Development of an AlphaLISA assay to detect weak interactions between lectins and glycans on antibodies.

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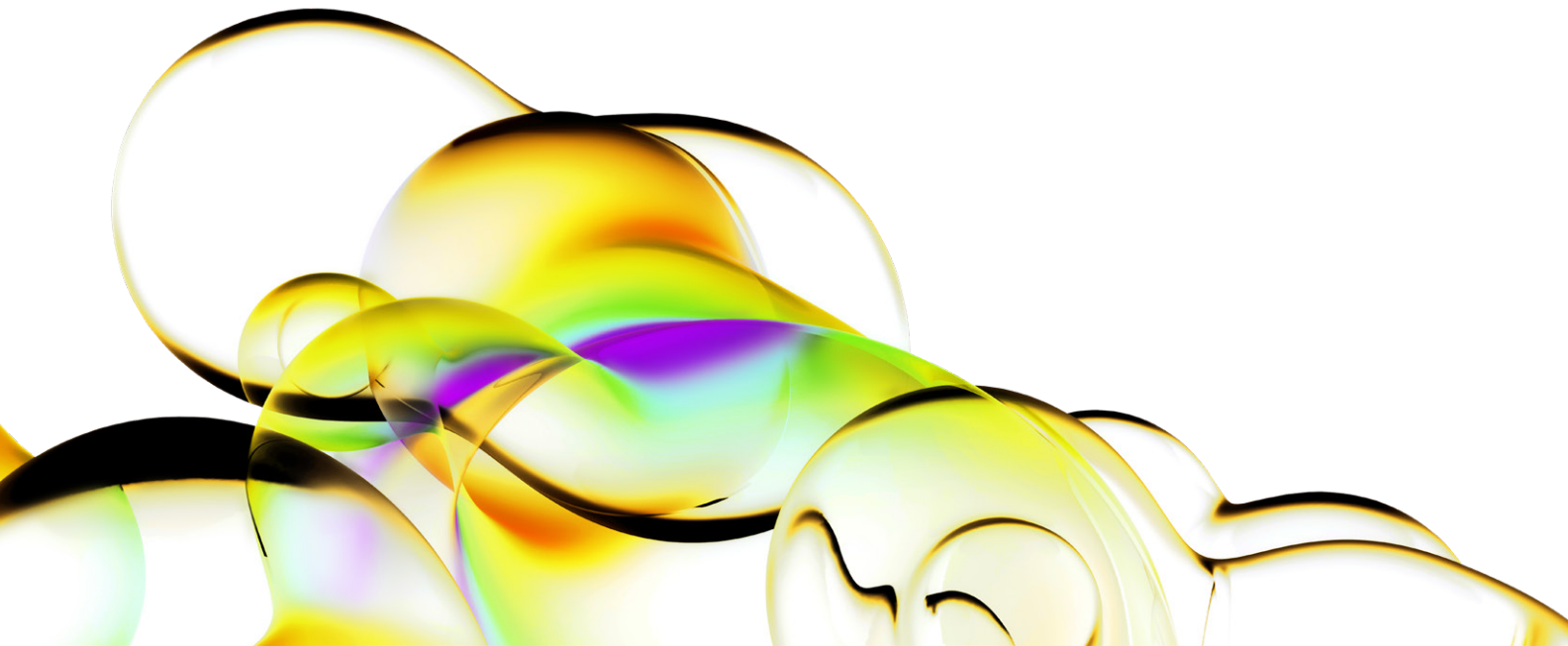
Introduction

Biomolecular interactions, or binding events between biomolecules, are important components of biological processes such as transcription, translation, and post-translational modifications used for cell signaling. A number of these binding events have been targeted for the development of novel therapeutic drugs. There are a variety of assay formats to measure binding events; however, efficient, highly sensitive assays that can be used to study a large range of binding affinities are extremely important for fully validating therapeutic drugs.

AlphaLISA® is a bead-based assay technology that can be used to study a large range of biomolecular interactions in a microplate format. Alpha assays require two types of beads: Donor beads and Acceptor beads. Typically, each bead is conjugated to a protein or antibody used to capture one of the targets in the biomolecular interaction assay. When the two biomolecules interact, the Donor bead is brought into proximity of the Acceptor bead and excitation of the Donor bead results in a luminescent signal from the Acceptor bead. AlphaLISA can detect a broad range of affinities with dissociation constants (K_D) ranging from picomolar to low millimolar. In wash-based methods, weak interactions may be disrupted. Since AlphaLISA is homogeneous (no wash steps required), transient interactions can easily be measured. Since each bead has multiple binding sites, when one antibody-analyte binding event takes place, this facilitates the other antibody-analyte bindings. Low nanomolar concentrations of binding partners can generate high local concentrations of protein complexes reaching micromolar levels between beads (Figure 1).



