APPLICATION NOTE

Measuring strong to weak binding interactions with AlphaLISA.

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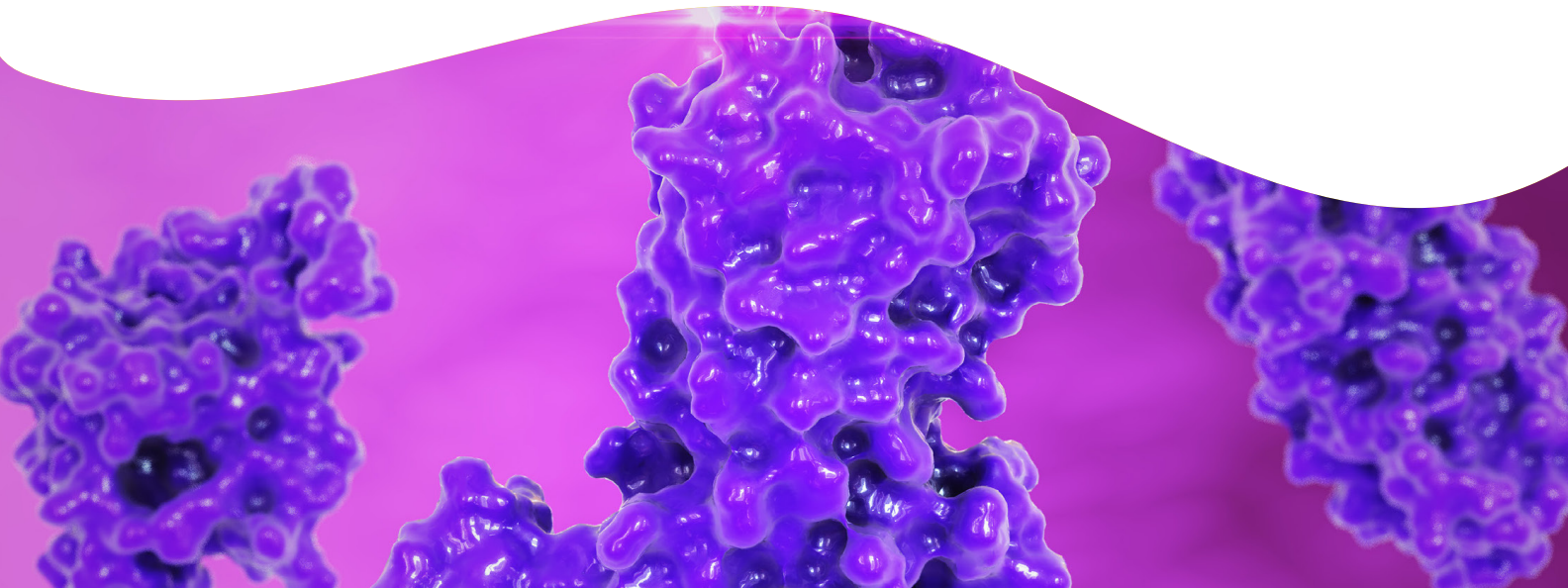
Introduction

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 commonly used to measure binding events *in vitro* (e.g. filtration assays, fluorescence polarization, ELISA-like assays, and TR-FRET). However, a limitation of these techniques is that they only have the utility of measuring binding events between proteins with a limited range of dissociation constants (K_D), often in the nanomolar range. Since biological processes involve binding events that range from very high (K_D < nM) to very low (K_D > µM) affinities, assay platforms that can be used to study a large range of binding affinities are extremely important for fully developing and 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. Since the assay is homogeneous (no wash steps required), transient interactions can easily be measured. Each bead has multiple binding sites, so 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). This allows the user to minimize the concentration of reagents required to perform the biomolecular interaction assay.

