

Utilizing AlphaLISA technology to screen for inhibitors of the CTLA-4 immune checkpoint.

Authors

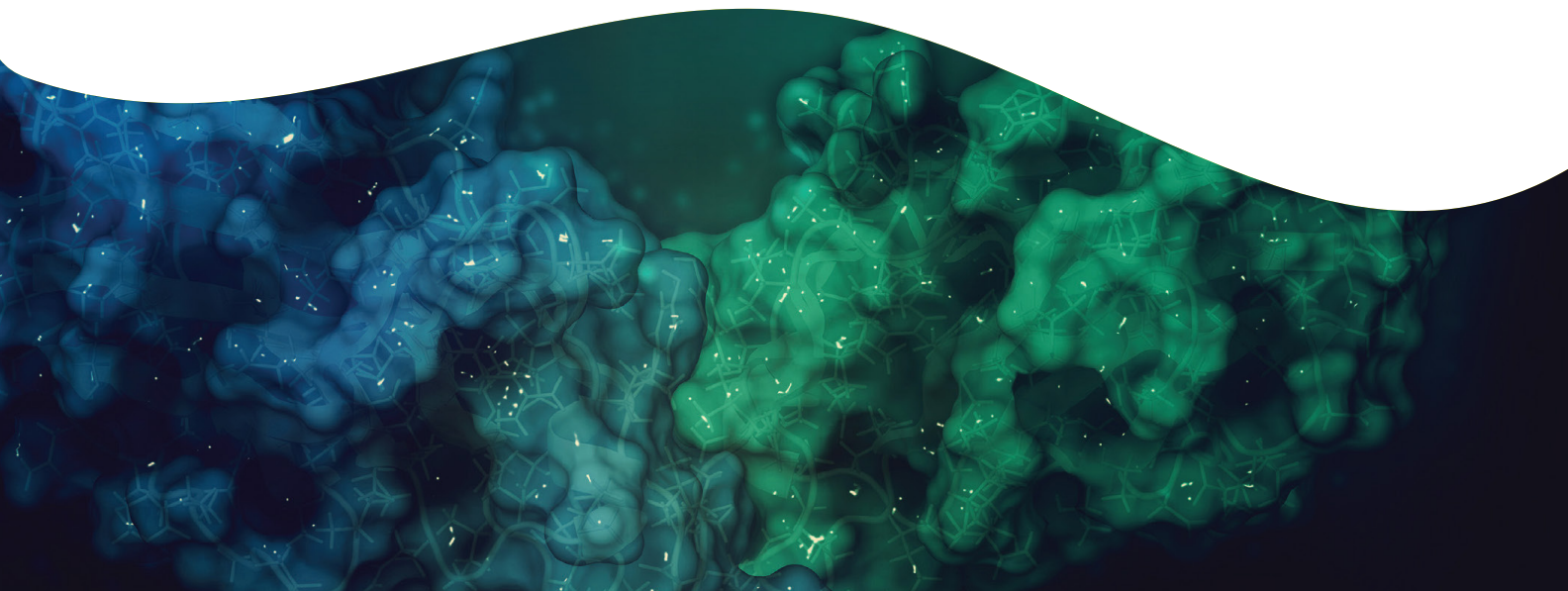
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Introduction

Immune checkpoints serve a critical role in the immune system to prevent autoimmunity and manage the degree and duration of an immune response. One immune checkpoint of significant interest involves the inhibitory (anti-inflammatory) transmembrane protein cytotoxic T-Lymphocyte-associated protein 4 (CTLA-4, or CD152) which competes with another transmembrane protein, CD28. CD28 is a co-stimulatory (pro-inflammatory) receptor present on T-lymphocytes, and is activated by binding to its membrane ligands CD80 and CD86 on antigen presenting cells. However, CTLA-4 also binds to CD80 and CD86, with a greater affinity, where CTLA-4 outcompetes CD28 for ligand binding. When CTLA-4 is expressed (after T-lymphocyte activation) and competes with CD28 for its ligands, the immune system response is downregulated. As a result of this immune system response balance, immune checkpoints provide an opportunity for therapeutic intervention to modulate immune system activity.

There is high demand for novel immune checkpoint inhibitors. Currently, only a few FDA-approved immune checkpoint drugs exist while only one targets CTLA-4, Ipilimumab. This particular method of treatment is called immunotherapy. In the case of some cancers, tumors gain the ability to modulate immune checkpoints and evade the immune system's natural tumor defense. Immunotherapy works by precisely controlling immune system responses through inhibiting specific immune checkpoints, leading to increased immune system activation and anti-tumor response. It was demonstrated that a CTLA-4 blockade led to increased T-lymphocyte infiltration at the tumor site in more than half of the biopsy specimens tested¹.