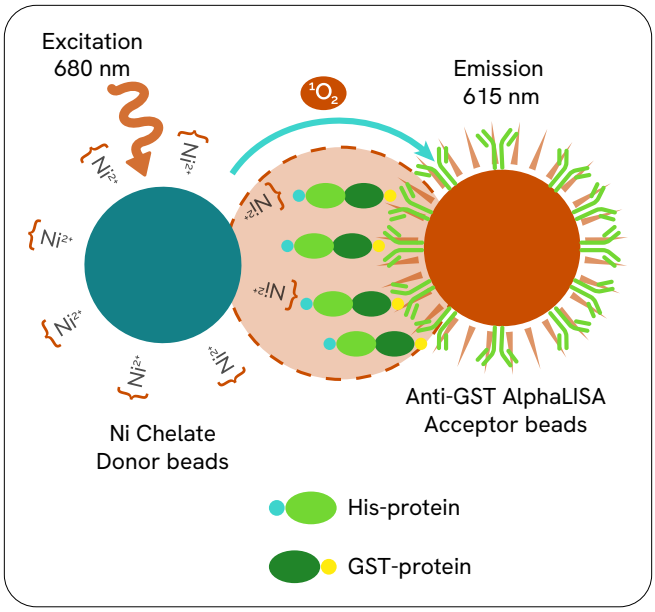


Figure 1: AlphaLISA bead-based technology enhances avidity between biomolecules.

One clear advantage of AlphaLISA over many other technologies is the ability to measure transient or weak binding interactions (K_D > 1 µM), such as detecting glycans on a glycosylated protein binding to lectins. N-glycosylation in the Fc region of monoclonal antibodies is involved in effector functions including antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cellular cytotoxicity (CDCC). The degree at which the antibodies are modified, and the type of glycosylation, can be variable and dependent on the host. Antibodies that are used as therapeutics can show a high degree of heterogeneity in these N-linked oligosaccharides and therefore high throughput analysis of antibody glycosylation is an important tool for quality control of therapeutic antibodies. Certain N-linked glycans are known to bind specific types of lectins with weak affinity. For example, *Erythrina cristagalli* lectin (ECL) and *Griffonia simplicifolia* lectin II (GSL II) have been shown to bind specifically to different N-linked oligosaccharides using frontal affinity chromatography.[1,2] ECL binds galactosylated biantennary N-glycan with fucose (G2F) with a dissociation constant of 20 µM.[1] GSL II binds to N-acetylglucosamine (βGlcNAc) residues with a dissociation constant of 50 µM.[2] Onitsuka et al.[3] utilized AlphaLISA to detect these known weak binding interactions and developed an AlphaLISA assay to detect specific types of glycosylation on antibodies. Onitsuka et al. directly conjugated the antibody of interest to the AlphaLISA Acceptor beads.

Here we modified their protocol by using Protein G AlphaLISA Acceptor beads to capture the antibody of interest and utilized the same biotinylated lectins. Figure 2 shows the schematic used to detect the glycosylation state of the antibody using AlphaLISA.

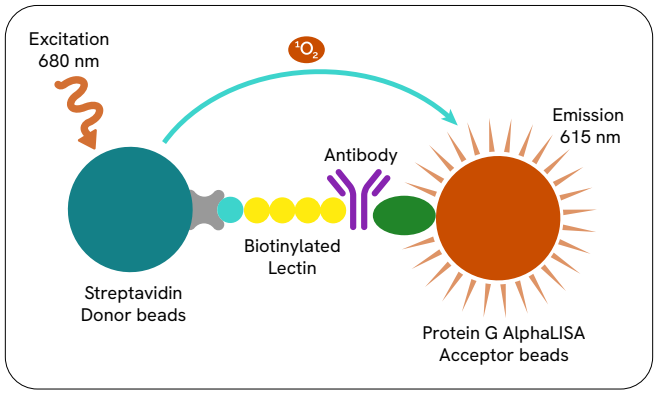


Figure 2: Detection of glycan binding lectins using AlphaLISA.

In this technical note, we show how to develop an AlphaLISA assay to look at a weak binding interaction between a glycosylated antibody and a lectin.

Materials and methods

Instrumentation

All AlphaLISA measurements were performed on the Revvity 2105 EnVision® multimode plate reader using standard Alpha settings.