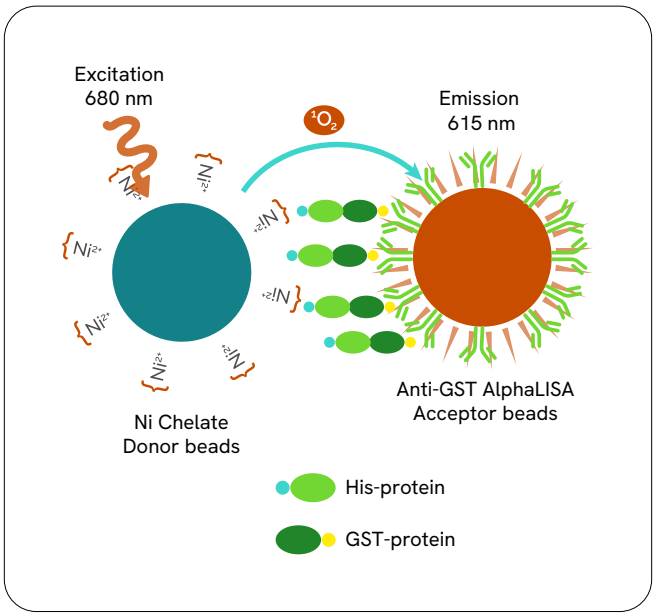


Figure 1: AlphaLISA bead-based technology enhances avidity between biomolecules.

In this application note, we demonstrate how AlphaLISA can be used to measure three very different types of biomolecular interactions with three very different binding affinities: TDP-43 protein binding to TAR-32 DNA oligo (K_D < 1 nM), MDM2 protein binding to p53 protein (K_D= 0.3 µM), and two different lectins binding a glycosylated antibody (K_D > 10 µM).

Materials and methods

Instrumentation

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

TDP-TAR-32 binding assay (tight binding)

GST-tagged TDP-43 (Abnova, #H00023435-P02) + biotinylated TAR-32 (5′- CTG CTT TTT GCC TGT ACT GGG TCT CTG TGG TT-3′ synthesized by Integrated DNA Technologies) or biotinylated TAR-32 reverse complement (5′-AA CCA CAG AGA CCC AGT ACA GGC AAA AAG CAG-3′ synthesized by Integrated DNA Technologies) were added to a gray 384-well AlphaPlate™ (Revvity, #6005350) with anti-GST Acceptor beads (Revvity, #AL110) (20 µg/mL final) for 1 hour. Streptavidin Donor beads (Revvity, #6760002) (20 µg/mL final) were then added for a final 1-hour incubation.

For competition and Z′ assays, non-biotinylated TAR-32 + GST-tagged TDP-43 (50 ng/mL final) + anti-GST Acceptor beads (20 µg/mL final), + biotinylated TAR-32 (0.5 nM final) were added to the plate for 1 hour. Streptavidin Donor beads (20 µg/mL final) were then added and incubated for 1 hour prior to reading the plate.

MDM2-p53 binding assay (moderate binding)

GST-tagged MDM2 (Boston Biochem, #E3-202), His-p53 (Boston Biochem, #SP-405), and GSH AlphaLISA Acceptor beads (Revvity, #AL109) (20 µg/mL final) were added to the AlphaPlate and incubated for 1 hour. Then, Ni chelate Donor beads (Revvity, #AS101) were added and incubated for 1 hour prior to reading the plate. For competition assays, MDM2 (1 nM final), Nutlin-3a (SelleckChem, #S8059), and His-p53 (1 nM final) were added to the plate and incubated for 1 hour. Then, GSH AlphaLISA Acceptor beads (20 µg/mL final) were added for 1 hour. Finally, Ni chelate Donor beads (20 µg/mL final) were added and incubated for 1 hour prior to measurement. Z′ measurements used the same assay set-up as the competition format with 50 µM Nutlin-3a.

Glycosylated antibody-lectin binding assay (weak binding)

Biotinylated GSL II (Vector Labs, #B-1215) or ECL (Vector Labs, #B-1145) (5 µL) and a goat polyclonal antibody (Jackson Laboratories, #123-005-021) were added to an AlphaPlate and incubated for 30 minutes. Next, Protein G AlphaLISA Acceptor beads (Revvity, #AL102) (20 µg/mL final) were added and incubated for 1 hour. Finally, Streptavidin Donor beads (20 µg/mL final) were added and incubated for 30 minutes prior to measurement.

