

Development of an AlphaLISA assay to measure a DNA-protein interaction with a strong affinity.

Authors

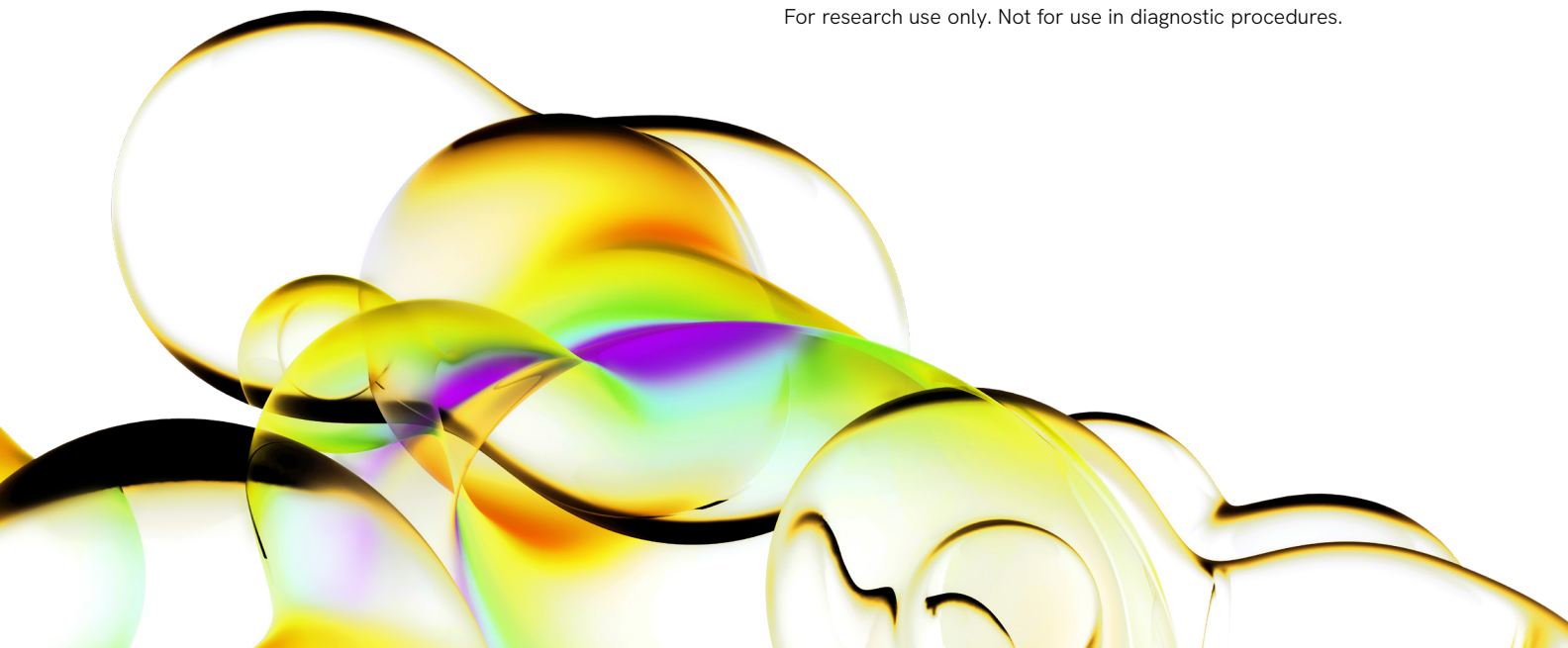
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Introduction

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. Specifically, transcription of many genes relies heavily on the interactions of transcription factors with DNA promoters and other DNA elements. Therefore, DNA-protein interactions are popular targets for the development of novel therapeutic drugs. There are a variety of assay formats to measure binding events; however, efficient, highly sensitivity assays that can be used to study a large range of binding affinities are extremely important for fully validating therapeutics drugs.