Reagents

- Biotinylated GSL II (Vector Labs, #B-1215)
- Biotinylated ECL (Vector Labs, #B-1145)
- GSL II (Vector Labs, #L-1210)
- ECL (Vector Labs, #L-1140)
- A goat polyclonal antibody (Jackson Laboratories, #123-005-021)
- Protein G AlphaLISA Acceptor beads (Revvity, #AL102)
- Streptavidin Donor beads (Revvity, #6760002)
- Immunoassay Buffer (Revvity, #AL000)

AlphaLISA assay protocols

Various concentrations of 10X biotinylated GSL II or ECL (5 µL) were cross-titrated with the 5X antibody (10 µL) for 30 minutes. Then the reaction was incubated with 10 µL of Protein G AlphaLISA Acceptor beads (20 µg/mL final) for one hour. Finally, 25 µL of Streptavidin Donor beads (20 µg/mL final) were added and incubated for 30 minutes (Protocol 1). For Protocol 2, 5 µL 10X biotinylated GSL II or ECL and 10 µL of Protein G AlphaLISA Acceptor beads (20 µg/mL final) were added to the plate followed by 10 µL of 5X antibody and the reaction incubated for 1 hour. Then, 25 µL of Streptavidin Donor beads (20 µg/mL final) were added and incubated for 30 minutes. For competition and Z′ assays, 15 µL 3.3X non-biotinylated GSL II or non-biotinylated ECL were incubated with 5 µL of biotinylated GSL II or ECL respectively, 5 µL of 10X antibody (1 nM final), and 5 µL of Protein G AlphaLISA Acceptor beads (20 µg/mL final) for 1.5 hours. Finally, 20 µL Streptavidin Donor beads (20 µg/mL final) were added and incubated for 30 minutes.

Data analysis

The data were analyzed using GraphPad Prism® software. Binding curves were generated using nonlinear regression, using a four-parameter logistic equation (sigmoidal dose-response curve with variable slope). The inhibition curves were generated using log (inhibitor) vs. response - variable slope (four parameters).

For Z′ determination, we used the equation:

$$Z' = \frac{1 - (3*(\text{standard deviation (high signal)} + \text{standard deviation (low signal)})}{(\text{average (high signal)} - \text{average (low signal)})}$$

Results

Optimizing reagent concentrations and assay protocol

We chose a polyclonal goat antibody from Jackson Laboratories for development of the assay. We first did a cross-titration of the biotinylated lectins and the antibody (Figure 3). Since the Protein G AlphaLISA Acceptor beads have an approximate bead capacity of 1 nM, we tested 0.5, 1, and 2 nM of the antibody. We examined seven different concentrations of the biotinylated lectins and chose to move forward with 0.5 nM of GSL II and 0.8 nM of ECL.

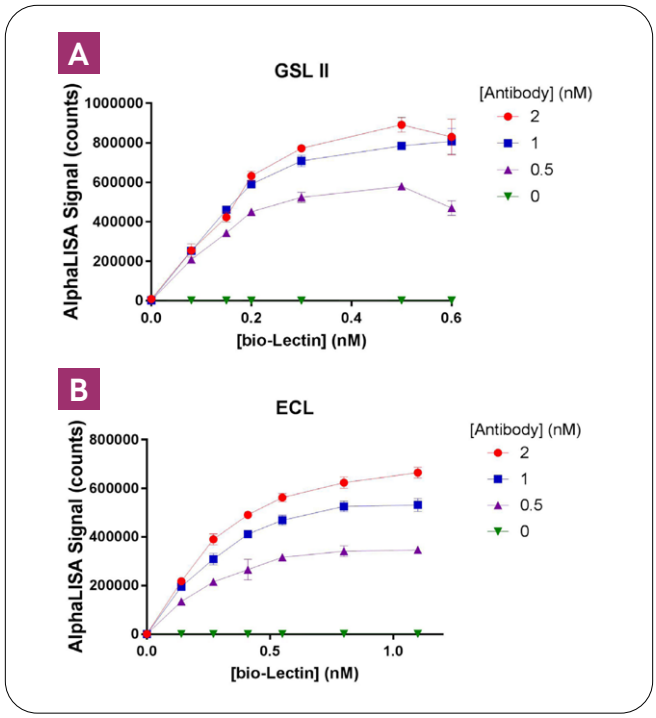


Figure 3: Biotinylated lectin and antibody cross-titration for GSL II (A) and ECL (B).