For Z′ determination, non-biotinylated ECL (Vector Labs, #L-1140) was incubated in the AlphaPlate with biotinylated ECL, antibody (1 nM final) and 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 prior to measurement.

Data analysis

The data were analyzed using GraphPad Prism® software. The binding curves were generated using nonlinear regression (assuming one binding site and no non-specific binding), 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).

Results and discussion

Using AlphaLISA to detect tight binding interactions

AlphaLISA can be used to measure tight binding interactions, such as those with K_Ds below 1 nM. Cassel et al. developed a robust AlphaScreen assay to screen for inhibitors of oligonucleotide binding to TDP-43 (TAR DNA binding protein 43).[1] The authors used an Alpha assay to screen more than 7000 compounds for inhibitors of oligonucleotide binding to TDP-43. They determined the K_D for TAR-32 binding to TDP-43 using both saturation binding curves (K_D = ~0.7 nM) as well as measuring association and dissociation rate constants and calculating a K_D from those values (K_D= ~0.4 nM). They also determined a Z′ value of 0.55 from their screen. Figure 2 shows a schematic of the AlphaLISA assay used in this application note, based on the AlphaScreen assay used by Cassel et al.

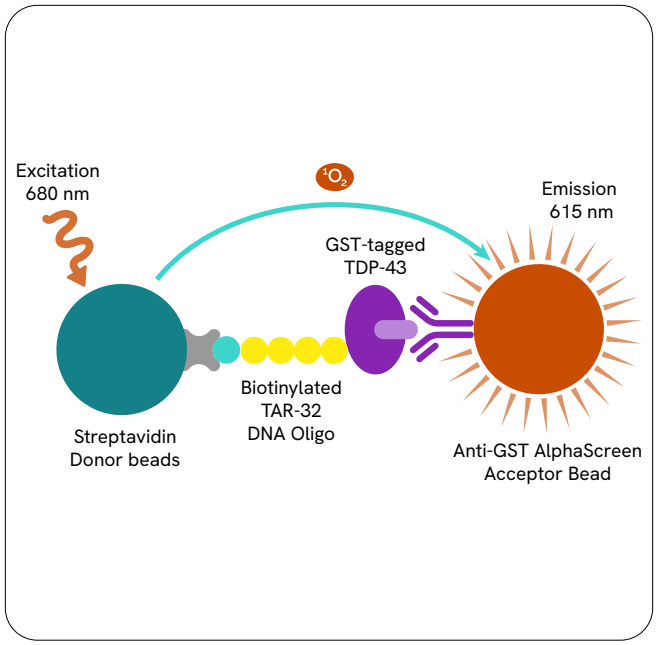


Figure 2: Detection of TDP-43 binding to TAR-32 DNA using AlphaLISA.

Using a slightly modified protocol (see technical note)[2], we measured the K_D for binding to the biotinylated TAR-32 DNA oligo using a saturation binding curve and confirmed specificity by showing no binding to a biotinylated reverse complement of TAR-32 DNA oligo (Figure 3A). We also show robustness of the AlphaLISA assay by calculating a Z′ of 0.89 using TDP-43 (50 ng/mL) bound to biotinylated TAR-32 (0.5 nM) in the presence or absence of competing 1 µM of a non-biotinylated TAR-32 in the binding reaction (Figure 3B). This system is an excellent example of how AlphaLISA can easily be used to measure very tight binding interactions and screen for inhibitors between a DNA oligo and a protein.

Using AlphaLISA to detect moderate binding interactions

AlphaLISA can also be used to investigate protein-protein interactions with intermediate binding affinities (e.g., K_D of 1 nM to 1 µM). It has previously been shown that p53 and MDM2 proteins bind with a dissociation constant (K_D) in the range of 300 nM.[3] We developed and optimized an AlphaLISA assay to look at this moderate protein-protein interaction shown in a previous technical note[4] (Figure 4). Figure 5 shows the initial cross-titration of the two proteins. We see excellent signal to background (> 500) at 10 nM MDM2 and 30 nM p53.

