

Optimizing a ternary complex assay with AlphaLISA Toolbox reagents for targeted protein degradation applications.

Authors:

Katie Graham
Bagna Bao
Adam Carlson
Sara Bdioui
Fabienne Charrier Savournin

Revvity, Inc.

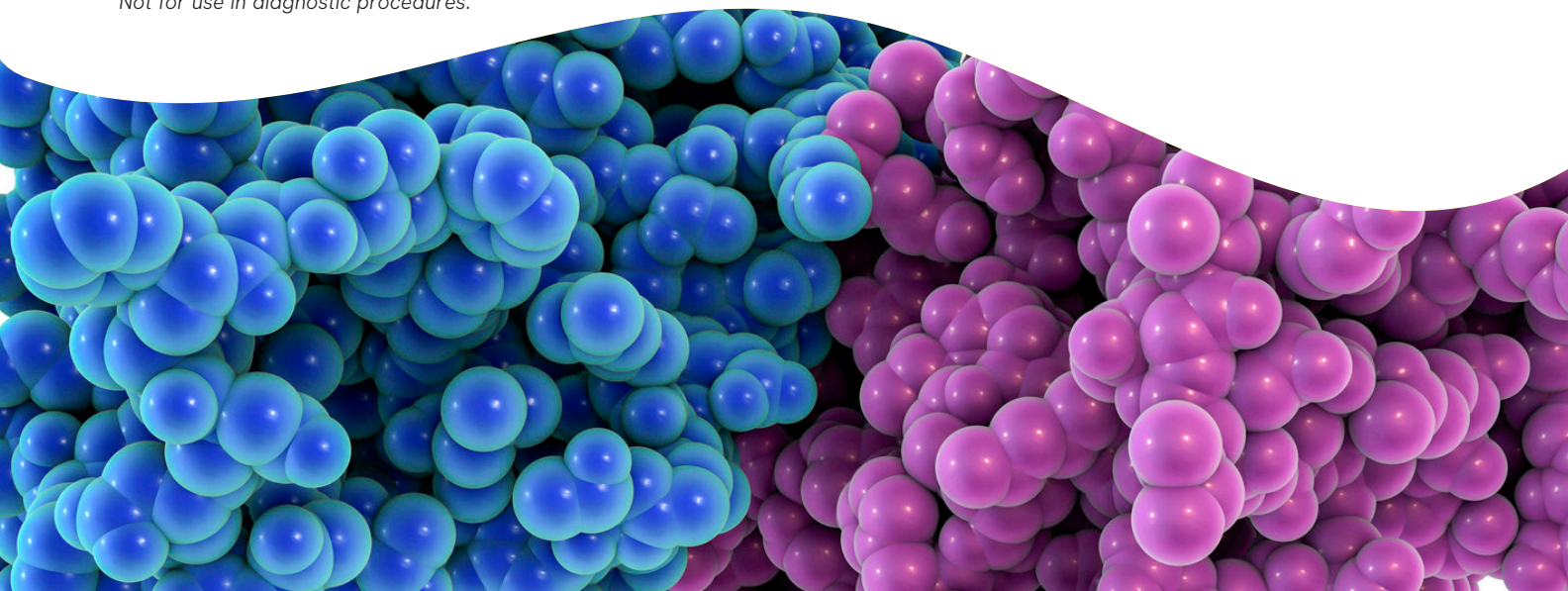
Introduction

Protein degradation is a tightly regulated process that plays a critical role in homeostasis. Degradation occurs via two pathways, the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal system (ALS). Targeted protein degradation (TPD) is a therapeutic strategy which harnesses natural protein degradation pathways to moderate protein concentrations. TPD offers several advantages to traditional pharmacological treatments, including reduced incidence of drug resistance and the ability to target diverse, and in some cases, previously undruggable proteins.¹

Proteolysis targeting chimeras (PROTAC[®]s), which exploit the UPS to moderate protein levels, are a commonly applied TPD technology.² PROTACs are small heterobifunctional molecules consisting of two ligands joined by a chemical linker—one ligand engages an E3 ubiquitin ligase and the other ligand, called the warhead, selectively binds a protein of interest (POI). The joining of the E3 ligase with the target protein via the PROTAC results in the formation of a ternary complex, which is an important step toward catalyzing ubiquitination and, ultimately, degradation of the protein (Figure 1).

Thus, there is a need for tools that can monitor the occurrence of these structures within the protein degradation pathway, thereby allowing researchers to screen potential candidate molecules. In particular, assessing formation of the ternary complex is critical, as increasing evidence suggests the potency of a PROTAC is heavily dependent on the stability of the ternary complex and associated protein-protein interactions.³