These findings help explain why metastatic melanoma patients undergoing immune checkpoint treatments experience improved survival rates². As a result there is immense interest in discovering novel drugs to modulate the CTLA-4 immune checkpoint and others.

There is significant need to discover new drugs to block CTLA-4 and modulate immune system activity, but the necessary tools are required. Amplified luminescence proximity homogeneous assay (Alpha) technology is a highly useful tool to quickly and precisely screen many potential drug candidates for novel CTLA-4 immune checkpoint inhibitors. Alpha technology allows for the robust detection of protein-protein interactions using a simple and homogeneous no-wash format. Figure 1 provides an example of AlphaLISA™ technology, where streptavidin-conjugated Donor beads bind to biotinylated CD80 molecules and Anti-6xHIS Acceptor beads bind 6xHIS labeled CTLA-4 molecules. When both CD80 and CTLA-4 molecules are present, they bind together and bring the Donor and Acceptor beads within close proximity. Upon excitation with a 680 nm laser, the Donor beads generate singlet oxygen molecules that transiently diffuse in solution to activate nearby Acceptor beads, which through a series of reactions emit light at 615 nm. The light emission (AlphaLISA signal) is then detected on an Alpha-enabled microplate reader.

Herein, AlphaLISA technology was demonstrated in a screen for novel CTLA-4 blocking drugs by utilizing the AlphaLISA CTLA-4/CD80 binding assay. First, to demonstrate the binding assay, a titration of CTLA-4 was performed with a fixed amount of CD80 protein. Further, unlabeled proteins CTLA-4, CD80, and a negative control were used to block CTLA-4/CD80 binding. Next, a variety of antibodies were tested to try to block CTLA-4/CD80 binding. Utilizing a successful blocking antibody, Ipilimumab, different buffers, media and DMSO were tested to show the effect on Alpha technology performance. Finally, the Z-prime (Z') was determined using an anti-CD80 antibody to demonstrate the exceptional quality of the assay for drug screening.

Materials and methods

Antibodies and other reagents

Seven antibodies were tested:

- Ipilimumab, a fully humanized, FDA-approved anti-CTLA-4 monoclonal antibody (*In vivo*Gen #hctla4-mab1)
- Human IgG1 isotype control (Biolegend #12160)
- Anti-CD80, anti-CD86, and the corresponding isotype control (R&D Systems #MAB140, #MAB141 and #MAB002, respectively)
- Anti-mouse CTLA-4 and the corresponding Syrian hamster IgG (Biolegend #106202 and #402002, respectively)
- Non-6xHIS labeled CTLA-4-Fc (R&D Systems #7268-ct)
- Non-biotinylated CD80-Fc (BPS Bioscience #71125)

All antibodies and proteins were reconstituted and stored according to the data sheets provided by the supplier.

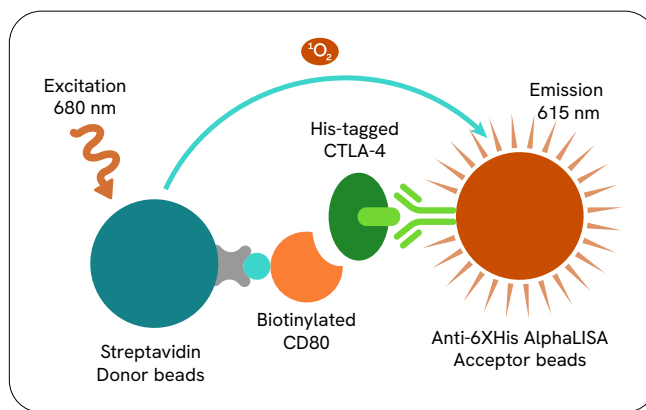


Figure 1: AlphaLISA CTLA-4/CD80 binding assay schematic. When both the biotinylated CD80 and 6xHIS tagged CTLA-4 bind, the Donor beads and Acceptor beads are brought within close proximity. Upon excitation, singlet oxygen from the Donor beads diffuses and activates nearby Acceptor beads to generate light that is proportional to the amount of binding.

