Application Note

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Microvolume Determination of Labeling Efficiency Using Spectral Scanning Analysis in a Standard Microplate Reader

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Abstract

Various 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 many 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. This application note describes the analysis of protein-antibody conjugates labeled with biotin or digoxigenin using microvolume spectral-scanning analysis in a microplate reader.

Introduction

Spectrophotometric analysis has been widely used in the biological sciences for decades for the detection and quantification of many 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 to 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, many 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. This 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 ensure the success of downstream applications. Several commercially available products have been developed to simplify labeling efficiency. This application note describes methods for labeling antibodies (and the subsequent quantification of product yield and labeling efficiency required for validation of an immunogenicity assay) using a model system for detection of antidrug 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.

MSR ratios from 2 to 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 bisaryl 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 µL of sample using an Agilent BioTek Take 3 microvolume plate and a standard absorbance capable microplate reader.

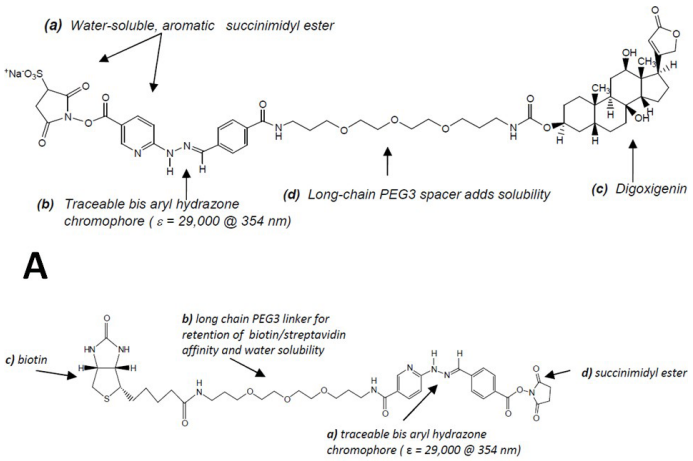

Figure 1. ChromaLink labeling reagents. The incorporation of the UV-traceable bisaryl hydrozone chromophore allows rapid quantification of label incorporation while the long-chain PEG3 spacer aids in retention of biotin/streptavidin kinetics and increases solubility in aqueous solutions. (A) ChromaLink digoxigenin and (B) ChromaLink biotin.

Materials and methods

Two different drug antibody-labeling reactions were performed for use in the assay format described previously. For the AlphaLISA ADA assay, drug antibody biotinylation was performed using the ChromaLink Biotin reagent (Solulink, Inc., San Diego, CA) according to the manufacturer's 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). Briefly, before labeling, the drug antibody was concentrated to meet labeling requirements using an Amicon Ultra-0.5 Centrifugal Filter device with a 100 K nominal molecular weight limit (NMWL) cut-off 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 ZebaTM 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 µL and incubated for two hours at room temperature, ~23 °C. Purification of the product was performed using a ZebaTM 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 to 380 nm in 1 nm increments using a 2 µL sample on an Agilent BioTek Take 3 microvolume plate read on an Agilent BioTek Epoch microplate spectrophotometer. Data were collected using Agilent BioTek Gen5 microplate reader and imager software. The biotinylation ratio of the product was calculated according to 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 µL using the appropriate modification buffer. ChromaLinkTM Digoxigenin labeling reagent was prepared by addition of 5 µL of DMF to the vial containing the reagent. The entire volume of drug antibody (100 µL at 1 mg/mL) was added to the reagent vial with mixing and incubated for 60 minutes at room temperature, ~23 °C. The reaction was quenched with 10 µL of 1 M tris, pH 8.9. The quenched reaction was subjected to centrifugation at 1,500 × g for 30 seconds and added to a Zeba Desalt spin column pre-equilibrated with PBS as described previously for purification. The product was analyzed as described previously to determine MSR and quantification of product recovery.

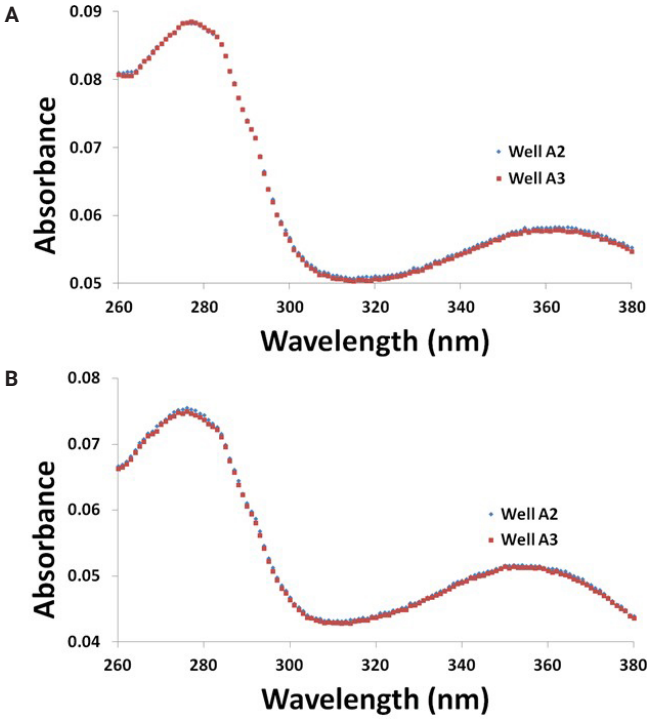

Figure 2. Microvolume spectral scanning analysis. Postlabeling analysis of drug antibody-labeling efficiency was performed using 2 µL of product. Data were collected in 1 nm increments from 260 to 380 nm in duplicate. (A) Digoxigenin-labeled drug antibody and (B) Biotin-labeled drug antibody (representative data).

Results and discussion

Several drug antibody-labeling experiments were performed then analyzed by microvolume absorbance spectral scanning using 2 µL of recovered product (Figure 2). Recovery was typically ≥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 ≈90 µL. The MSR depends on several factors including antibody mass in reaction, reaction buffer, incubation time, and the reaction stochiometry. When following standard methods using a 15-fold mole-equivalent of linker in the labeling reaction, the typical MSR can range from 2 to 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 microwell following analysis-minimizing sample loss during analysis.

Conclusion

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

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