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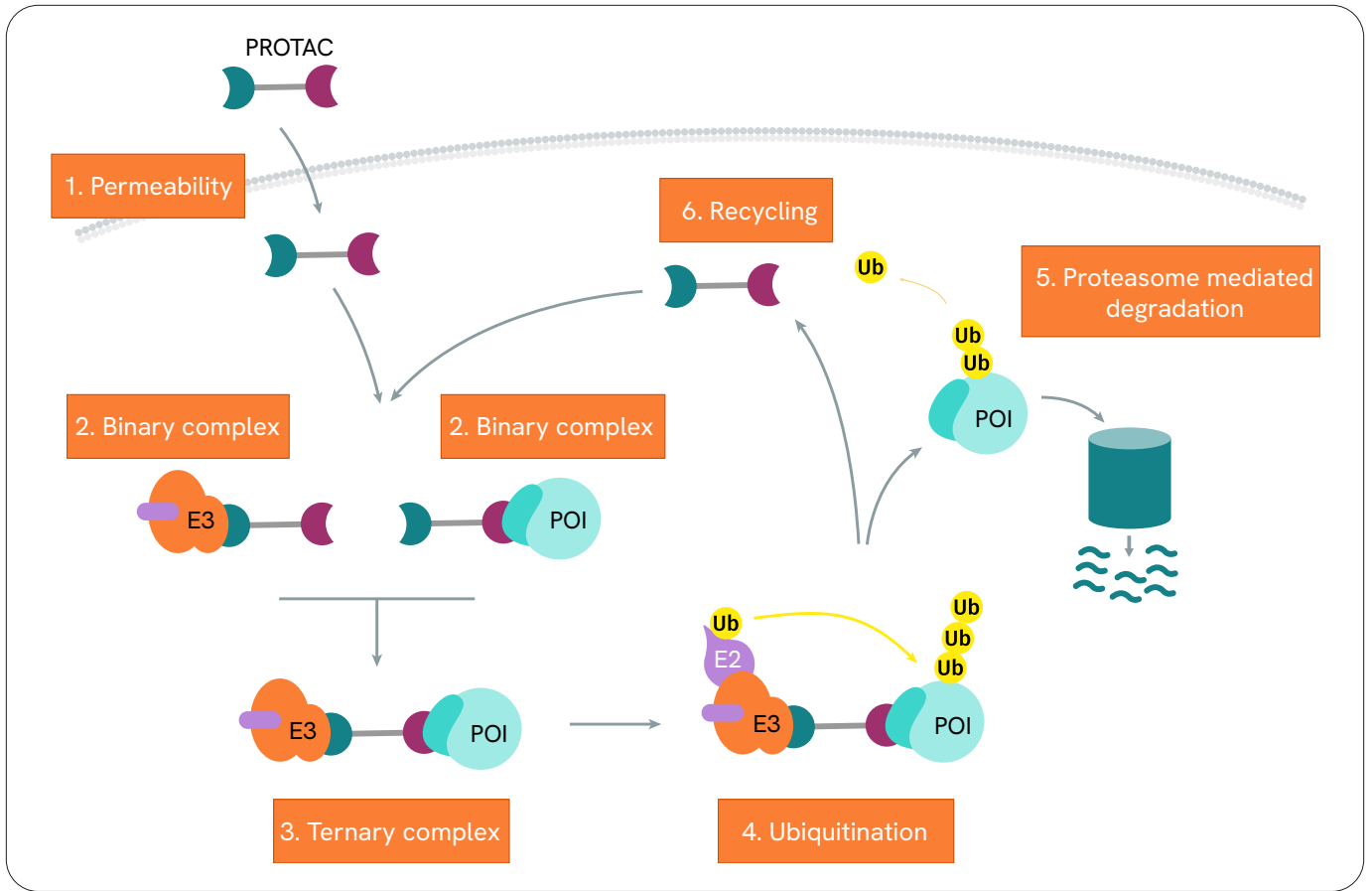


Figure 1: Schematic of targeted protein degradation pathway utilizing PROTACs. This multi-step pathway requires formation of several intermediary structures (e.g., binary complex, ternary complex) which can be detected using immunoassay technologies.

Revvity's AlphaLISA™ Toolbox reagents meet this research need by providing the flexibility to customize assays for detection of a ternary complex with variable ligases, protein partners, and PROTAC molecules. AlphaLISA is a homogenous, no-wash, bead-based luminescent amplification assay. Anti-analyte Donor and Acceptor beads bind to the target analyte and upon excitation at a specific wavelength, the Donor bead transfers a singlet oxygen to the Acceptor bead if they are within proximity (≤ 200 nm). As a result, light is produced by the Acceptor beads which can be measured for quantitation of the analyte. In the case of a ternary complex assay, when the POI and E3 ligase are successfully joined via the PROTAC in a ternary complex formation, anti-tag Donor and Acceptor beads (which bind to tags on the target POI and E3 ligase) are within range to produce the Alpha signal (Figure 2). The customizable nature of AlphaLISA Toolbox reagents comes from the variety of anti-tagged Donor and Acceptor beads available, which can be interchanged depending on the tags present on the target protein and E3 ligase.

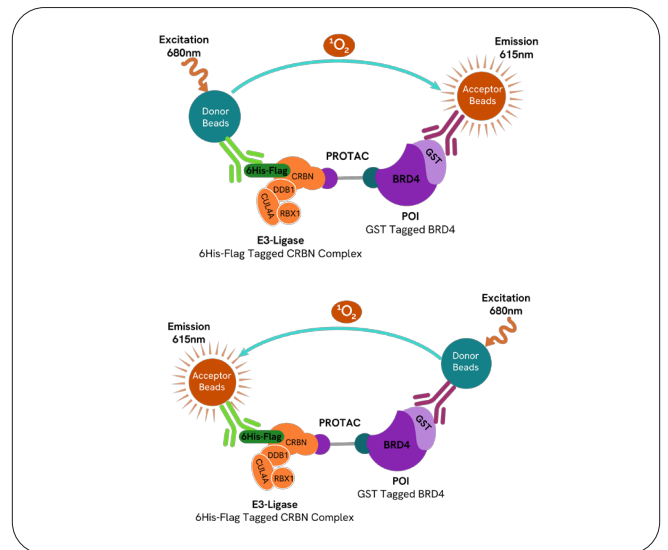


Figure 2: Schematic of AlphaLISA setup for detection of a ternary complex using Toolbox reagents. When the tagged E3 ligase and POI are joined by the PROTAC forming the ternary complex, the anti-tag Donor and Acceptor beads are within range to produce signal. Anti-tag bead types and the direction of the reaction (e.g. Donor bead attachment to either E3 ligase or POI) can be exchanged depending on the tags in use.

