APPLICATION NOTE

Rapid no wash assays for characterizing a mouse TIGIT/ PD-L1 bispecific antibody.

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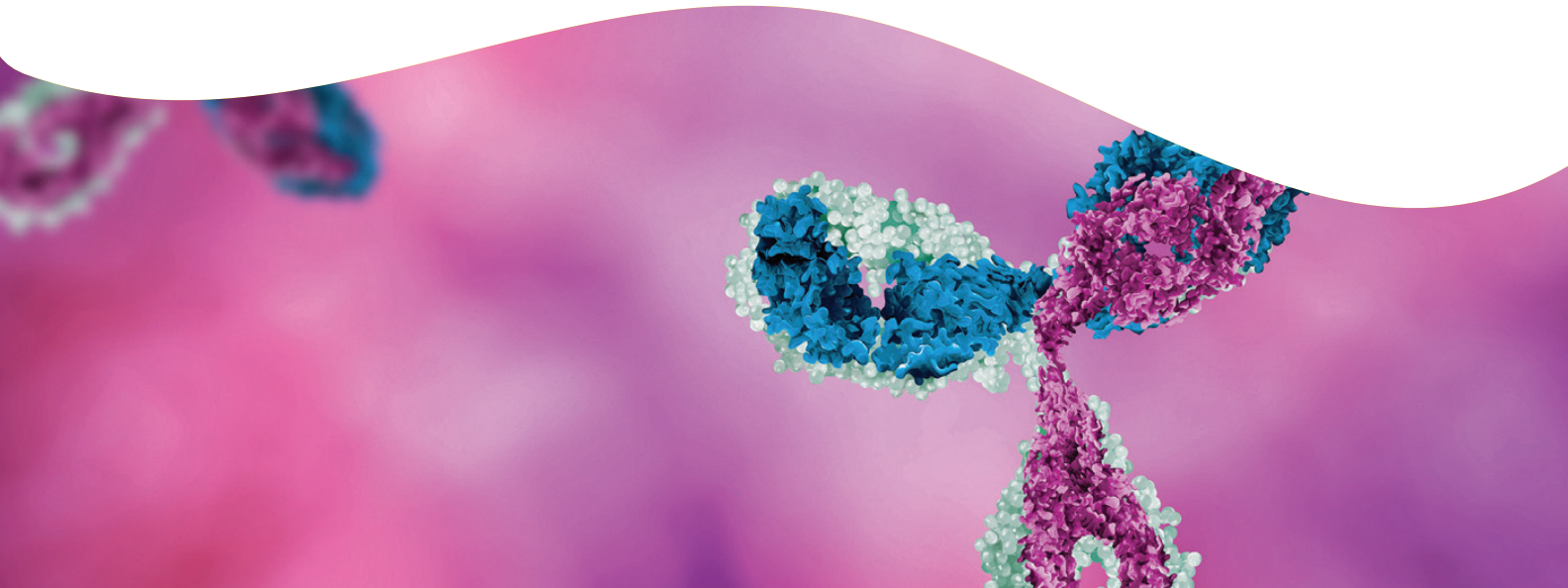
Introduction

Therapeutic antibodies as a drug class has been growing over the last decade, where the world's top selling therapeutics are antibody drugs such Pembrolizumab and Adalimumab. These antibody therapeutics are what some would consider as classically structured antibodies composed of identical heavy and light chain variable domains on both antigen binding sites. This approach to monoclonal antibody drug discovery and development has been the paradigm for the last 30 years. Recently, technical advancements in antibody engineering has brought about greater interest in more novel antibody therapeutic design. Blinatumomab (approved in 2015) and emicizumab (approved in 2018) are new classes of antibody therapeutics called bispecific antibodies (bsAbs). Unlike classical antibody formats in which the heavy and light chain are identical on both antigen binding sites, bsAbs are engineered to have two separate antigen binding sites. They can be fragment-based such as blinatumomab which is a bispecific T-cell engager (BiTE®) targeting CD19 and CD3 for treatment of advanced acute lymphoblastic leukemia (Kantarjian et al.); or a full monoclonal bispecific antibody such as emicizumab targeting factor X and factor IX for treatment of Hemophilia A (Oldenburg et al.).

The principle behind bispecific antibody design is to create an antibody / antibody fragment to two or more binding sites to help with the treatment of complex diseases. These therapeutic antibodies can bind two different antigens expressed on two different cells, bringing those cells together as an effective treatment. For example, a bispecific antibody targeting a receptor



on a cytotoxic cell and one on a tumor cell can be used to bring the cytotoxic cell near the tumor cell, allowing for faster targeting of the tumor cell for cell death. There are also bispecific antibodies that can simultaneously bind and interfere with two different receptor-ligand interactions for a more effective blockage of proliferative or angiogenesis-associated pathways. Currently, there are a few bispecific antibody drugs on the market, however these molecules are entering the clinical pipeline faster and use patented platforms such as bispecific T-cell engager (BiTE®), Duobody, Dual variable-domain antibody (DVD-Ig), Dual affinity retargeting (DART), and Trispecific Killer Engager (TriKE) - just to name a few (Suurs et al.) (Figure 1).

Rapid no-wash type assays have been previously used to characterize bispecific antibodies during the discovery process (Mazor et al.). As more bsAbs are produced as therapeutics, fast and accurate methods for functionally evaluating and characterizing the stability of these antibodies are necessary during both discovery and development stages, as well as during formulation and quality analysis.

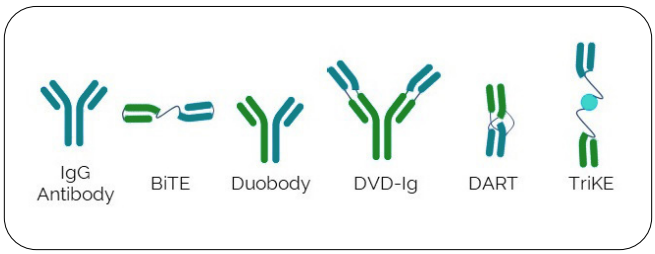


Figure 1. Examples of some bsAb constructs currently approved or in clinical trials as compared with an IgG antibody.