Next, we optimized the order of addition using 1 nM antibody. It was unclear whether the lectin and Protein G would compete for binding to the antibody. Therefore, we compared a 3-step protocol (Protocol 1, see Materials and Methods) where we first pre-incubated the antibody with the biotinylated lectins and then added the Protein G AlphaLISA Acceptor beads later, to a 2-step protocol (Protocol 2) where we added the Protein G AlphaLISA Acceptor beads at the same time as the biotinylated lectin (Figure 4). Slightly higher signal was seen with Protocol 2 suggesting the biotinylated lectins may partially block Protein G binding, therefore adding them at the same time gave the best assay window.

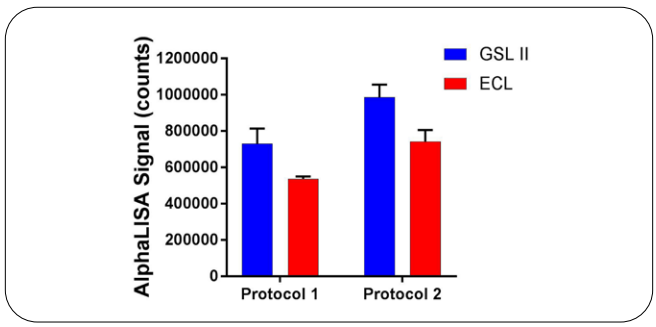


Figure 4: **Protocol 1**: Pre-incubation of the biotinylated-lectin with antibody for 30 minutes prior to addition of Protein G AlphaLISA Acceptor beads for 1 hour. Then incubating with Streptavidin Donor beads for 30 minutes. **Protocol 2**: Incubation of biotinylated-lectin, Protein G AlphaLISA Acceptor beads, and antibody for 1 hour, then incubating with Streptavidin Donor beads for 30 minutes.

Next, we performed full dose response curves for the goat polyclonal antibody to detect either galactosylated biantennary N-glycan with fucose using biotinylated ECL or βGlcNAc using biotinylated GSL II utilizing Protocol 2. As shown in Figure 5, we observed a nice dose response for the antibody titration detecting both types of glycans.

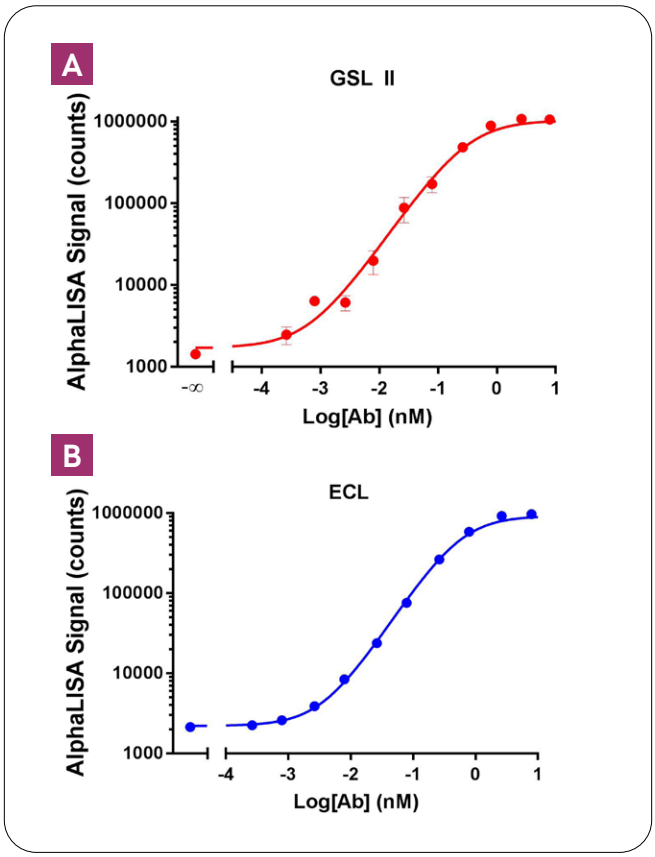


Figure 5: Full dose response curves. A) Using biotinylated GSL II to detect βGlcNAc on the goat polyclonal antibody. B) Using biotinylated ECL to detect G2F on the goat polyclonal antibody.

Specificity of interaction

To confirm specificity of the signal, we competed the signal off by adding non-biotinylated versions of the lectins into the reaction. We used 1 nM antibody and 0.1 nM of each biotinylated lectin and competed the signal off with the respective non-biotinylated lectins. Competition was successful (Figure 6); however, we were unable to use this type of experiment to determine a K_D because the exact concentration of the glycans on the antibody is unknown.