TDP-43 is a protein that regulates gene transcription by binding to TAR DNA sequences and functions as a repressor of HIV-1 gene transcription.¹ Cassel et al. developed a robust AlphaScreen™ assay to screen for inhibitors of oligonucleotide binding to TDP-43 (TAR DNA binding protein 43).² They determined the K_D for TAR-32 binding to TDP-43 using saturation binding curves ($K_D = \sim 0.7$ nM) as well as measuring association and dissociation rate constants and calculating a K_D from those values ($K_D = \sim 0.4$ nM). In Alpha assays, saturation binding curves can be used to measure the K_D for a tight binding interaction if all protein concentrations used to derive the K_D fall below the bead binding capacities of both beads. The authors used the Alpha assay to screen more than 7000 compounds for inhibitors of oligonucleotide binding to TDP-43. They also determined a Z' value of 0.55 from their screen.

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AlphaLISA™ and AlphaScreen are bead-bead homogeneous assay technologies 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 this technical note, we show how to easily develop a robust AlphaLISA assay that measures a tight binding interaction between a protein and a DNA oligonucleotide using the same model system as Cassel et al.²

Materials and methods

Instrumentation

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

Reagents

- N-terminal GST-tagged TDP-43 (TARDBP) protein (Abnova, #H00023435-P02)
- Biotinylated and non-biotinylated TAR-32 (5'- CTG CTT TTT GCC TGT ACT GGG TCT CTG TGG TT-3' synthesized by Integrated DNA Technologies)
- Biotinylated TAR-32 reverse complement (5'-AA CCA CAG AGA CCC AGT ACA GGC AAA AAG CAG-3' synthesized by Integrated DNA Technologies)
- Anti-GST Acceptor beads (Revvity, #AL110)
- Streptavidin Donor beads (Revvity, #6760002)
- AlphaPlate™-384, light gray (Revvity, #6005350)

AlphaLISA protocol

10 μ L of TDP-43 was incubated with 10 μ L of biotinylated TAR-32 or biotinylated TAR-32 complement and 10 μ L anti-GST Acceptor beads for 1 hour in 384-well AlphaPlates. Then 20 μ L of Streptavidin Donor beads were added and incubated for 1 hour. For competition and Z' assays, 5 μ L of 10X non-biotinylated TAR-32 were incubated with 10 μ L of GST-tagged TDP-43 (50 ng/mL final),

10 μ L of anti-GST Acceptor beads (20 μ g/mL final), and 5 μ L of biotinylated TAR-32 (0.5 nM final) for 1 hour. Then 20 μ L of Streptavidin Donor beads (20 μ g/mL final) were added and incubated for 1 hour.

Data analysis

The data were analyzed using GraphPad Prism® software. Binding curves were generated using nonlinear regression, using a 4-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

Assay optimization and K_D determination

The first step in developing an AlphaLISA assay is to decide on an assay set-up. For a GST-tagged TDP-43 protein and a biotinylated DNA oligo, there are a few different ways to set-up the AlphaLISA assay. For GST-tagged proteins, either anti-GST or glutathione AlphaLISA Acceptor beads can be used with streptavidin Donor beads. Alternatively, glutathione Donor beads could be used with streptavidin AlphaLISA Acceptor beads. Cassel et. al showed excellent data using streptavidin Donor beads and anti-GST AlphaScreen Acceptor beads.² We chose to use a similar assay setup, but with anti-GST AlphaLISA Acceptor beads for this technical note (Figure 1).

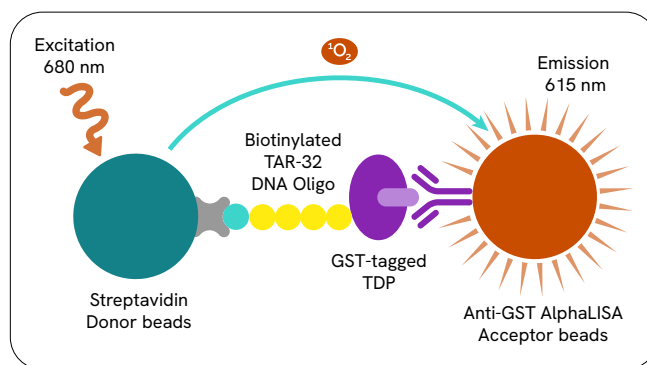


Figure 1: Detection of TDP-43 binding to TAR-32 DNA using AlphaLISA.