In this application note, we demonstrate how to characterize the binding and specificity of a mouse bispecific antibody targeting mouse TIGIT and mouse PD-L1. Both TIGIT and PD-L1 play an important role in immune suppression of cancer cells. In fact, antibodies to TIGIT and PD-L1 have both been shown to suppress tumor progression (Harjunpää et al.; Sun et al.) and recently, anti- TIGIT and anti-PD-L1 antibodies have shown promising results in combination therapy (Harjunpää et al., Grapin et al.). We show how to design quick and easy experiments to measure binding of the bispecific antibody to the target proteins. In addition, we developed a no-wash multiplex assay to show direct binding of each site to its respective target simultaneously in one well. Finally, using forced degradation experiments, we show how these assays could be used to evaluate the stability and functional reproducibility between batches of the bsAb preparations.

Materials and methods

AlphaLISA assay

Alpha technology is a fast, easy, highly sensitive, homogeneous (no-wash) assay platform that can be performed in a microplate format. Alpha assays require two bead types: Donor beads and Acceptor beads. The Donor beads convert ambient oxygen to singlet oxygen upon illumination at 680 nm. The singlet oxygen can then diffuse approximately 200 nm in solution. If an Acceptor bead is within that distance, energy is transferred to the Acceptor bead, resulting in light production. If an Acceptor bead is not in proximity of a Donor bead, little to no signal is produced over background. In the bispecific antibody assay, one of the target proteins binds the Donor bead and the other target protein binds the Acceptor bead. If both arms of the bispecific antibody are functional, they will bind both targets bringing the beads into proximity. If either arm is not functional, no signal will be generated. An example of a bispecific antibody bridging Alpha assay used in this application note is shown in Figure 2.

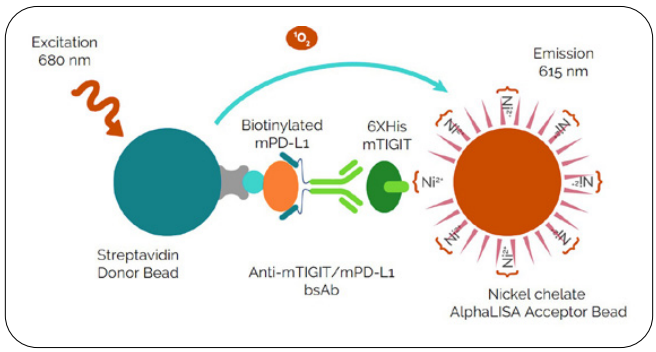


Figure 2. Schematic for bispecific antibody detection using a bridging AlphaLISA assay.

Reagents

The bispecific antibody used in the experiments described here (anti-mTIGIT/mPD-L1) was purchased from Absolute Antibody (cat# Ab00973-1.69). Recombinant mouse His-TIGIT (ACRO Biosciences, cat# TIT-M52E6-50ug) and recombinant mouse Biotinylated Avi-PD-L1-Fc (ACRO Biosciences, cat# PD1- M82F5-25 ug) were used for the assays. For testing specificity, recombinant mouse TIGIT-Fc (ACRO Biosciences, cat# TIT-M5257- 50 ug) and mouse PD-L1-Fc (ACRO Biosciences, cat# PD1- M5251) were used for the competition experiments.

All AlphaLISA™ assays were performed using Revvity standalone Donor or Acceptor beads in either AlphaPlate-384 (cat# 6005350) or ½ Area AlphaPlate-96 (cat# 6002350).

His-tagged proteins were captured by Ni chelate AlphaLISA Acceptor (cat# AL108C) or Ni chelate Donor beads (cat# AS101M). The biotinylated proteins were captured by either Streptavidin AlphaLISA Acceptor beads (cat# AL125C), Streptavidin Donor beads (cat# 6760002) or Streptavidin AlphaPlex 545 beads (cat# AP125SM-C). For multiplexing, the bispecific antibodies were captured by anti-mouse Alpha Donor beads (cat# AS104D). Protocols for each assay tested are listed in the text or in the figure legend.

Data analysis

Data were analyzed using GraphPad® version 7 software. The binding curves were fitted using nonlinear regression (assuming there is one binding site and none of the signal comes from non-specific binding) with a 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting. Inhibition curves were generated using log (inhibitor) vs. response - Variable slope (four parameters) function.

The major emission lines of the lanthanide chelate used in AlphaLISA and AlphaPlex™ are generally narrow, however, small amounts of light from minor secondary emissions can pass the filters of the other channels in a multiplex assay. Therefore, a correction factor is necessary for accurate data collection. Spectral overlap correction (SOC) is quantified as a percentage of the signal of emission on one detection channel that is detected on a second channel (e.g. the percentage of an AlphaLISA (Eu) signal that is detected through a Tb filter relative to that detected through a Eu filter) (For more details, see the AlphaPlex Development Guide). In order to calculate the SOC for the bsAb assays, only one of the proteins was added to the assay with two high concentrations of the bispecific antibody (0.3 nM and 0.1 nM). For determining the terbium into europium channel SOC, the His-mTIGIT protein was left out of the reaction. The background (no bispecific antibody) counts were subtracted and then the amount of signal in the europium channel/terbium channel * 100% was calculated as the percent overlap of the terbium signal into the europium channel (Tb_{Corr}). For determining the europium into terbium channel SOC, the Bio-mPD-L1 protein was left out of the reaction. The background (no bispecific antibody) counts were subtracted and then the amount of signal in the terbium channel/europium channel * 100% was calculated as the percent overlap of the europium channel into the terbium channel (Eu_{Corr}). The data was then corrected using the following equations:

545 (corrected) = $545 - ((615-(545*Tb_{Corr})) * Eu_{Corr})$

615 (corrected) = $615 - ((545-(615*Eu_{Corr})) * Tb_{Corr})$

The Eu_{Corr} was calculated as ~2% and the Tb_{Corr} was calculated as ~5% for the experiments presented in this application note.