AlphaLISA CTLA-4/CD80 binding assay

AlphaLISA binding assay for CTLA-4/CD80 (Revvity #AL3046) was performed according to the recommended protocol provided with the kit (Figure 2). Each kit supplies the necessary Acceptor beads, 6xHIS labeled CTLA-4, biotinylated CD80, Donor beads, and the recommended assay buffer to perform the assay. Lyophilized proteins were prepared in the appropriate diluent according to the protocols. To perform the assays, 10 µL of drug dilution, or sample to be tested, was added to each well of a 384-well OptiPlate (Revvity #6007290). Then 10 µL of 6xHIS tagged CTLA-4 was added, followed by the addition of 10 µL of biotinylated CD80, to each well. Finally, a mixture of 10 µL of Anti-6xHIS Acceptor beads and Streptavidin Donor beads were added to each well of the plate under subdued light or under green filters (Rosco 389) to prevent photobleaching. The plate was covered with TopSeal-A-PLUS (Revvity #6050185), covered with a black lid (Revvity #6000027), and incubated for 90 minutes at room temperature prior to measuring AlphaLISA signal on an Alpha-enabled microplate reader. Plates can be read with or without the TopSeal-A-PLUS with no measureable difference. If the solutions do not appear to be settled at the bottom of the well (i.e. droplets on well wall), the plate can be gently tapped or briefly centrifuged at 500 x g for about 10 seconds.

Instruments

All Alpha assays were measured on the Alpha-enabled EnVision™ multilabel plate reader (Figure 3), using the 640as mirror module (#444) and the M570w emission filter (#244). Standard Alpha measurement settings were used: total measurement time 550 ms and excitation time 180 ms. Importantly, white 384-well OptiPlates (Revvity #6007290) were used for all assays. The specific plate type should be selected in the Alpha protocol settings in the EnVision software, which is located under Select Protocol → Protocol-General settings → Plate type → 384 OptiPlate.

Data analysis

All curves were plotted in GraphPad Prism version 7.0 fitting nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) with 1/Y² weighting method. IC₅₀ and IC₈₀ values were calculated using the GraphPad Prism software. The Z-prime was calculated between the IC₈₀ and the max inhibition values.

$$Z\text{-prime (Z')} = 1 - \left[\frac{3 \times (\text{standard deviation of IC}_{80} + \text{standard deviation of max inhibition})}{|\text{mean of IC}_{80} - \text{mean of max inhibition values}|} \right]$$

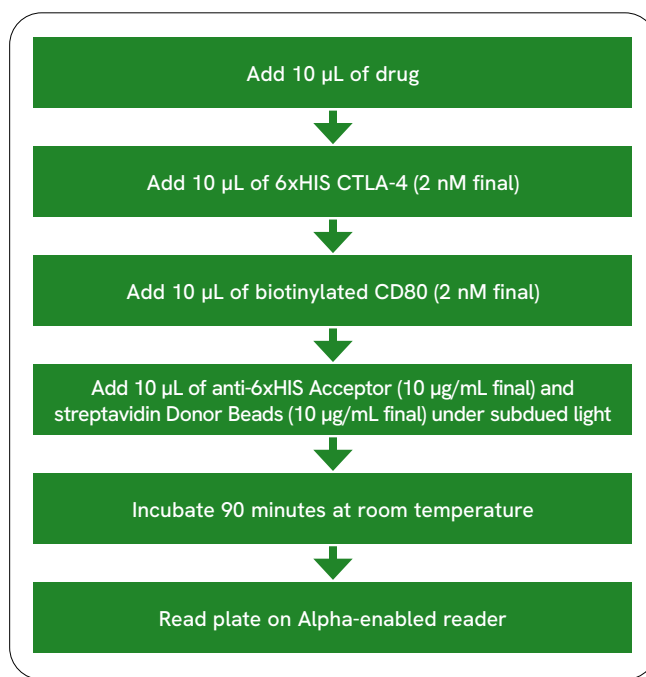


Figure 2: CTLA-4/CD80 binding assay potocol workflow.



Figure 3: EnVision multilabel plate reader.