This application note demonstrates the use of AlphaLISA Toolbox reagents to detect ternary complex formation using a common target for TPD. A series of experiments were conducted to test ternary complex formation and optimize assay signal for detection (Figure 3). The components of the ternary complex tested here include glutathione S-transferase-tagged recombinant human BRD4 protein (GST-BRD4) as the target protein; recombinant human Cereblon/DDB1/Cul4A/Rbx1 Complex (CRBN Complex) with N-terminal FLAG-tags on the Cereblon and DDB1 subunits and N-terminal 6x His tags on the Cul4A and Rbx1 subunits as the E3 ligase; and dBET6 as the PROTAC. BRD4 is a BET (Bromodomain and Extra-Terminal domain) class chromatin reader protein which has a role in transcription and epigenetic regulation, and its downregulation is associated with a reduction in cancer development, making it a prime anticancer drug target.⁴ The E3 ligase: CRBN Complex, is a multi-subunit molecule—the Cereblon subunit, which features a binding region for the PROTAC, is linked via DNA binding protein 1 (DDB1) with the scaffolding protein Cullin 4A (Cul4A) and its regulator ring box proteins (Rbx1), and together these form the complete E3 ligase which is responsible for ubiquitination of the target protein.⁵ The PROTAC, dBET6, is a potent and selective degrader of BET bromodomains, and features a JQ1-derivative warhead linked to a thalidomide E3 ligand.

Experimental setup and results

Testing ternary complex formation with broad titration

In an initial experiment, a broad range of target protein, E3 ligase, and PROTAC concentrations were cross titrated to establish the concentration ranges of compounds needed for ternary complex formation. Concentrations of 5 and 1 nM GST-BRD4 protein were cross-titrated with 10, 5, and 1 nM CRBN Complex, and 1000 or 200 nM dBET6 PROTAC. All reagents were prepared in Binding Assay Buffer and a single AlphaLISA bead pair: Anti-FLAG Donor beads with Anti-GST Acceptor beads was used in this preliminary experiment. This broad titration was conducted first using only one bead pairing to reduce the amount of E3 ligase and protein needed for development of the Toolbox assay (if the target protein and E3 ligase are available in sufficient quantities, testing multiple Alpha Donor and Acceptor bead pairings across a broad range of concentrations can be undertaken simultaneously).

The broad titration produced AlphaLISA signal indicative of ternary complex formation, with results dependent on the concentration of the compounds tested. With 200 nM dBET6, concentrations ≥ 1 nM BRD4 and ≥ 1 nM CRBN Complex showed an increase in AlphaLISA signal 4.3-6.3 times greater than background signal, depending on the BRD4 and CRBN Complex concentration (Figure 4). With 1000 nM dBET6, AlphaLISA signal was depressed across all concentrations of BRD4 and CRBN Complex, likely due to the hook effect from an overabundance of binary complexes resultant from excess PROTAC. With 200 nM dBET6, AlphaLISA signal increased between concentrations of 1 and 5 nM CRBN Complex, but did not appreciably increase between 5 nM and 10 nM, indicating a suitable concentration of CRBN Complex would likely be 5 nM or less for the assay. For BRD4 protein, AlphaLISA signal showed similar patterns across both 1 nM and 5 nM concentrations, indicating an increase in protein concentration did not translate into an improvement of signal. Therefore, concentrations below 1 nM BRD4 and 5 nM CRBN Complex were explored in subsequent experiments, as these ranges produced moderate to strong signal.

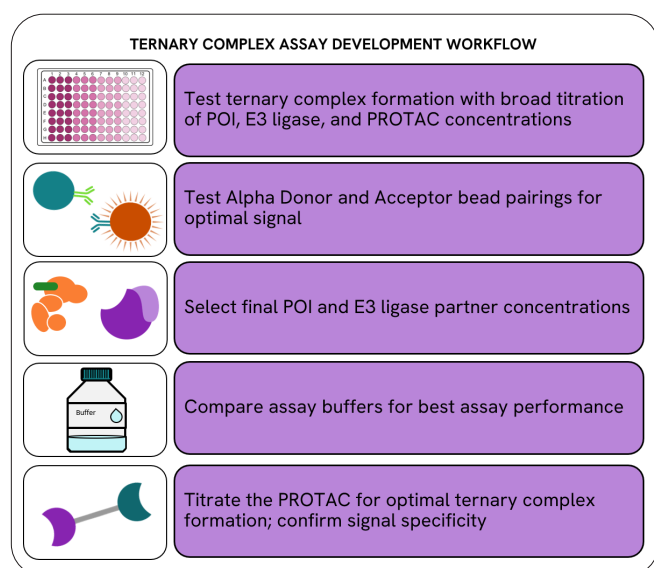


Figure 3: Experimental workflow for developing a ternary complex assay using Alpha Toolbox reagents.