Instrumentation

Alpha assays were measured using a Revvity EnVision™ 2105 multilabel plate reader. Barcode #444 mirror (#2101-4010) and AlphaScreen 570/100 filter barcode #244 (cat #2100-5710) was used for the AlphaLISA assay. The terbium barcode #701 filter (cat# 2100-5930), europium barcode #203 filter (cat# 2100-5090), and barcode #605 (cat# 2102-5910) mirror was used for the AlphaPlex assays. For the multiplexing assays, the plate was read sequentially with the terbium channel read first.

Results and discussion

I. Development of a bridging assay for bispecific antibody characterization

In order to characterize the bispecific antibodies using a biochemical assay, it is important to first find good recombinant proteins for binding and determine the best binding buffer. In preliminary experiments, we identified two good recombinant proteins, His-tagged TIGIT and biotinylated Avi-tagged PD-L1 and identified Immunoassay Buffer (cat# AL000) as a buffer where each of the recombinant proteins bind the bispecific antibody (data not shown). After identifying the appropriate recombinant proteins and the binding buffer, the next step is to test a crosstitration of the two proteins to make sure that they can bind the bispecific antibody simultaneously and determine the optimal protein concentrations for the assay. Six concentrations of protein were tested using the assay schematic shown in Figure 2 and the protocol in Figure 3A. Two different bead orientations were tested, Streptavidin Donor beads with Ni chelate AlphaLISA Acceptor beads (Figure 3B) and Ni chelate Donor beads with Streptavidin AlphaLISA Acceptor beads (Figure 3C). As shown in Figure 3B, the Streptavidin Donor and Ni chelate AlphaLISA Acceptor beads showed the best signal to background. At protein concentrations higher than 1 nM, the signal begins to plateau and/or hook in the assay. Therefore, 1 nM of each recombinant protein was used for further assay development steps.

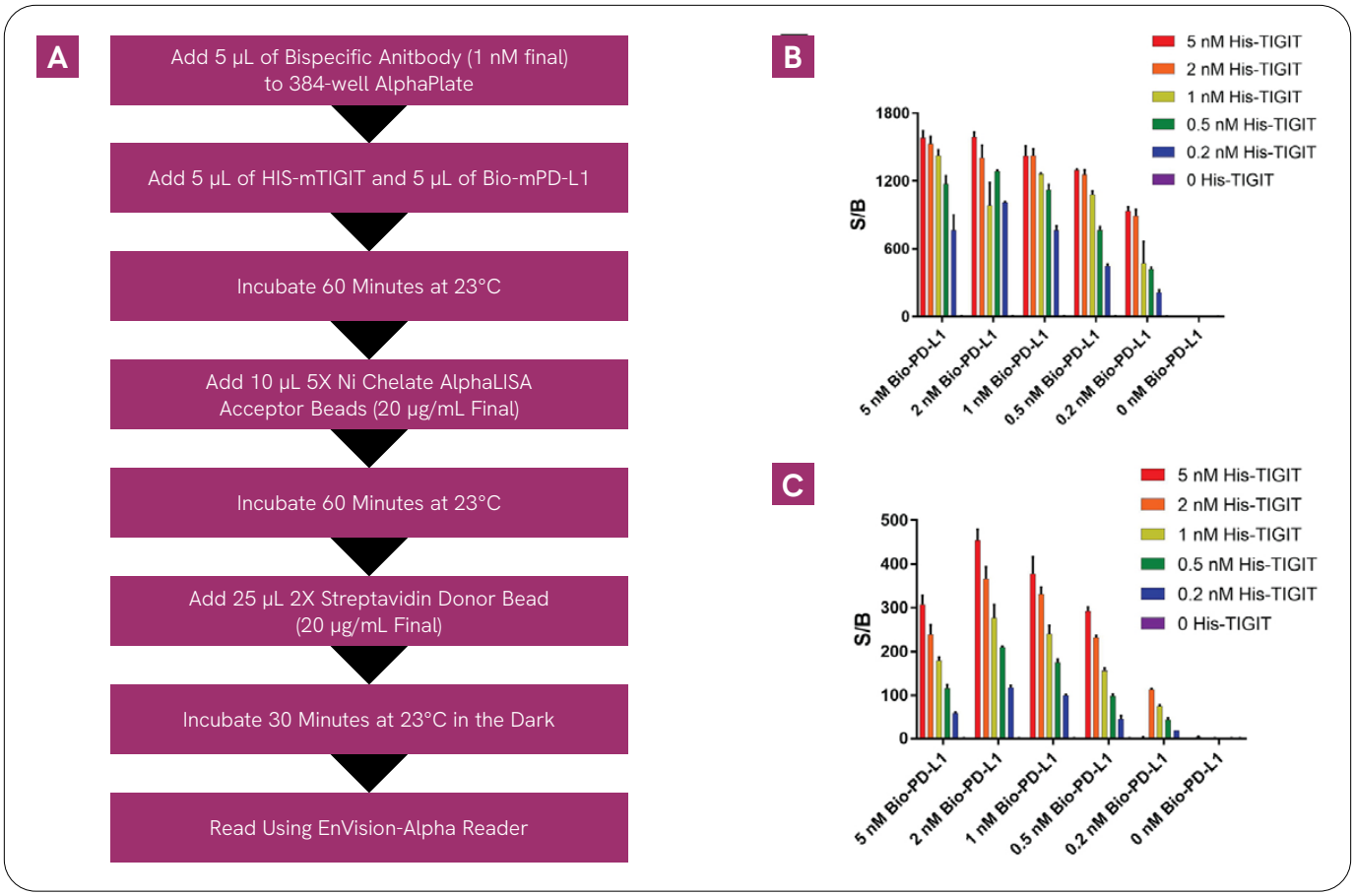


Figure 3. **Cross-titration of recombinant mouse TIGIT and PD-L1 proteins.** A) Protocol used for cross-titration. B) Streptavidin Donor beads with Ni chelate AlphaLISA Acceptor beads. C) Ni chelate Donor beads with Streptavidin AlphaLISA Acceptor beads.