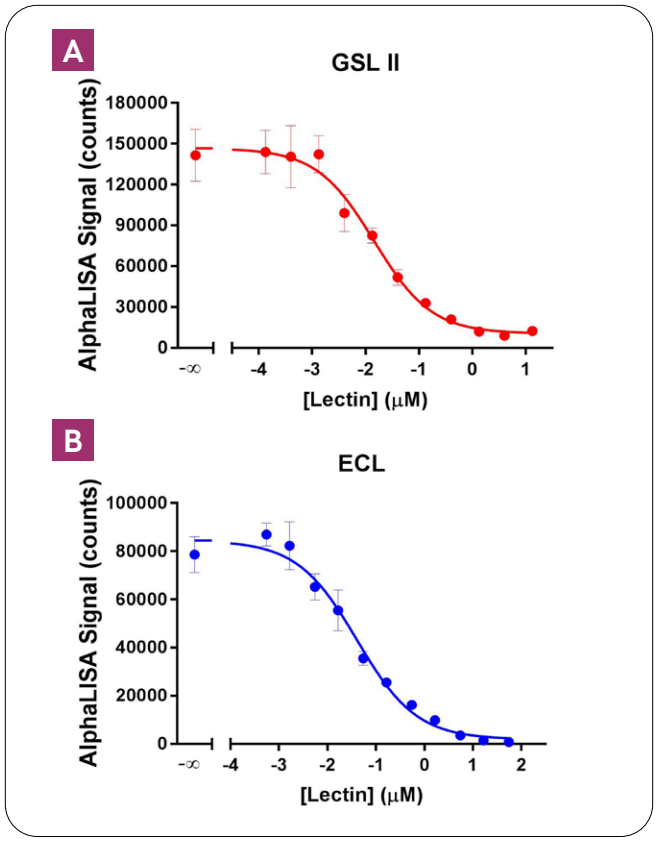


Figure 6: Specificity of interaction. Titration of unlabeled GSL II (A) or ECL (B) in the presence of 1 nM goat polyclonal antibody and 0.1 nM of biotinylated GSL II (A) or ECL (B).

Assay robustness

Finally, the robustness of this type of assay was tested using biotinylated ECL and the goat polyclonal antibody. The addition of 55.5 nM non-biotinylated ECL to the binding reaction was used to generate the low signal required to calculate a Z′. Figure 7 shows a Z′ of > 0.5 for this assay (Z′= 0.58). Therefore, the data presented here shows that AlphaLISA is a robust assay to detect weak interactions between biomolecules.

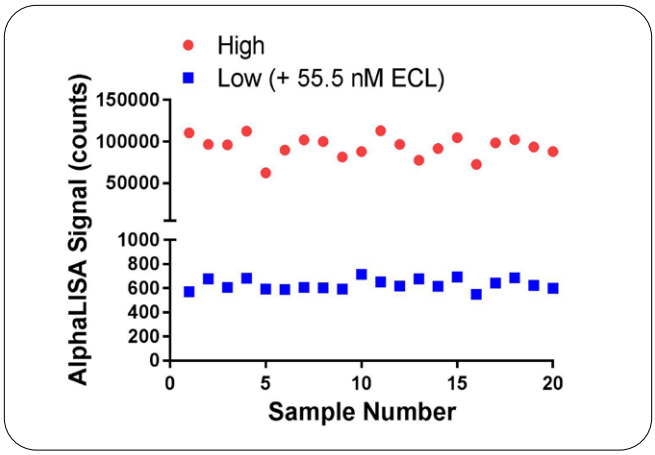


Figure 7: Robustness of assay (Z'). 0.1 nM of biotinylated ECL was incubated with 1 nM antibody (high signal, red). The same assay set-up was used for the low signal measurement except for the addition of 55.5 nM of non-biotinylated ECL (low signal, blue). Z'= 0.58.

Summary

In this technical note, we demonstrate the utility of AlphaLISA technology for detection of protein-protein interactions with weak affinities. Specifically, we show how to optimize an assay to detect the binding of glycans on an antibody to two different lectins. These assays could be used to profile the glycosylation state of a therapeutic antibody. The versatility and flexibility of AlphaLISA technology means that the user can measure weak interactions using a simple homogeneous assay platform.

References

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2. Nakamura-Tsuruta et al. Comparative analysis by frontal affinity chromatography of oligosaccharide specificity of GlcNAcbinding lectins, Griffonia simplicifolia lectin-II (GSL-II) and Boletopsis leucomelas lectin (BLL). J. Biochem (2006); 140:285-291.

3. Onitsuka et al. Rapid evaluation of N-glycosylation status of antibodies with chemiluminescent lectin-binding assay. Journal of Bioscience and Bioengineering (2015); 120(1): 107-110.

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