The order of addition and the timing are also parameters that can be optimized. For this assay, we chose a two-step assay, where the GST-TDP-43 protein, biotinylated TAR-32 DNA oligo, and anti-GST Acceptor beads were incubated for an hour and then the streptavidin Donor beads were added and incubated for an additional hour. This is a slight modification from the assay designed by Cassel et al. where the biotinylated DNA oligo was pre-incubated with the streptavidin Donor beads and the GST-TDP-43 was pre-incubated with the anti-GST AlphaScreen Acceptor Beads for 30 minutes and then the whole reaction incubated for 3 hours.²

The next step is to perform a cross-titration of the protein and the biotinylated DNA oligo. We chose four different GST-TDP-43 protein concentrations and titrated the biotinylated TAR-32 DNA oligo. As shown in Figure 2, we see a dose-dependent increase in Alpha signal as a function of biotinylated TAR-32 DNA. As the concentrations are well below the binding capacities for the streptavidin Donor beads and the anti-GST AlphaLISA Acceptor beads, the EC_{50} can approximate a dissociation constant (K_D). The values shown measured in Figure 2 are consistent with the data published.²

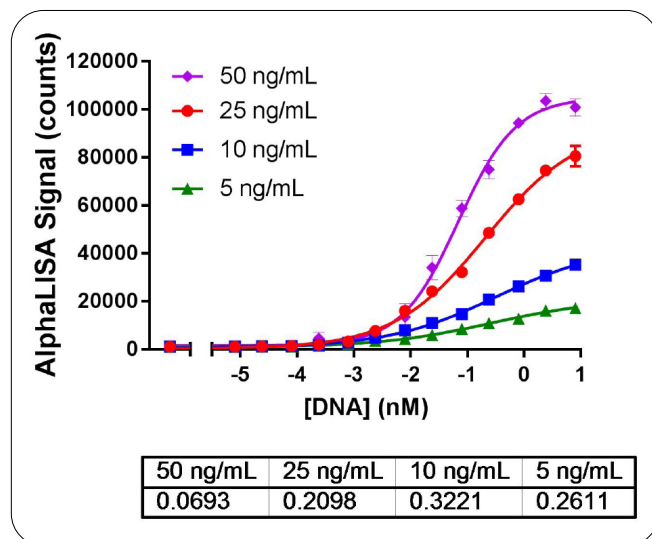


Figure 2: Saturation binding of four different concentrations of TDP-43 to TAR-32 DNA. The EC_{50} values at each protein concentration are listed in the table.

Specificity of the interaction

To confirm specificity of the signal, we tested binding to a biotinylated reverse complement of TAR-32 DNA oligo using 50 ng/mL of TDP-43 protein. The TDP-43 protein has a high affinity for TG/UG-rich DNA and RNA oligonucleotides and therefore, the reverse complement should have little to no binding at the same low concentrations as the TAR-32 DNA oligo.^{1,2} As shown in Figure 3, when compared with the TAR-32 DNA, we see no specific binding of the reverse complement TAR-32 DNA. We next showed specificity of the signal by performing a competition assay. The binding curve using 50 ng/mL TDP-43 gave excellent signal to background of 85 at the EC_{85} for the reaction (0.5 nM biotinylated TAR-32). Therefore, using the EC_{85} for the binding reaction, we successfully competed off the signal with a non-biotinylated version of TAR-32 DNA (Figure 4). This is consistent with the previously published data comparing the ability of a variety of different oligos to compete with the interaction.²

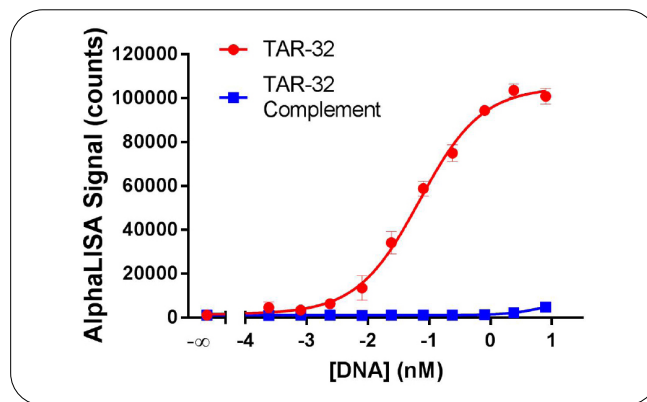


Figure 3: Specificity of the TDP-43-TAR-32 interaction. Comparison between binding of a biotinylated TAR-32 oligo and a biotinylated reverse complement TAR-32 DNA oligo to TDP-43 protein (50 ng/ μ L).