Once suitable protein concentrations are determined, a titration of the bispecific antibody can be performed. The EC_{50} and signal window should remain constant if both binding sites on the antibody are intact and functional. Incubating the Acceptor beads prior to the addition of the Donor beads was found to be unnecessary (data not shown), so the protocol was simplified by adding the beads at the same time and incubating for two hours using a 96-well ½ Area AlphaPlate. As shown in Figure 4B, titration of anti-mTIGIT/mPD-L1 bispecific antibody in this assay produces a nice curve with a high signal to background and an EC_{50} of 0.27 nM.

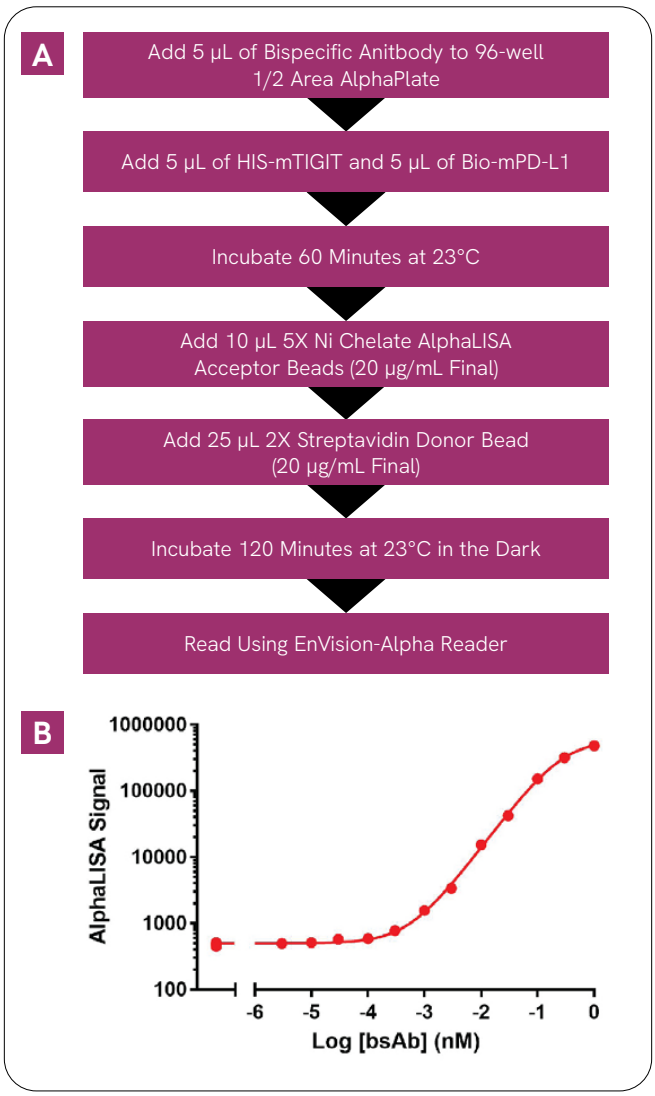


Figure 4. Bispecific antibody characterization assay. A) Protocol used for titration. B) Titration of anti-mTIGIT/mPD-L1 bispecific antibody.

Finally, to assess the signal specificity and to show that each site is binding similarly in the presence of the other protein, unlabeled proteins were used to compete for binding of the bsAb. As shown in Figure 5B, there is a dose-dependent decrease in signal when either mTIGIT-Fc or mPD-L1-Fc is incubated in the assay. We see similar IC_{50} values for both proteins (mTIGIT= 0.4 nM; mPD-L1= 0.5 nM). This data shows that the signal is specific to the interaction of the bispecific antibody with both proteins.