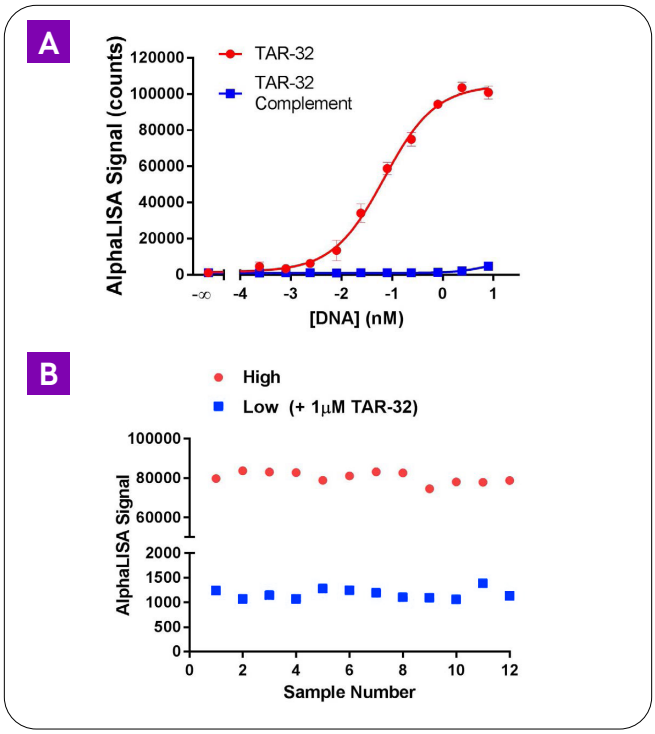


Figure 3: Saturation Binding and Specificity of the TDP-43/TAR-32 Interaction. A) Comparison between binding of a biotinylated TAR-32 oligo and a biotinylated reverse complement TAR-32 DNA oligo to TDP-43 protein (50 ng/µL. B) Robustness of TDP-43/TAR-32 Assay (n=12) by Z′ determination.

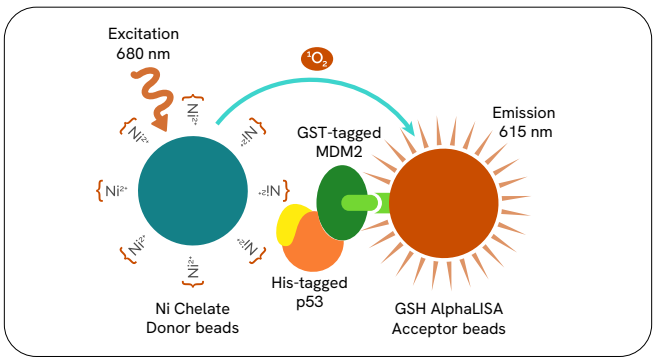


Figure 4: Detection of p53-MDM2 Interaction using AlphaLISA.

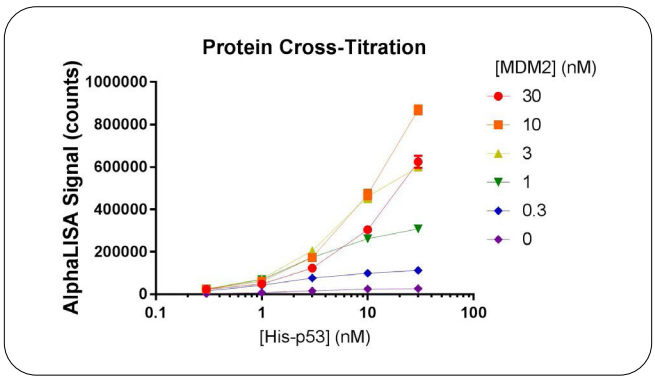


Figure 5: Protein cross-titration for p53-MDM2 binding assay.

