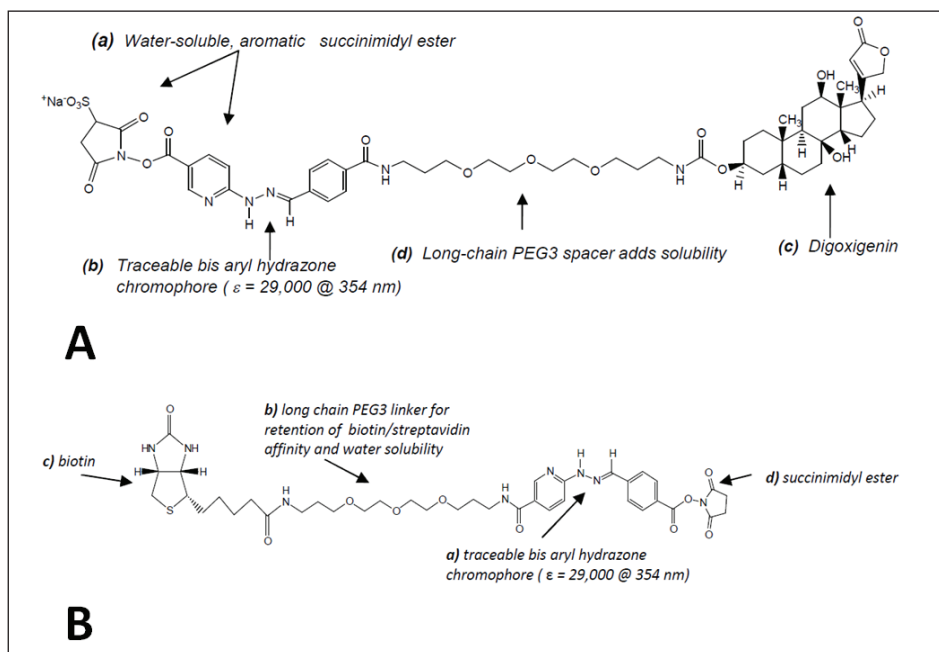


Figure 1. ChromaLink Labeling Reagents. The incorporation of the UV-traceable bis-aryl hydrazone chromophore allows for rapid quantification of label incorporation while the long-chain PEG3 spacer aids in retention of biotin/streptavidin kinetics and increases solubility in aqueous solutions. A) ChromaLink Digoxigenin and B) ChromaLink Biotin.

Materials and Methods

Two different drug antibody labeling reactions were performed for use in the assay format described above. For the AlphaLISA® ADA assay, drug antibody biotinylation was performed using the ChromaLink Biotin reagent (Solulink, Inc., San Diego, CA, USA) as per the manufacturers' recommendation and using standard purification procedures. For the solution based ELISA ADA assay, drug antibody digoxigenin was performed using the ChromaLink Digoxigenin One-Shot Antibody-Labeling Kit (Solulink, Inc., San Diego, CA, USA). Briefly, prior to labeling, the drug antibody was concentrated to meet labeling requirements using an Amicon® Ultra-0.5 Centrifugal Filter device with a 100K Nominal Molecular Weight Limit (NMWL) cutoff and exchanged into the appropriate modification buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 for biotin and 130 mM sodium phosphate, pH 8.0 for digoxigenin labeling) using a Zeba™ Desalt spin column.

For biotin labeling, 0.05 mg of drug antibody was incubated with NHS-ChromaLink-biotin prepared in DMF at a 30:1 molar ratio. The reaction volume was completed with modification buffer to a total volume of 100 μ L and incubated for 2 hours at room temperature, ~23 °C. Purification of the product was performed using a Zeba™ Desalt spin column preequilibrated with phosphate buffered saline (PBS) and spun to dryness.

Wells to be used for spectral scanning were first subjected to absorbance measurements at 280 and 354 nm with the blanking buffer, PBS, for background subtraction calculations following spectral scanning. An absorbance spectral scan was performed, in duplicate, for each sample from 260-380 nm in 1 nm increments using a 2 μ L sample on a Take3™ Micro-Volume Plate read on an Epoch™ Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Data was collected using Gen5™ Data Analysis Software (BioTek Instruments, Inc., Winooski, VT, USA). The biotinylation ratio of the product was calculated as per the manufacturers' protocol using the appropriate Kit Calculator from Solulink (<http://www.solulink.com/library#calcs>) and background subtracted absorbance readings at 354 nm and 280 nm to determine the MSR and product recovery, respectively.