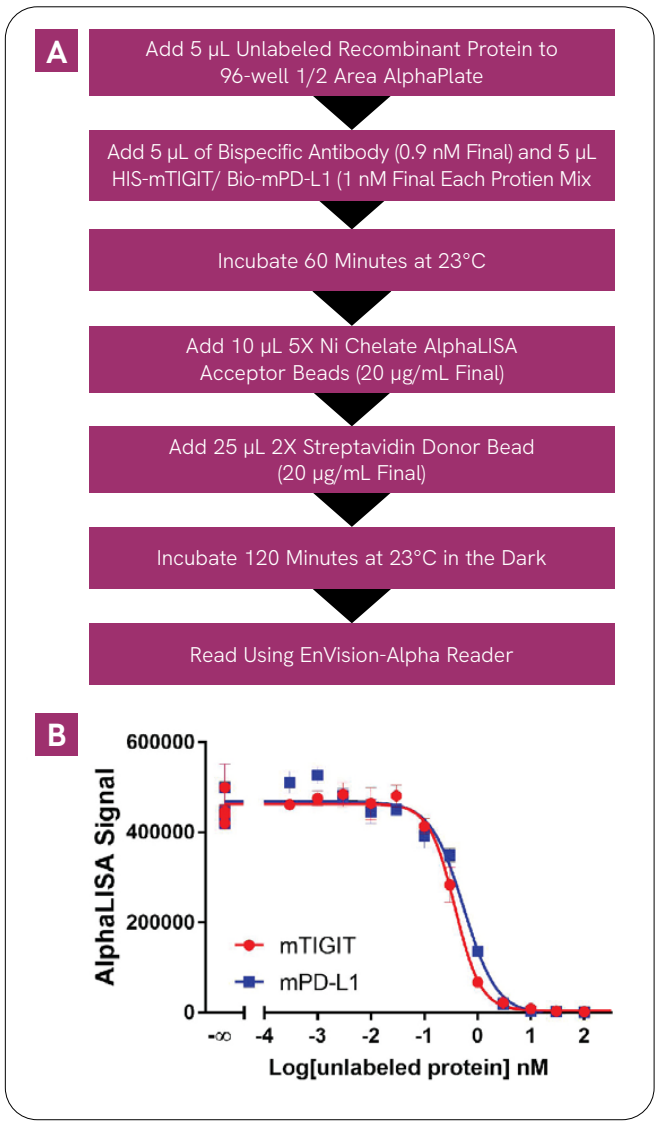


Figure 5. Determining Signal Specificity. A) Protocol B) Results of the competition experiment using unlabeled proteins.

II. Development of a multiplexing alpha assay for bispecific antibody characterization

Multiplexing can minimize sample use and provide more information about how each arm of the bispecific antibody is binding to its respective target. An Alpha-based multiplexing assay (AlphaPlex) enables the dual measurement of two proteins from the same well using two types of Alpha Acceptor beads that maximally emit at resolved wavelengths. The terbium containing AlphaPlex beads emit at 545 nm and the europium containing AlphaLISA beads emit at 615 nm. For a bispecific antibody assay, one recombinant protein is captured by the AlphaLISA Europium Acceptor beads and the other is captured by the AlphaPlex 545 Acceptor bead. The bispecific antibody binds each of the proteins and is captured by the Donor bead. This set-up allows detection of each individual arm binding to its respective recombinant protein in a single well. To set-up a multiplexing assay for characterizing the anti-mTIGIT/mPD-L1 bsAb, the bispecific antibody was captured with anti-mouse Donor beads. Then, Ni chelate europium containing AlphaLISA Acceptor beads (emission at 615 nm) were used to capture the His-tagged mTIGIT and terbium containing Streptavidin AlphaPlex beads (emission at 545 nm) to capture the biotinylated mPD-L1. If only one arm can bind, there will only be signal in that channel and if both can bind, signal will be produced in both channels (Figure 6).

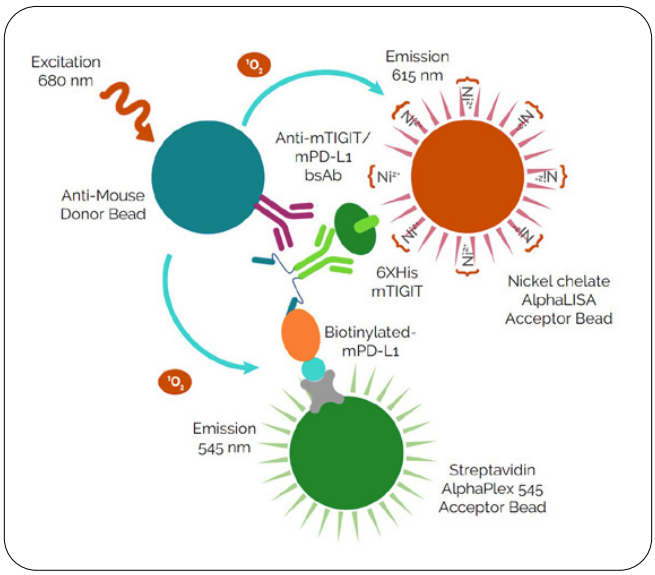


Figure 6. Schematic for detecting the bsAb using multiplexing with AlphaLISA and AlphaPlex 545 Acceptor beads.

Multiplexing requires some optimization steps. Since it was already confirmed the bispecific antibody can bind both target proteins simultaneously, the next step is to test the binding to multiplex Alpha Acceptor beads. The optimal protein concentrations in the multiplexing assay may not be the same as in the single emission assay (using only AlphaLISA Acceptor beads). This is because the signal in each channel is dependent only on each individual arm binding to its target and not a function of both arms binding as is the case for the single emission AlphaLISA assay. In addition, AlphaPlex beads may have different binding capacities to the AlphaLISA beads. A cross-titration of each of the proteins in the presence of both europium and terbium beads was performed first. The EC_{80} for the bispecific antibody titration from Figure 4 (0.9 nM) was used; a concentration that falls below the binding capacity of the anti-species Donor beads.

Figure 7 shows the results of the protein cross-titration with the raw signal corrected for spectral overlap between the europium and terbium channels (see Materials and Methods). In Figure 7B, the terbium channel shows that as the bio-mPD-L1 concentration increases from 0 to 2 nM (final), there is an increase in signal with a plateau between 1 and 2 nM final. Additionally, we see very little difference in signal across the His-mTIGIT concentrations in the terbium channel. This confirms that neither the mTIGIT protein nor any signal from the europium channel (after spectral overall correction) is interfering with the binding of mPD-L1 to the bispecific antibody as detected by the terbium signal. The same is true for the detection of the binding of the mTIGIT protein in the europium channel (Figure 7C). A steady increase in signal can be seen from 0 to 10 nM of HismTIGIT and very little change in signal up to 1 nM as we titrate in the Bio-mPD-L1 protein. A small decrease can be seen in the europium signal at 2 nM Bio-mPD-L1, which may indicate that near the hook point for mPD-L1 binding to the terbium bead, there may be interference in the europium channel. This assay shows that each site on the bispecific antibody binding to its respective target can be independently detected in a single well. From this data, 1 nM (final) Bio-mPD-L1 and 10 nM (final) HismTIGIT was used for the bispecific antibody titration.