Next, we show how this AlphaLISA assay can be used to screen for inhibitors of the interaction, such as Nutlin-3a. As shown in Figure 6A we see a competition curve indicative of disrupting this interaction. The IC_{50} for this assay at these concentrations was 1.3 µM. Finally, the robustness of the assay for screening was measured using 1 nM of each protein with or without 50 µM Nutlin-3a. Figure 6B shows that this assay has a high Z′ (0.70) value indicating its suitability for high throughput screening.

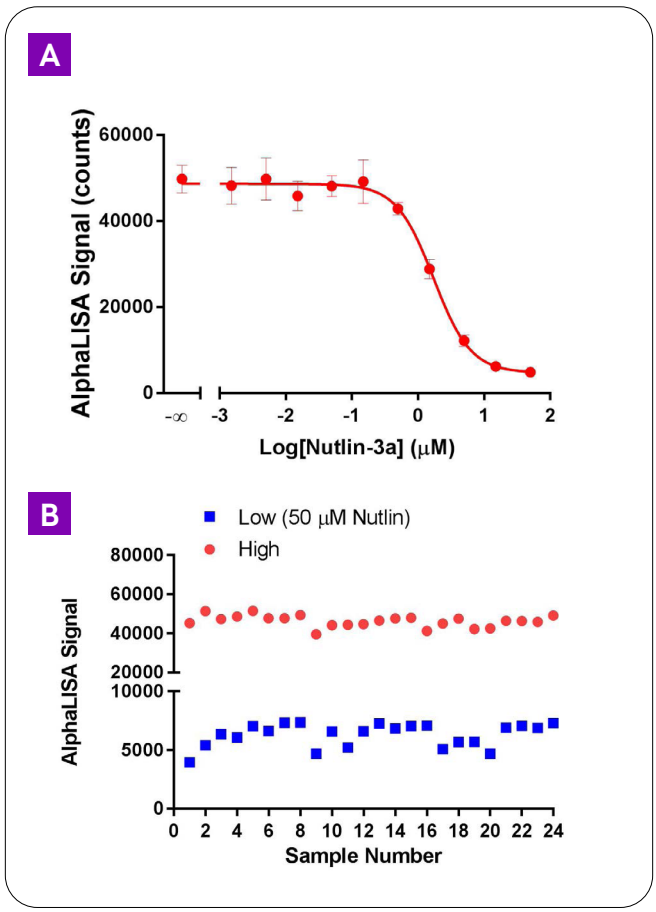


Figure 6: A) Specificity of AlphaLISA MDM2-p53 binding assay (inhibitor titration). B) Robustness of the AlphaLISA MDM2-p53 binding assay (Z′).

Using AlphaLISA to detect weak binding interactions

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 lectin binding to a glycoprotein. Certain N-linked glycans on glycosylated proteins 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.[5,6]

ECL binds galactosylated biantennary N-glycan with fucose (G2F) with a dissociation constant of 20 µM.[5] GSL II binds to βGlcNAc residues with a dissociation constant of 50 µM.[6] Onitsuka et. al utilized AlphaLISA to detect these known weak binding interactions and developed an AlphaLISA assay to detect specific types of glycosylation on antibodies.[7] They directly conjugated the antibody of interest to the AlphaLISA Acceptor beads. Here we modified their protocol by using Protein G beads to capture the antibody of interest and utilized the same biotinylated lectins. Figure 7 shows the schematic used to detect the glycosylation state of the antibody using AlphaLISA.