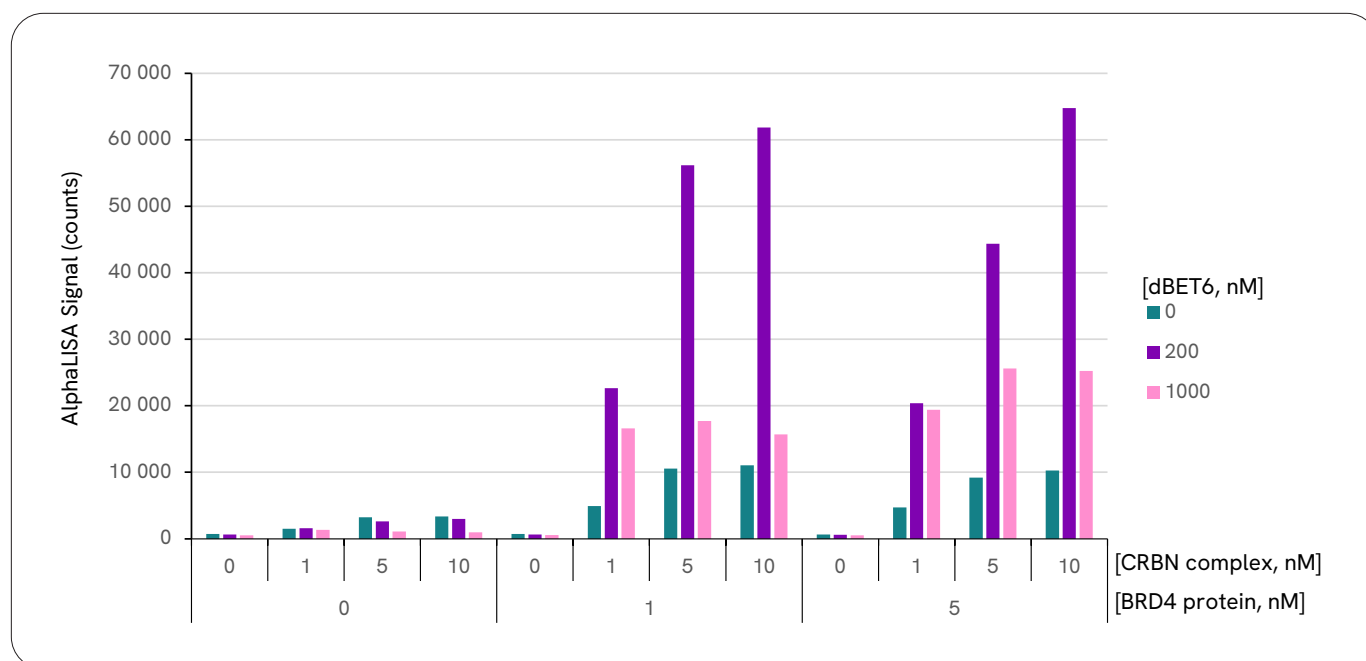


Figure 4: Comparison of AlphaLISA signal for ternary complex detection across a broad range of BRD4 protein, CRBN Complex, and dBET6 PROTAC concentrations. Differences in AlphaLISA signal based on PROTAC concentration are shown by the colored bars (pink: 1000 nM; purple: 200 nM; teal: 0 nM (i.e. background signal (absence of PROTAC/no ternary complex formation)).

Testing Alpha Donor and Acceptor bead pairs and final protein optimization

When developing a ternary complex assay, a comparison of different Donor and Acceptor bead combinations should be conducted to determine the best pairings to maximize S/B ratio. In this study, the target protein was GST-tagged, while the CRBN Complex featured both FLAG- and 6x His-tagged subunits. This allowed for four different bead combinations to be tested: 1) Anti-FLAG Donor beads with Anti-GST Acceptor beads; 2) Glutathione Donor beads with Anti-FLAG Acceptor beads; 3) Glutathione Donor beads with Anti-6x His Acceptor beads; and 4) Anti-6x His Donor beads with Anti-GST Acceptor beads. Absolute signal and S/B ratios were compared across a select range of protein and E3 ligase concentrations (chosen based on results of the broad titration experiment) for each bead pairing. Tested concentrations included: 1, 0.5, and 0.25 nM GST-BRD4 and 5, 2.5, and 1 nM CRBN Complex with 100 nM dBET6. Comparing the S/B ratios across these concentrations also enabled selection of the optimal target protein and E3 ligase concentrations for use in the final ternary complex assay setup (a full titration of PROTAC concentrations was conducted later).

Donor and Acceptor bead pairs showed variable success for detection of the BRD4, CRBN Complex, dBET6 ternary complex, highlighting the importance of testing different bead combinations when working with AlphaLISA Toolbox reagents. For this assay, Anti-6x His beads, when used either as the Donor or Acceptor bead, resulted in poor signal-to-background ratios (Figure 5, Table 1). With Anti-6x His beads, elevated background signal (high signal when no PROTAC present) made it impossible to differentiate the signal resulting from ternary complex formation. Given the poor S/B ratios, Anti-6x His Donor and Acceptor beads were not further explored in this specific case study, but may be useful in other ternary complex assays, as bead combinations may perform differently depending on the tag-types, protein partners, and buffers used in the ternary complex assay.