Labeling with digoxigenin was performed as described above with the following modifications. The drug antibody concentration was adjusted to 1 mg/mL in a volume of 100 μ L using the appropriate modification buffer. ChromaLink™ Digoxigenin labeling reagent was prepared by addition of 5 μ L DMF to the vial containing the reagent. The entire volume of drug antibody (100 μ L @ 1 mg/mL) was added to the reagent vial with mixing and incubated for 60 minutes at room temperature, \sim 23° C. The reaction was quenched with 10 μ L of 1 M Tris, pH 8.9. The quenched reaction was subject to centrifugation @ 1,500 x g for 30 seconds and added to a Zeba™ Desalt spin column pre-equilibrated with PBS as described above for purification. The product was analyzed as described above to determine MSR and quantification of product recovery.

Results and Discussion

Several drug antibody labeling experiments were performed and subsequently analyzed by micro-volume absorbance spectral scanning using 2 μ L of recovered product (Figure 2). Recovery was typically \geq 80% of the starting sample as determined by quantification using absorbance at 280 nm. Product loss is likely due to residual hold-up in the Zeba column resin as recovered volume was generally \approx 90 μ L. The MSR is dependent on several factors including antibody mass in reaction, reaction buffer, incubation time and the reaction stoichiometry. When following standard methods using a 15-fold mole-equivalent of linker in the labeling reaction the typical MSR can range from 2-8 labels per target molecule. For the drug antibody labeling experiments the MSR ranged from 3.34 to 6.84 and 2.07 to 3.1, for biotin and digoxigenin labeling, respectively; well within the expected range. The sample was recovered from the Take3™ micro-well following analysis minimizing sample loss during analysis.

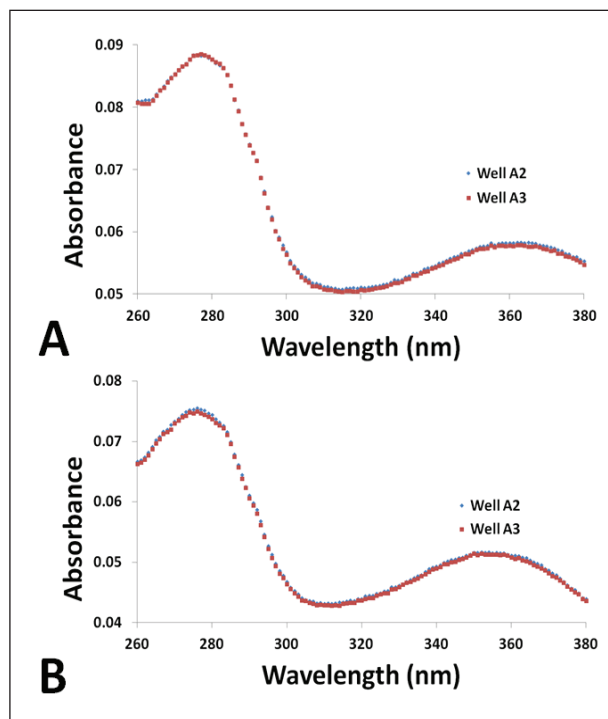


Figure 2. Micro-Volume Spectral Scanning Analysis. Post-labeling analysis of drug antibody labeling efficiency was performed using 2 μ L of product. Data was collected in 1 nm increments from 260 to 380 nm in duplicate. A) Digoxigenin labeled drug antibody and B) Biotin labeled drug antibody (representative data).

Conclusion

The ability to efficiently label specialized biological reagents for use in assay development was shown to be easily achieved by use of a commercially available labeling reagent with a UV traceable tag. Biotin and digoxigenin labeling of a drug antibody for use in an immunogenicity anti-drug antibody assay was performed and quantified using micro-volume absorbance spectral scanning analysis. Typical recovered volumes of labeled product were \sim 90 μ L and determined to be pure by SDS-PAGE analysis (data not shown). Duplicate measurements of 2 μ L samples were used for MSR analysis representing \sim 4-5% of the sample. Due to the limited amount of recoverable labeled product the ability to perform micro-volume analysis that allows for sample recovery was important. Replicate data showed excellent reproducibility resulting in recovery, labeling efficiency and associated MSR values agreeing well with expected values.