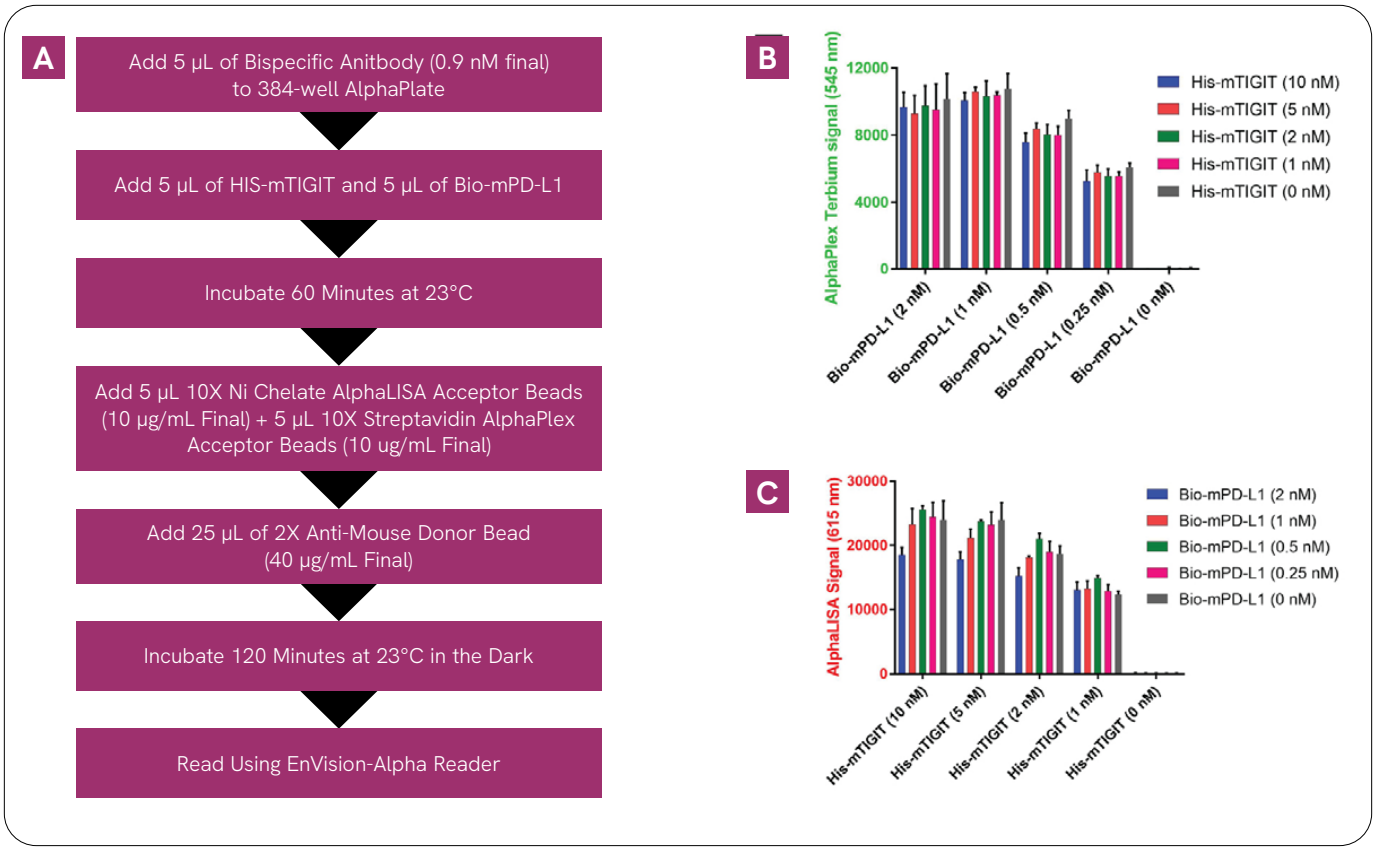


Figure 7. Cross-titration of proteins with multiplexing beads. A) Protocol used to test cross-titration of proteins in the AlphaPlex assay. B) Signal in the terbium channel for the cross-titration. C) Signal in the europium channel for the cross-titration.

Figure 8 shows the results of the bispecific antibody titration. In a single well, binding of the bispecific antibody to each protein is detected using both the terbium channel and the europium channel. For the bispecific antibody binding to bio-mPD-L1, the EC_{50} is 0.1 nM and for binding to His-mTIGIT, the EC_{50} is 0.6 nM. The differences in the EC_{50} values could reflect differences in the binding affinities to each target or it could be that bsAb binding to the bio-mPD-L1 hooks at a lower concentration due to lower amounts of protein in the assay.

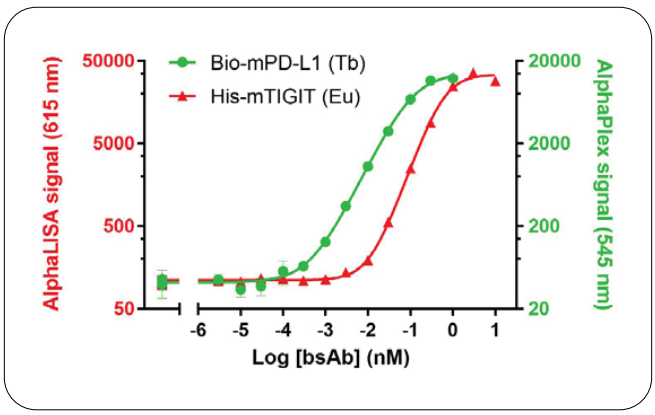


Figure 8. Bispecific antibody titration multiplexing. BsAb titration as detected in both the europium (615 nm) and terbium (545 nm) channels. Protocol used is the same as Figure 7A.