In contrast, bead pairings of Anti-FLAG Donor beads with Anti-GST Acceptor beads or Glutathione Donor beads with Anti-FLAG Acceptor beads showed moderate to strong AlphaLISA signal and S/B ratios at most

concentrations of BRD4 and CRBN Complex (Figure 5, Table 1), demonstrating these combinations of beads can be successfully applied to detect the ternary complex. The combination of Anti-FLAG Donor beads with Anti-GST Acceptor beads had S/B ratios ranging from 4.1-5.9, compared to the Glutathione Donor beads with Anti-FLAG Acceptor beads which had S/B ratios spanning from 3.6-11.3 (Table 1). In general, a minor increase in background signal was observed with the

Anti-FLAG Donor/Anti-GST Acceptor beads compared to the Glutathione Donor/Anti-FLAG Acceptor beads which contributed to reduced S/B ratios. Despite this, the AlphaLISA signal was sufficiently high to distinguish formation of the ternary complex with both bead pairings. However, due to the improved S/B ratio when using Glutathione Donor beads with Anti-FLAG Acceptor beads, this combination was selected for further use.

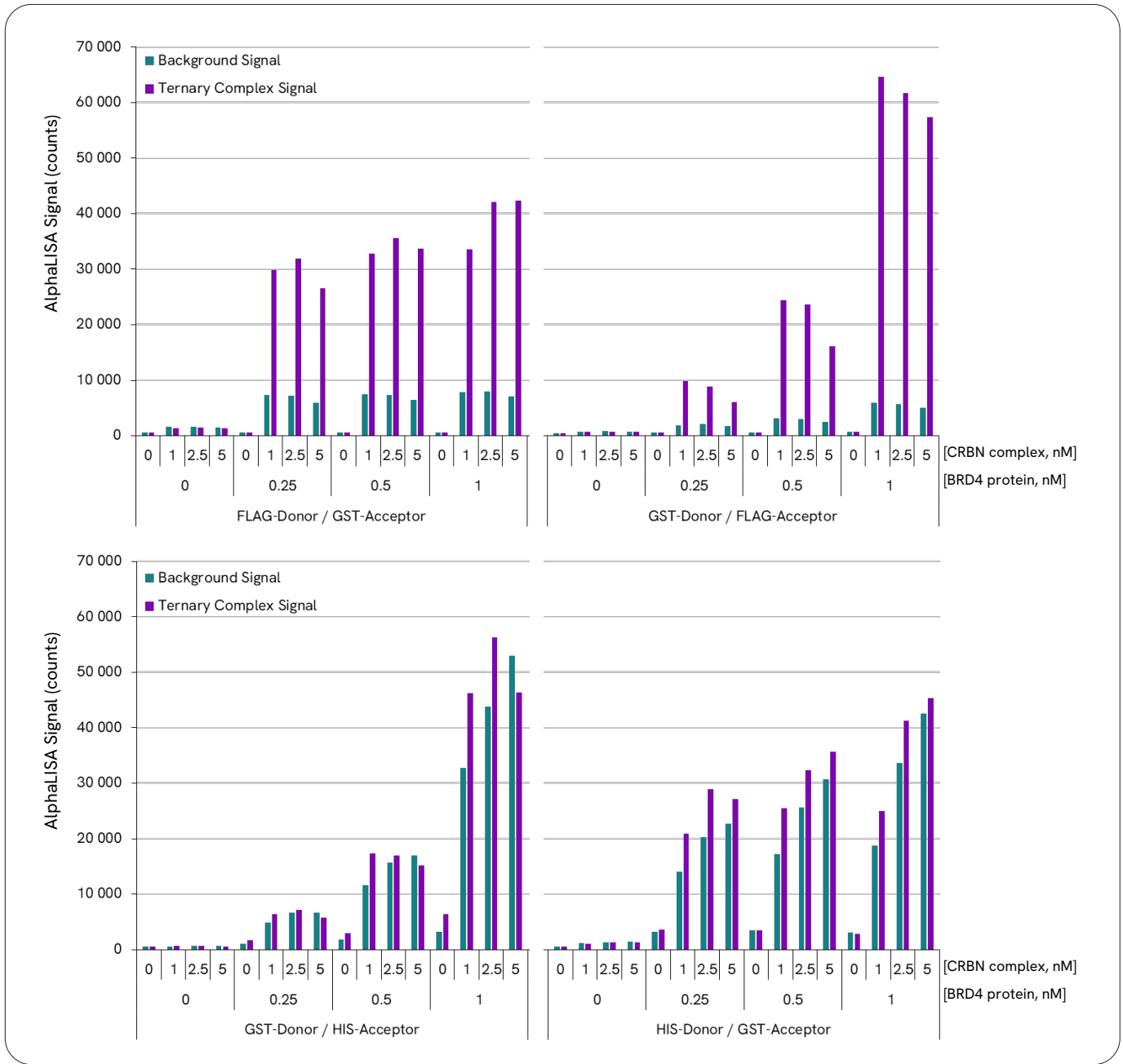


Figure 5: Comparison of AlphaLISA Donor and Acceptor bead pairings tested for detecting the ternary complex of GST-BRD4, FLAG-6x His-CRBN Complex, and dBET6. Signal produced from the ternary complex (using 100 nM dBET6 PROTAC) is shown by the purple bars versus background signal (0 nM dBET6) depicted by teal bars. Four combinations of Donor and Acceptor beads were tested. Top left: Anti-FLAG Donor and Anti-GST Acceptor; Top right: Glutathione Donor and Anti-FLAG Acceptor; Bottom left: Glutathione Donor and Anti-6x His Acceptor; Bottom right: Anti-6x His Acceptor and Anti-GST Acceptor.