Results

Demonstration of CTLA-4/CD80 binding assay functionality

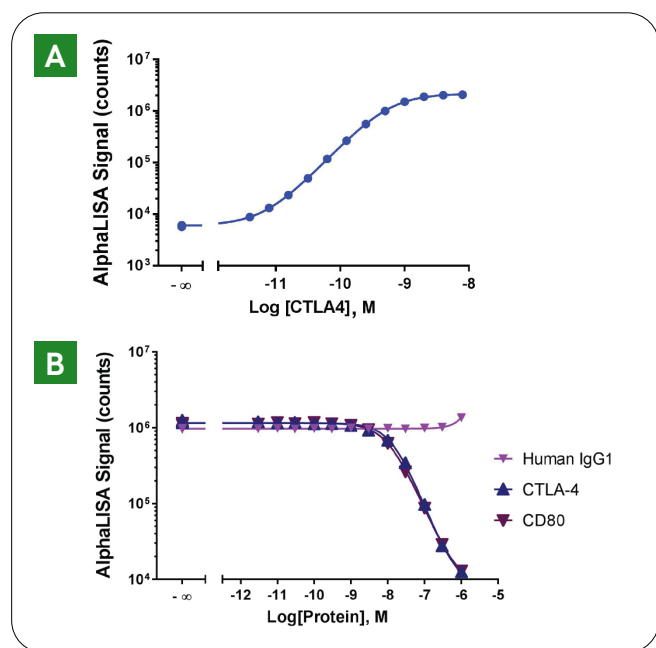


Figure 4: Titration of CTLA-4 and use of unlabeled proteins demonstrate the functionality of the protein-protein binding assay. A: A range of concentrations of 6xHIS CTLA-4 were tested with 2 nM biotinylated CD80. The increase in signal is proportional to the quantity of binding between CTLA-4 and CD80. B: Inhibition curves are shown for a range of unlabeled CTLA-4, CD80, and a human IgG1 control. Proteins were titrated to displace the binding between the labeled CTLA-4 and CD80 molecules. Biotinylated CD80 and CTLA-4 were used at the optimal concentrations of 2 nM.

AlphaLISA technology is a straightforward and robust bead-based immunoassay technology that utilizes a homogeneous format that does not require washing. Assays are quick and amenable to small and large scale screening procedures. The binding kits are validated, optimized and designed to aid in the discovery or confirmation of drugs by affirming specific blocking mechanisms. To demonstrate the performance of the CTLA-4 and CD80 binding kit, 2 nM of biotinylated CD80 was used with a titrated amount of 6xHIS CTLA-4 from 8 nM down to 3.9 pM. The curve in Figure 4A displays a sigmoidal binding curve where 50% and 90% maximal response were achieved at 0.55 nM and 2 nM of CTLA-4, respectively, relative to the 2 nM biotinylated CD80 protein used. To further demonstrate the kit's functionality, the binding assay was performed using the optimized and recommended concentrations of 6xHIS CTLA-4 (2 nM) and biotinylated CD80 (2 nM). The protein-protein interaction was then exposed to unlabeled CTLA-4 and CD80 molecules or a Human IgG1 control antibody. The human IgG1 antibody

did not have any effect on the CTLA-4/CD80 binding represented by the flat line in Figure 4B, however, unlabeled CTLA-4 and CD80 effectively competed with the labeled proteins and inhibited AlphaLISA signal when a labeled molecule was displaced. IC₅₀ values for unlabeled CTLA-4 and CD80 were 14.2 nM and 11.1 nM, respectively.

Only CD80- and CTLA-4-specific antibodies blocked the binding of CTLA-4/CD80

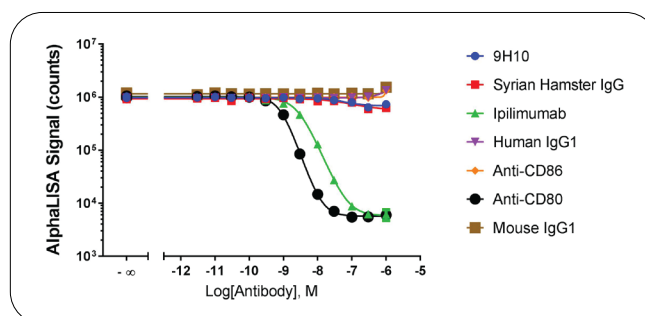


Figure 5: Antibodies targeting either CTLA-4 or CD80 potentially inhibited binding between the proteins. Inhibition curves were performed for seven different antibodies. The maximum concentration tested was 1 μM. Antibodies were diluted in half-log increments to reach as low as 3 pM. The associated isotype controls were used for each antibody tested. 6xHIS CTLA-4 and biotinylated CD80 were used at 2 nM final concentrations. All dilutions were performed in Universal buffer.