Next, to show signal specificity of the binding to target protein, a competition experiment with each of the untagged proteins was performed. Figure 9A shows the protocol used for this experiment. Figure 9B shows the data collected in the terbium channel. A clear decrease in the terbium signal is shown as mPD-L1-Fc (IC_{50} = 0.6 nM) is titrated and no change in the signal is seen as mTIGIT-Fc is titrated. Figure 9C show the same trend for the europium channel, a strong decrease in signal with titrating in mTIGIT-Fc (IC_{50} = 0.6 nM) and very little change when titrating in mPD-L1-Fc. There is a small decrease in signal at high concentrations of mPD-L1-Fc which may be due to some steric hindrance at high concentrations that interferes with mTIGIT binding. The similar IC_{50} values suggest similar binding affinities to the target proteins.

III. Evaluating the assays using forced degradation

In order to evaluate the ability of the assays to characterize bispecific antibody stability, a forced degradation experiment was performed. For this, aliquots of the anti-mTIGIT/mPD-L1 bispecific antibody were stored at either 37°C or 4°C for 12 days. The single emission AlphaLISA bispecific antibody assay was run on both samples (using the same protocol as Figure 4) and the EC_{50} and the window for

each assay was compared. As shown in Figure 10, the EC_{50} does not significantly change between the two samples, but the maximum signal at each bsAb concentration decreases significantly for the 37°C sample. This suggests that one or both binding sites of the bispecific antibody has started to degrade during the incubation at 37°C. To estimate how much of the antibody has degraded when incubated at high temperature, the signal generated by the 37°C sample can be interpolated from the curve generated from the sample stored at 4°C. Two concentrations of the bispecific antibody (0.1 and 0.03 nM) were chosen, run in triplicate, and concentration of functional bsAb was interpolated from the 4°C standard curve. The data shown in Table 1 shows an average of 37% recovery for the antibody stored at 37°C, indicating significant degradation.

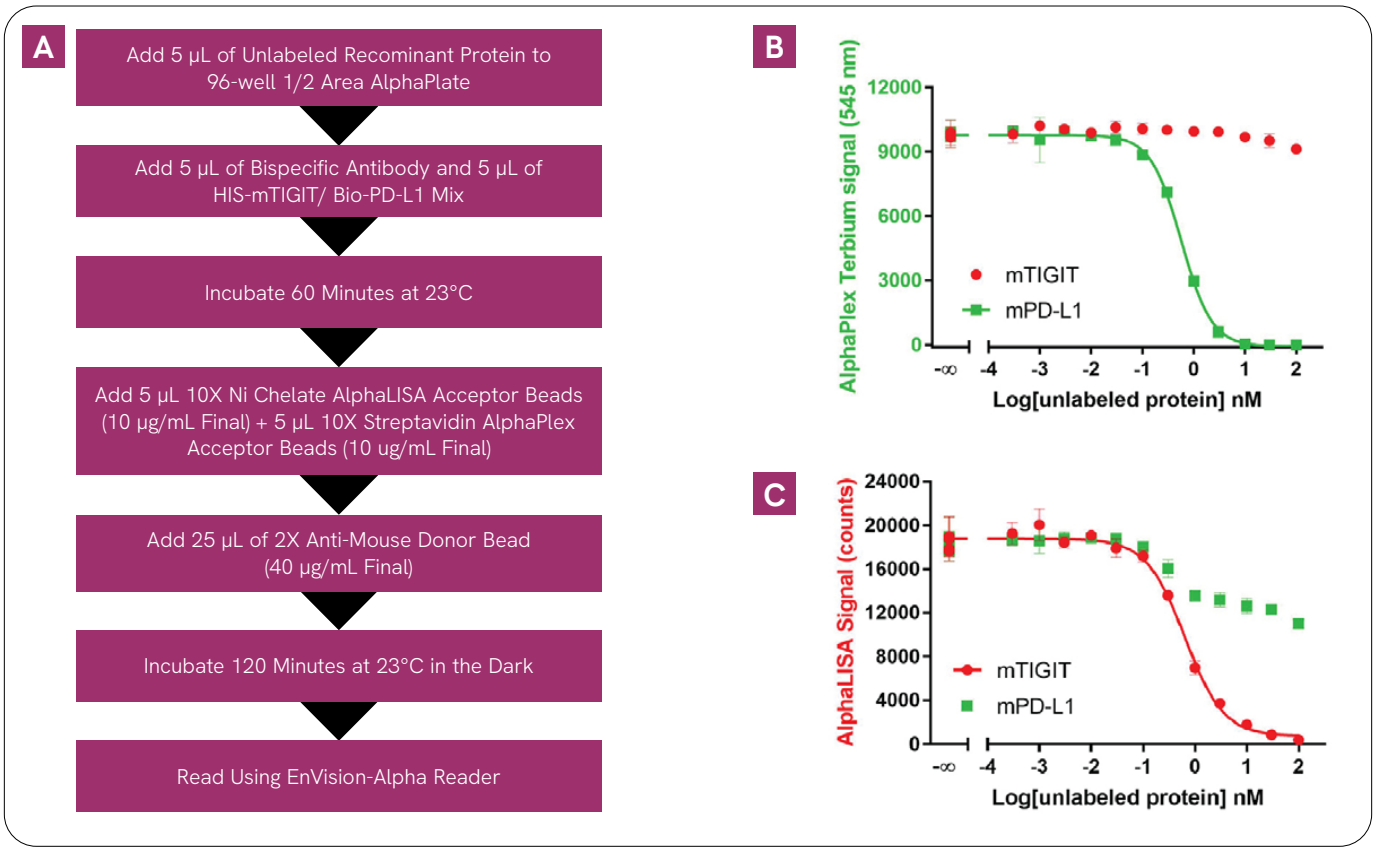


Figure 9. Signal specificity with the multiplexing assay. A) Protocol used to determine specificity of signal in the AlphaPlex assay. B) Titration of each of the unlabeled proteins and the signal measured in the terbium channel C) Titration of each of the unlabeled proteins and the signal measured in the europium channel.