Table 1: Signal-to-background (S/B) ratios for four combinations of AlphaLISA Donor and Acceptor bead pairs across varying concentrations of BRD4 and CRBN Complex. Green shading denotes possible useable combinations of beads and concentration ranges for the assay.

	S/B	Concentration BRD4 protein [nM]				Alpha Bead Pairing	Conclusion
		0	0.25	0.5	1		
Concentration CRBN Complex [nM]	0	1.02	0.95	0.97	0.99	Anti-FLAG Donor Anti-GST Acceptor	Moderate ternary complex signal; slightly elevated background signal resulted in lower S/B ratios
	1	0.86	4.08	4.39	4.27		
	2.5	0.93	4.40	4.83	5.29		
	5	0.86	4.46	5.19	5.94		
	0	1.03	1.00	1.06	1.01	Glutathione Donor Anti-FLAG Acceptor	Ternary complex signal is moderate to high & can be differentiated from background signal; highest S/B ratios
	1	0.98	5.28	7.94	10.81		
	2.5	0.94	4.16	8.02	10.84		
	5	0.88	3.56	6.59	11.33		
	0	1.00	1.56	1.67	1.99	Glutathione Donor Anti-6x His Acceptor	Ternary complex cannot be differentiated due to high background signal; poor S/B ratios
	1	1.09	1.33	1.48	1.41		
	2.5	1.01	1.08	1.08	1.29		
	5	0.89	0.86	0.89	0.88		
	0	0.94	1.13	0.97	0.90	Anti-6x His Donor Anti-GST Acceptor	Ternary complex cannot be differentiated due to high background signal; poor S/B ratios
	1	0.94	1.49	1.48	1.33		
	2.5	1.01	1.43	1.27	1.23		
	5	0.89	1.20	1.16	1.07		

After selecting the Glutathione Donor and Anti-FLAG Acceptor bead pairing for further use, a final determination could be made on BRD4 and CRBN Complex concentrations to be used in the assay. Based on results of the cross titration of these two compounds (Figure 5, Table 1), 1 nM BRD4 with 1 nM CRBN Complex were selected, as they produced strong signal with a good S/B ratio, while conserving the amount of protein needed in the assay. Increasing the concentrations of BRD4 and CRBN Complex above 1 nM did not increase signal.

Selection of assay buffer

The assay buffer matrix plays an important role in protein interaction assays, thus an experiment was conducted to test assay performance with various types of buffers. Three buffers were selected for comparison, including Protein-Protein Interaction (PPI) Buffer, Binding Assay Buffer, and

Universal Assay Buffer. A complete set of ternary complex assay reagents was prepared in each buffer type (1 nM GST-BRD4 protein and 1 nM CRBN Complex with 100 nM dBET6 PROTAC; Glutathione Donor beads with Anti-FLAG Acceptor beads used for detection).

As seen in Figure 6, AlphaLISA signal was significantly impacted by the buffer matrix. Binding Buffer produced much higher absolute signal compared to PPI and Universal Buffer and demonstrated lower background signal compared to PPI Buffer. Results of this experiment emphasize the importance of testing various buffer types when building a ternary complex assay, as certain buffers may improve signal or impede detection of the ternary complex due to the components that make up each assay buffer (e.g. the concentration of detergent or BSA in the matrix). Based on its performance, Binding Buffer was used in all subsequent experiments.

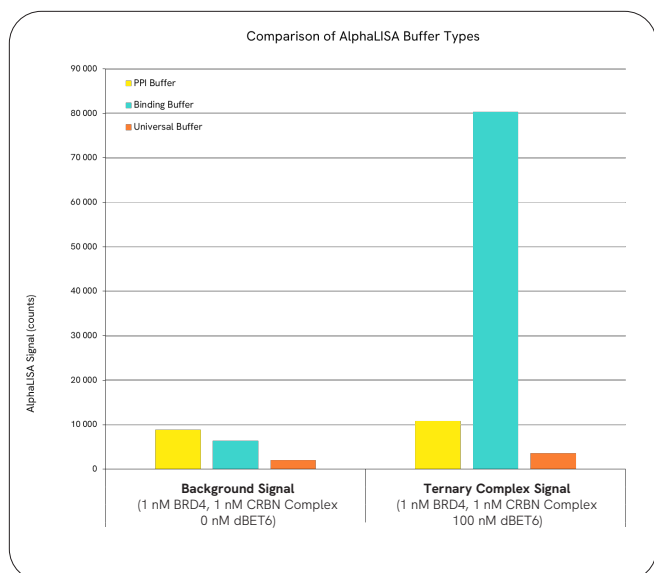


Figure 6: Comparison of AlphaLISA signal based on assay buffer type (yellow bars: PPI Buffer; turquoise: Binding Buffer; green: Universal Buffer). Results depict signal from the ternary complex (1 nM BRD4, 1 nM CRBN Complex, and 100 nM dBET6) versus background signal.