Several known antibodies were tested to potentially block CTLA-4/CD80 binding. This experiment was designed to mimic the screening of unknown proteins and small molecules. To assess whether the CTLA-4/CD80 binding assay can identify specific antibodies to block this interaction, the assay was performed using the optimized concentrations of 2 nM 6xHIS CTLA-4 and 2 nM biotinylated CD80. For positive control antibodies, an anti-CD80 antibody and an anti-CTLA-4 (Ipilimumab) antibody were used. Further, several suspected negative controls were selected: anti-CD86, anti-mouse CTLA-4 and associated mouse IgG1 isotype (anti-CD80 and anti-CD86 control), human IgG1 isotype (Ipilimumab control) and the Syrian hamster IgG (anti-mouse CTLA-4 control) isotype controls. The antibodies were titrated in half-log increments from 1 μM to 3 pM. As expected, both Ipilimumab and anti-CD80 potentially inhibited CTLA-4 and CD80 binding in a dose-response fashion. The observed IC₅₀ values for Ipilimumab and anti-CD80 were 2.8 nM and 0.86 nM, respectively. Further, each of the suspected negative controls did not show any blocking of CTLA-4/CD80 binding. This supports the specificity of this protein-protein interaction assay in discovering drugs that inhibit only this binding interaction (Figure 5).

Solvent controls are necessary to limit false positives

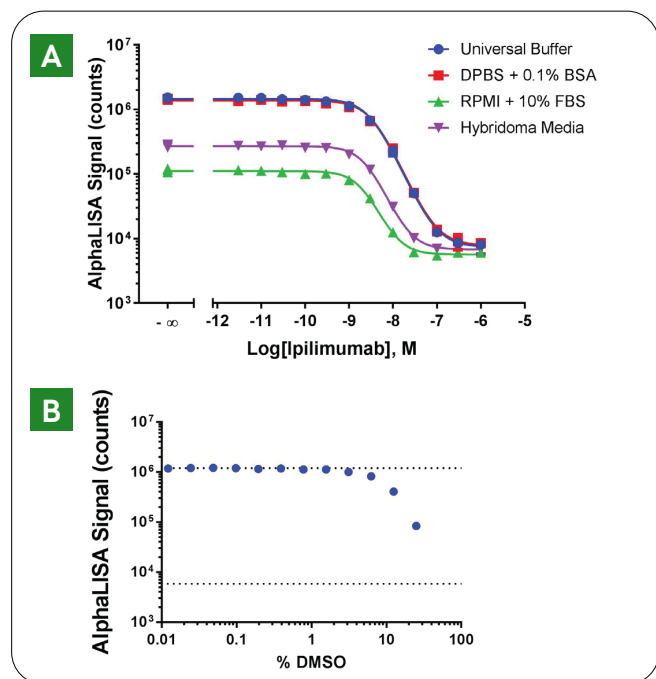


Figure 6: Drugs prepared in different buffers, media, or solvents must have the necessary controls to prevent false positives. A: Different buffers and media may have highly variable effects on AlphaLISA signal counts. Inhibition curves were performed using 2 nM biotinylated CD80 and 2 nM 6xHIS CTLA-4. Ipilimumab dilutions were prepared in AlphaLISA Universal buffer, DPBS + 0.1% BSA, RPMI + 10% FBS and Hybridoma media. B: DMSO can inhibit Alpha assays. DMSO tolerance was assessed by testing a range of DMSO concentrations from 25% to 0.01% final.

Drugs can be prepared in a variety of different buffers, media, or solvent. Each of the different solutions may perform slightly or very differently with Alpha technology. It is critically important to ensure any of these differences are taken into account to minimize the chances of false positives. In order to show how different solutions affect Alpha technology, the CTLA-4/CD80 assay was performed using 2 nM of each protein. Ipilimumab dilutions were prepared in AlphaLISA Universal buffer, DPBS + 0.1% BSA, RPMI + 10% FBS, or Hybridoma media. It is important to note for this assay that all protein and bead dilutions were performed in 1x AlphaLISA Universal Buffer (provided in the kit). By comparing the maximum counts (uninhibited signal) between the different media and buffer tested, AlphaLISA Universal Buffer gave the highest counts (~1.5 million), while DPBS + 0.1% BSA, Hybridoma media, and RPMI + 10% FBS were reduced by ~6%, 82%, and 92%, respectively. Thus, when performing a drug screen, it is important to prepare negative controls in all of the drug solutions used. Although alterations to the maximum counts may be observed, it is important to highlight that the IC₅₀ for ipilimumab remained unchanged in

the different solutions tested. Furthermore, a very common solvent used for small molecule drug screens, dimethyl sulfoxide (DMSO), can inhibit Alpha assays when used at high concentrations. To determine the binding assay's tolerance, DMSO was titrated from 25% to 0.01% final concentration in the well. As shown in Figure 6B the assay was very tolerant to DMSO up to a concentration of 1.6% DMSO (final) in the well where only about 6% inhibition of maximum signal was observed. Thus, in a 10 µL addition, compounds may contain up to 6.4% DMSO and only minimally affect Alpha signal, however, it is still important to have the proper controls to represent any variations caused by DMSO alone.