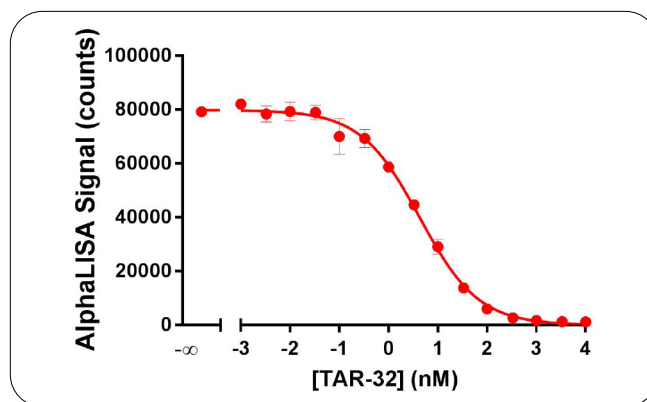


Figure 4: Specificity of the TDP-43-TAR-32 interaction. Competition of a non-biotinylated TAR-32 DNA oligo using 50 ng/ μ L (final) TDP-43 and 0.5 nM (final) biotinylated TAR-32 DNA.

Assay robustness

Finally, we show robustness of the AlphaLISA assay by calculating a Z' using TDP-43 (50 ng/mL) bound to biotinylated TAR-32 (0.5 nM) in the presence of absence of 1 μM of a non-biotinylated competing TAR-32 (Figure 5). We calculated a Z' of 0.89 for this assay set-up with a signal to background of 69. This demonstrates 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.

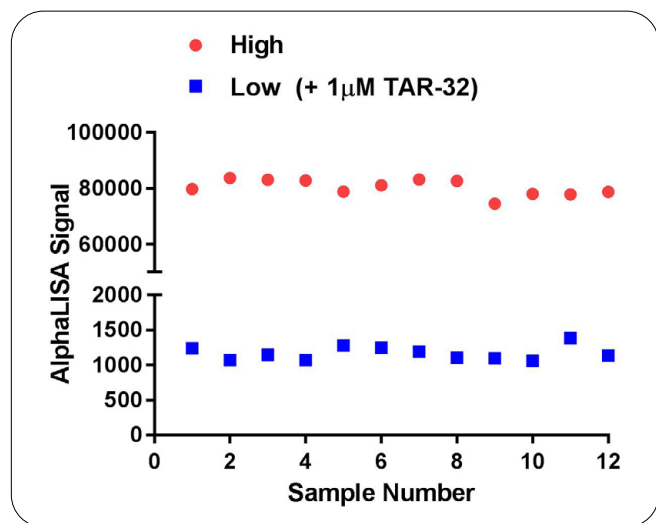


Figure 5: Robustness of TDP-43-TAR-32 assay (n=12). Z' value is 0.89 with a signal to background value of 69.

Summary

In this technical note, we demonstrate how AlphaLISA technology can be used for setting up assays that measure and screen for inhibitors of a tight binding interaction between a DNA oligonucleotide and a protein. We confirmed previous data measuring a tight binding interaction between a DNA oligo (TAR-32) and a protein (TDP-43). We also show how carefully designed assays can be used to measure dissociation constants (K_D) for DNA-protein binding interactions. The versatility of AlphaLISA technology allows the user the ability to utilize the same assay whether screening inhibitors with a large range of affinities or looking at a variety of binding partners of a single biomolecule.

References

1. Ou SH et al. Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type I TAR DNA sequence motifs. *J. Virol* 1995; 69: 3584-3596.
2. Cassel et al. Development of a novel nonradiometric assay for nucleic acid binding to TDP-43 suitable for high-throughput screening using AlphaScreen™ technology. *Journal of Biomolecular Screening* 2010; 15(9): 1099-1106.