Titration of PROTAC in a ternary complex and signal specificity tests

Following optimization of the assay components, a full range of dBET6 concentrations (2.5-fold dilution from 62,500 nM to 0.42 nM) were titrated against fixed concentrations of 1 nM BRD4 and 1 nM CRBN Complex to determine the optimal concentration of PROTAC for ternary complex formation. Titration of the PROTAC is a critical step in developing a ternary complex assay, as excess PROTAC can result in the formation of binary complexes (PROTAC bound only to either the target protein or E3 ligase) which can impede formation of the ternary complex and, subsequently, protein degradation. By titrating the PROTAC with the target protein and E3 ligase, results can be plotted as a bell-shaped curve, where concentrations greater than the peak of the curve demonstrate a hook effect resulting from an overabundance of binary complexes. A related PROTAC: dBET1, was also serially diluted following the same procedure for comparison of affinity and assay hook points of the two PROTAC molecules.

To demonstrate the AlphaLISA signal measured was specific to detection of the ternary complex formation between BRD4, CRBN Complex and the PROTAC, several control molecules were tested in place of dBET6. In these experiments, the control molecules bind with one partner (either the target protein or E3 ligase), thereby inhibiting any interactions between the protein and E3 ligase that would result in an increase in AlphaLISA signal. Control molecules included thalidomide and JQ1 (which bind to the CRBN Complex and BRD4, respectively). An irrelevant PROTAC molecule: MT-802 was also tested for signal specificity. MT-802 does not recognize the BRD4 protein and thus, ternary complex formation and associated AlphaLISA signal should not occur.

As expected, a full titration of the PROTAC dBET6 demonstrated a bell-shaped curve, with a hook point observed at ~100 nM (Figure 7). The PROTAC dBET1 also produced a bell-shaped curve demonstrating successful formation of a ternary complex with this PROTAC, however, the hook point was slightly increased (~250 nM), suggesting dBET1 to be less efficient at forming ternary complexes compared to dBET6. Previous studies have proposed dBET6 to be a more potent degrader than dBET1 (possibly due in part to the longer linker length), with protein degradation found to be significantly greater in cells treated with dBET6 compared to dBET16. In contrast, the irrelevant PROTAC, MT-802, showed low AlphaLISA signal that was not different from background. This pattern is indicative of a lack of ternary complex formation (as expected) and illustrates the specificity of the Toolbox assay developed for the target protein and E3 ligase used in this study. Similarly, no increase in signal was observed with the control molecules thalidomide and JQ1, further demonstrating specificity of the AlphaLISA signal for ternary complex detection.

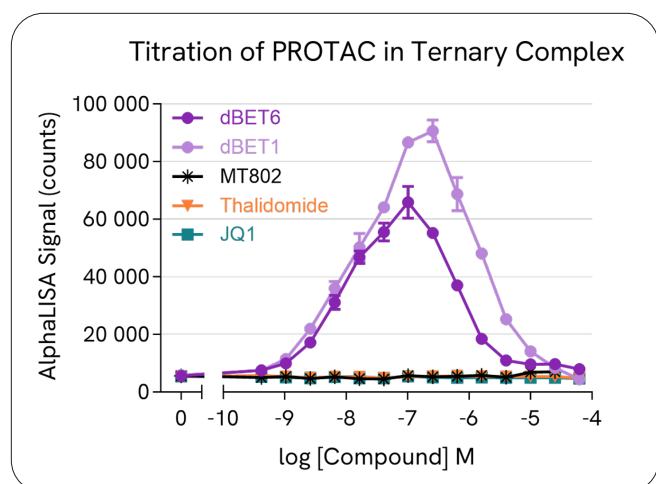


Figure 7: Titration of PROTACs in the ternary complex. Titrating a PROTAC molecule with its target protein and E3 ligase results in a bell-shaped curve which can be used to determine the hook point of the assay. The study PROTAC, dBET6 (dark purple circles) was compared with a related PROTAC, dBET1 (light purple circles) and found to be more efficient at forming ternary complexes due to a lower hook point concentration. In contrast, an irrelevant PROTAC, MT-802 (black stars), and the inhibitor molecules thalidomide (orange triangles) and JQ1 (teal squares) showed no increase in AlphaLISA signal, demonstrating specificity of the Alpha signal for ternary complex formation.

Conclusion

This application note demonstrates that AlphaLISA Toolbox reagents can be successfully applied to detect the formation of ternary complexes for targeted protein degradation applications, such as screening PROTAC molecules for drug discovery and development. The interchangeable nature of AlphaLISA Toolbox reagents offers users the powerful ability to build custom assays; however, testing should be conducted to determine the optimal assay setup for the targets of interest. Results from this study highlight the importance of testing different bead combinations to ensure signal from the ternary complex can be distinguished from background, as well as to determine the best pairings of Donor and Acceptor beads to maximize the S/B ratio. In addition, the buffer matrix plays a critical role in the ternary complex assay. In this case, Binding Buffer performed much better compared to other buffers, but results may vary depending on the components of the ternary complex. If initial attempts at a

ternary complex assay are unsuccessful, a change in buffer type should be considered while troubleshooting. Further optimizations beyond those presented in this study can also be conducted, including testing different tag-types on the target protein or E3 ligase, altering the concentration of Donor and/or Acceptor beads, modifying incubation times, or altering the order of reagent additions, which may offer additional improvements to the ternary complex assay.