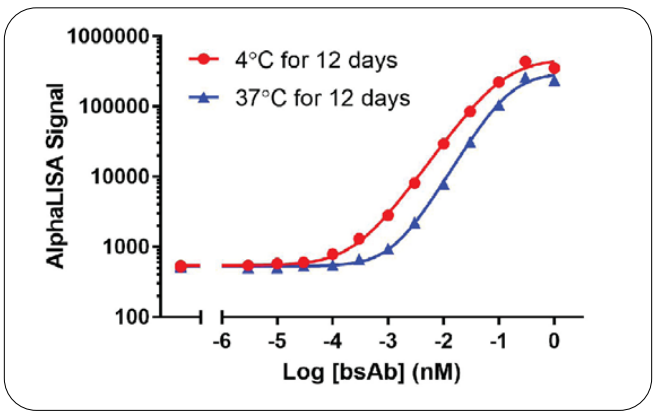


Figure 10. Assay for monitoring bsAb thermal stability using single emission AlphaLISA assay. BsAb titration comparing antibody incubated at 4°C and 37°C for 12 days. Protocol used is the same as Figure 4A.

Table 1. Percent recovery of thermally degraded bsAb (incubated at 37°C for 12 days).

Input concentration (nM)	Interpolated concentration (37°C) (nM)	% Recovery
0.1	0.039	39
0.1	0.037	37
0.1	0.037	37
0.03	0.011	37
0.03	0.010	33
0.03	0.011	37
	Average recovery	**37**

In order to determine which portion of the bsAb is degrading, we tested the same two antibodies in the multiplexing assay. As shown in Figure 11A, the signal in the terbium channel overlaps nicely, suggesting that the mPD-L1 binding site on the antibody is intact and has not degraded significantly during the time incubated at 37°C. For percent recovery, we chose 0.01 and 0.003 nM as these concentrations were high on the curve and not above the hook point for the assay. Table 2 shows the interpolated values and the percent recovery calculated as 114% verifying that the mPD-L1 binding site on the antibody is intact. Conversely, the signal in the europium channel decreased (Fig 11B), suggesting that the mTIGIT binding site on the bispecific antibody has partially degraded. Again, the EC_{50} does not change significantly, but the window of the assay decreases for the sample stored at 37°C. Table 3 shows the percent recovery in the europium channel calculated as an average of 44%, confirming that the decrease in the single emission AlphaLISA assay is due to the mTIGIT binding site on the bispecific antibody degrading when stored at 37°C over time.

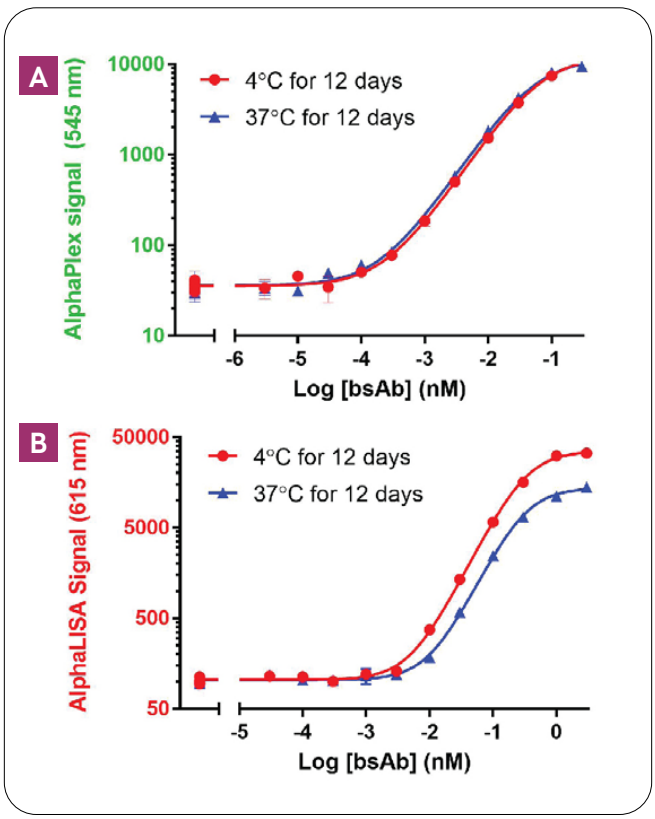


Figure 11. Multiplexing assay for monitoring bsAb thermal stability. A. BsAb titration in the terbium channel. B) BsAb titration in the europium channel. Protocol used is the same as Figure 7A.

Table 2. Percent recovery of thermally degraded bsAb (incubated at 37°C for 12 days) in the terbium channel (anti-mPD-L1 portion of antibody).

Input concentration (nM)	Interpolated concentration (37°C) (nM)	% Recovery (Terbium channel)
0.010	0.012	120
0.010	0.010	100
0.010	0.013	129
0.0030	0.0033	110
0.0030	0.0033	110
0.0030	0.0034	113
	Average recovery	**114**

Table 3. Percent recovery of thermally degraded bsAb (incubated at 37°C for 12 days) in the europium channel (anti-mTIGIT portion of antibody).

Input concentration (nM)	Interpolated concentration (37°C) (nM)	% Recovery (Europium channel)
0.30	0.11	37
0.30	0.11	37
0.30	0.11	37
0.10	0.053	53
0.10	0.052	52
0.10	0.044	44
	Average recovery	**43**

Conclusions

Assays to functionally characterize bispecific antibodies can be used in the discovery process as well as for quality control during the manufacturing process. Here we show how to design and set-up mix and read, no-wash Alpha assays to functionally characterize bispecific antibodies. These assays can be used to verify that each arm of the bispecific antibody is functional in the presence of both target proteins. It can also be used to look at the stability of the antibody over time in various formulations. During quality control analyses, these assays can be used to compare lot to lot variations. In addition to providing critical information about each individual arm of the assay, a multiplexing assay also saves on time and material. Overall, the ease of use and the minimal sample consumption make Alpha assays ideal for bispecific antibody discovery, development, and quality control analyses.

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