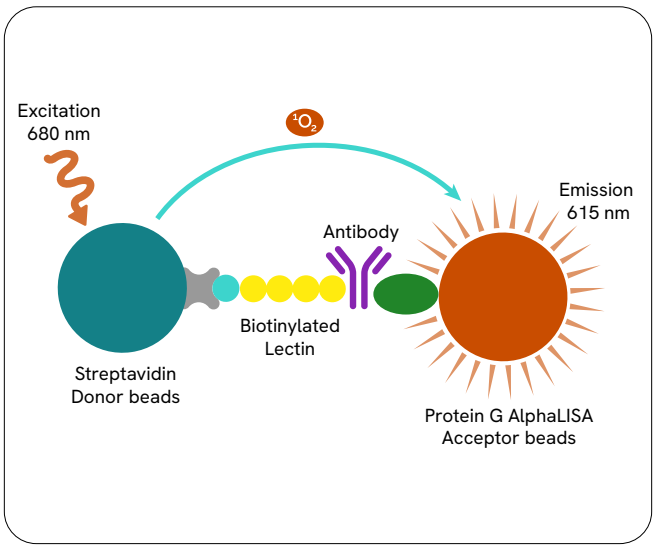


Figure 7: Detection of glycan binding lectins using AlphaLISA.

Using optimized conditions (see technical note)[8], we titrated a glycosylated goat polyclonal antibody to detect either galactosylated biantennary N-glycan with fucose (using biotinylated ECL) or βGlcNAc (using biotinylated GSL II). As shown in Figure 8A, we see a nice dose response for antibody titration detecting both glycans present on the antibody. To confirm specificity of the signal, we performed a competition assay by adding in non-biotinylated versions of the lectins. We were able to compete off the signal (see technical note).[8] 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 as the low signal to calculate a Z′. Figure 8B shows a Z′ of > 0.5 for this assay (Z′ = 0.58).

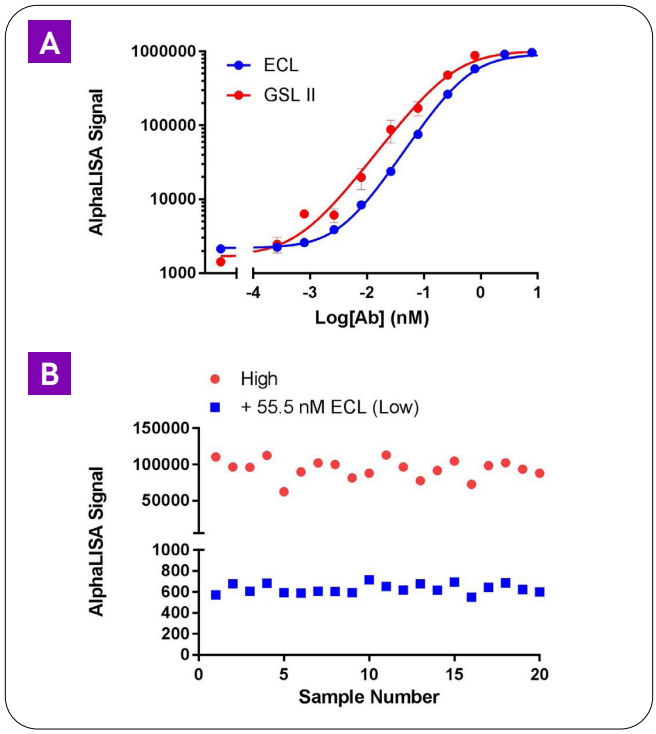


Figure 8: A) Full dose response curves using either biotinylated ECL to detect G2F or biotinylated GSL II to detect βGlcNAc on the goat polyclonal antibody. B) Robustness of glycan-binding assay (Z′). 0.1 nM of biotinylated ECL was incubated with 1 nM antibody or with the addition of 55.5 nM of non-biotinylated ECL. Measured Z′= 0.58.

Conclusions

AlphaLISA technology is a homogeneous bead-based assay platform that can be utilized to develop a variety of binding assays that provide robust S/B and high Z′ values. Here, we show three different AlphaLISA assays optimized to measure binding events between biomolecules with low, moderate, and high binding affinities. We confirmed previous data measuring tight binding affinities between a DNA oligo and a protein. We also show how AlphaLISA assays can be used to screen for inhibitors of protein-protein interactions of intermediate binding affinity. Finally, we demonstrate how weak binding interactions can be detected with AlphaLISA using low amounts of protein. The versatility and flexibility of AlphaLISA technology means that the user can screen inhibitors with a large range of affinities or measure different binding partners of a biomolecule with different binding affinities using the same assay platform.

References

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