CTLA-4/CD80 binding assay exhibits Z-prime > 0.8

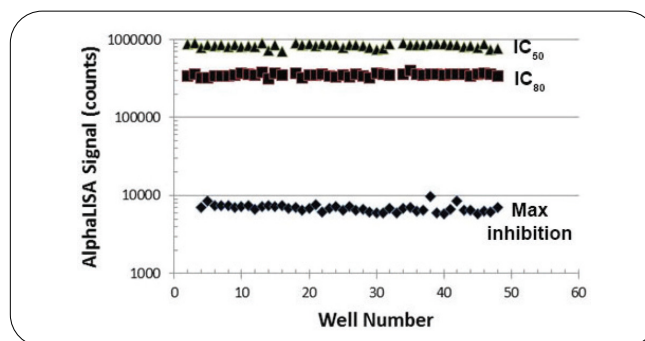


Figure 7: The CTLA-4/CD80 binding assay demonstrated excellent Z' value. Three different concentrations (0.86, 1.8, and 100 nM) of anti-CD80 antibody were tested with 48 well replicates. Z' was calculated using the IC₅₀ or IC₈₀ values with the maximum inhibition values.

A generally accepted method of assessing the overall quality of a screening assay is to calculate its Z-prime value (Z'). The Z' values of an assay fall into different quality ranges: Z' < 0 is a poor quality assay, Z' between 0 - 0.5 is a marginal assay, while the most desired is a Z' range between 0.5 - 1.0, which is representative of an exceptional quality screening assay. The Z' value is important because a Z' that approaches 1.0 indicates an assay that is better at distinguishing between true drug hits and normal assay variability. To determine the Z' for the CTLA-4/CD80 binding kit, the assay was performed using the recommended biotinylated CD80 (2 nM) and 6xHIS CTLA-4 (2 nM) concentrations and 1x Universal Buffer for the dilutions. The binding was inhibited to different degrees using an anti-CD80 antibody. In each case, 48-wells were used to calculate the IC₅₀, IC₈₀, and maximum inhibition, which were tested using 0.86 nM, 1.8 nM, and 100 nM of anti-CD80 antibody in 10 µL. The Z' at the IC₅₀ and IC₈₀ were calculated to be 0.83 and 0.85, respectively (Figure 7). The Z' values fell well within the 0.5 - 1.0 range and thus the assay can be considered of excellent quality when used to screen for potential drugs.

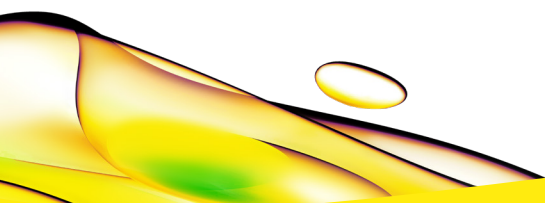
Conclusion

In this application note, AlphaLISA technology was used to screen for potential compounds that inhibit the CTLA-4 immune checkpoint. First, basic functionality of the binding assay was demonstrated by showing that increasing protein concentrations correlated with protein-protein binding assay signal. The assay was shown to be specific, where only drugs targeting CTLA-4, CD80 or the addition of unlabeled proteins led to the inhibition of the binding signal. It was demonstrated that it is critical to have proper controls for different buffer, media, and solvents in order to reduce the occurrence of false positives. Next, the binding assay was shown to be tolerant up to 1.6% DMSO final concentration. Finally, the binding assay was shown to be of excellent quality, with a $Z' > 0.8$.

AlphaLISA is a no wash and robust assay technology that is also compatible with liquid handling equipment, allowing different scales of high throughput screening. Therefore, the AlphaLISA CTLA-4/CD80 binding assay can provide the means to quickly and accurately screen and assess a variety of potential immune checkpoint inhibitors.

References

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