Materials and detailed methods

Assay protocol

A detailed list of all reagents and product numbers used in the study is in Table 2. A simple protocol was followed for all assays in which the three components of the ternary complex (target protein, E3 ligase, and PROTAC; 10 μ L each) were incubated together in the assay plate (384-well AlphaPlate (Revvity, #6005350)) for 90 minutes at room temperature (RT) to allow for formation of the ternary complex. Following incubation, 10 μ L of AlphaLISA Donor beads and 10 μ L of Acceptor beads (each at 20 μ g/mL final concentration) were added. The assay was incubated for an additional 90 minutes at RT in darkness to allow for binding of the beads to their targets on the ternary complex before reading the plate on an EnVision™ 2105 multimode plate reader (Revvity, #2105-0010) using Alpha settings. Wells were run in triplicate and data are reported as the average AlphaLISA Signal counts, or as AlphaLISA Signal-to-Background (S/B). For this study, background signal was defined as the signal measured when the target protein and E3 ligase were present in a well without PROTAC (0 nM PROTAC; *i.e.* no ternary complex formation) to account for any protein-protein interactions that may have occurred outside of the ternary complex.

Unless otherwise noted, all reagents were prepared in 1X AlphaLISA Binding Assay Buffer. Assay reagents were prepared to 5X concentration such that when added to the assay, the final concentration in the plate well was 1X (in 50 μ L total assay volume). Reagents were diluted such that the final concentration of DMSO in each well was kept below 0.5%, in order to prevent interference from DMSO on the Alpha signal.

| Table 2. Assay and AlphaLISA Toolbox reagents.

Reagent	Company	Product number	Purpose
BRD4 (BD1+BD2), GST-Tag Recombinant	BPS Bioscience	31044	Ternary complex component
Cereblon/DDB1/Cul4A/Rbx1 complex, Recombinant (FLAG & 6x His tags)	BPS Bioscience	100329	Ternary complex component
dBET6	MedChemExpress	HY-112588	Ternary complex component
dBET1	MedChemExpress	HY-101838	Ternary complex component
MT-802	MedChemExpress	HY-122562	Control/inhibitor molecule
JQ1	MedChemExpress	HY-78695	Control/inhibitor molecule
Thalidomide	R&D system	652	Control/inhibitor molecule
Binding Assay Buffer, 10X	Revvity	AL018	Assay buffer*
Protein-protein interaction (PPI) Buffer, 5X	Revvity	AL015	Assay buffer*
Universal Assay Buffer, 5X	Revvity	AL001	Assay buffer*
Anti-FLAG Donor beads	Revvity	AS103	Ternary complex detection*
Anti-6x His Donor beads	Revvity	AS116	Ternary complex detection*
Glutathione Donor beads	Revvity	6765300	Ternary complex detection*
Anti-GST Acceptor beads	Revvity	AL110	Ternary complex detection*
Anti-6x His Acceptor beads	Revvity	AL178	Ternary complex detection*
Anti-FLAG Acceptor beads	Revvity	AL112	Ternary complex detection*

*Indicates AlphaLISA Toolbox reagent

Titration of PROTAC in a ternary complex and signal specificity tests

To titrate the PROTAC, dBET6 was serially diluted 2.5-fold from 62,500 nM to 0.42 nM (6.25e-5 to 4.2e-10 M). To ensure comparability of results across concentrations, the dilution curve was prepared in a solution of Binding Buffer plus 1.25% DMSO, such that the entire dilution range contained the same final percentage (0.25%) of DMSO in the plate wells. The dBET6 dilutions were incubated with fixed concentrations of 1 nM BRD4 and 1 nM CRBN Complex (selected based on optimization experiments) following the assay protocol. Glutathione Donor beads and Anti-FLAG Acceptor beads (20 µg/mL final concentration) were used for detection. AlphaLISA signal counts were plotted to determine the hook point of the curve. A related PROTAC molecule: dBET1 was also serially diluted (62,500 nM to 0.42 nM) following the same procedure and separately incubated with 1 nM BRD4 and 1 nM CRBN Complex to compare the hook points and affinities of the two PROTACs. The dBET1 PROTAC also features a JQ1-derivative warhead and thalidomide ligand but has a shorter linker than dBET6.

Control molecules of thalidomide, JQ1, and an irrelevant PROTAC: MT-802 were also individually tested in place of dBET6 in order to demonstrate the AlphaLISA signal was specific to the formation of the ternary complex with BRD4, CRBN Complex, and dBET PROTACs. Thalidomide binds to CRBN Complex and JQ1 binds to BRD4 thereby impeding any interaction between the POI and E3 ligase which could result in AlphaLISA signal. The irrelevant PROTAC MT-802, features a ligand for CRBN, but contains a warhead for the protein BTK (not present in the assay), thus MT-802 cannot bind with BRD4 and ternary complex formation would not occur. Control molecules were serially diluted 62,500 nM to 0.42 nM as described for dBET6 above and incubated with BRD4 and CRBN Complex (1 nM each) along with Glutathione Donor beads and Anti-FLAG Acceptor beads (20 µg/mL), as per the assay protocol.

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