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

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1 Overview

Revvity's AlphaLISA® Toolbox reagents are a customizable tool to study PROTACs for targeted protein degradation (TPD) applications. This study demonstrates the use of AlphaLISA Toolbox reagents to detect ternary complex formation within the TPD pathway and explores methods for optimizing ternary complex detection with AlphaLISA using a common TPD target.

- AlphaLISA (amplified luminescent proximity homogeneous assay) is a bead-based chemiluminescent assay technology which can be used to quantitate diverse targets.
- AlphaLISA Toolbox reagents offer customizability through interchangeable anti-TAG Donor and Acceptor beads that can bind to any tagged molecule of interest
- Toolbox reagents can be applied to investigate various stages (e.g. PROTAC binding) within the TPD pathway.

2 Introduction

As the field of TPD and PROTACs expands, there is a need for immunoassay technologies that can investigate key stages of PROTAC interaction. PROTAC mediated degradation is a multi-step process (Fig 1a). The formation and stability of E3 ligase-PROTAC-target protein ternary complexes is critical for effective protein degradation. AlphaLISA Toolbox reagents (anti-TAG Donor and Acceptor beads; Fig 1b) were tested for effectiveness in detection of the ternary complex for a common TPD target. Ternary complex components included:

- *Protein of interest (POI):* recombinant (GST-tagged) BRD4
- *E3 ligase:* recombinant (FLAG- and 6xHis-tagged) Cereblon/DDB1/Cul4A/Rbx1-Complex (CRBN-Complex)
- *PROTAC:* dBET6 (thalidomide ligand; JQ1-derivative warhead)

Modifications to other assay components, such as the target protein (POI) and E3 ligase concentrations, as well as buffer types were explored to optimize assay signal and detection. A workflow for developing a ternary complex detection assay is shown in Fig 1c.

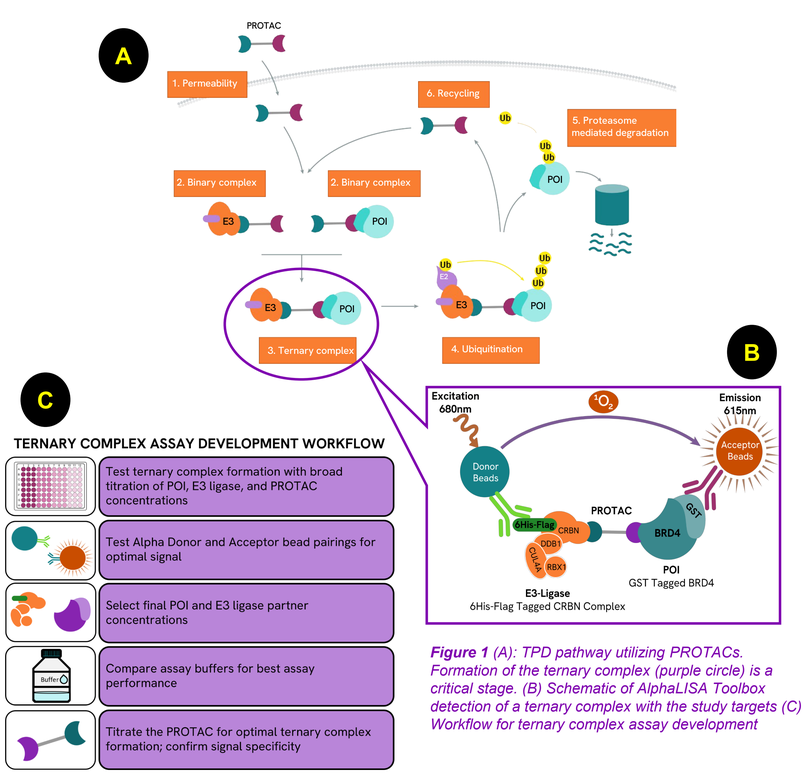


***Figure 1** (A): TPD pathway utilizing PROTACs. Formation of the ternary complex (purple circle) is a critical stage. (B) Schematic of AlphaLISA Toolbox detection of a ternary complex with the study targets (C) Workflow for ternary complex assay development*

3 Methods

General Assay Protocol:

- All three ternary complex components (10 µL each) were incubated together in a 384-well AlphaPlate for 90 min to allow formation of the ternary complex.
- Next, 10 µL each of AlphaLISA Donor and Acceptor beads (20 µg/mL final concentration) were added and incubated for 90 mins to allow binding of the beads to their targets on the ternary complex. The assay was then read on a EnVision multimode plate reader using AlphaLISA settings.

Testing Donor & Acceptor Bead Pairings / Protein Optimization:

- Four sets of Donor and Acceptor Bead Pairings (shown below) were tested to determine best performance in the assay, based on signal to background (S/B) ratio.

Donor Bead Type	Donor Bead Binding Partner	Acceptor Bead Type	Acceptor Bead Binding Partner
Anti-FLAG	CRBN Complex	Anti-GST	BRD4 protein
Glutathione	BRD4 protein	Anti-FLAG	CRBN Complex
Glutathione	BRD4 protein	Anti-6x His	CRBN Complex
Anti-6x His	CRBN Complex	Anti-GST	BRD4 protein

- Simultaneously, a narrowed range of BRD4 protein (0.25 - 1 nM) and CRBN Complex E3 ligase (1 - 5 nM) concentrations were tested to determine the optimal concentrations for use in the assay. [Narrowed range was determined based on an initial broad titration experiment; data not shown].

Comparison of Buffer Types:

- Three AlphaLISA buffers were tested for best performance in the assay based on S/B ratios: AlphaLISA Protein-Protein Interaction Buffer (PPI Buffer); Binding Buffer; and Universal Assay Buffer.

Titration of PROTACs in a Ternary Complex:

- Using the optimal protein concentrations (1 nM each of BRD4 & CRBN Complex), bead pair (Glutathione Donor Beads + Anti-FLAG Acceptor Beads), and buffer type (Binding Buffer), the PROTAC, dBET6, was titrated across a broad range of concentrations (0.42 – 62,500 nM) to determine the hook point of the assay.
- An additional PROTAC: dBET1, was tested, as well as control molecules (no signal expected) to demonstrate specificity of the assay for ternary complex detection.

4 Results

Testing Donor & Acceptor Bead Pairings / Protein Optimization:

- Glutathione Donor Beads & Anti-FLAG Acceptor Beads performed best in the assay, as indicated by the highest S/B ratios (Table 1).
  Anti-6x His beads, used either as the Donor or Acceptor, resulted in poor S/B ratios and were not useful in detection of the ternary complex. Anti-FLAG Donor Beads + Anti-GST Acceptor Beads had low to moderate S/B ratios.
- Based on results of the cross-titration of BRD4 and CRBN Complex, 1nM concentration was selected for both molecules, as this produced a strong signal, while conserving the amount of protein needed in 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		

Table 1. Comparison of Signal-to-Background (S/B) ratios of AlphaLISA Toolbox bead pairings for detection of the CRBN Complex-dBET6-BRD4 protein ternary complex. Ratios were compared across concentrations of CRBN complex & BRD4 protein to determine the best concentrations for use in the assay. Orange indicates poor S/B ratios, while green highlights acceptable S/B ratios.

Comparison of Buffer Types:

- Buffer type significantly impacted assay signal and ability to detect the ternary complex with Toolbox reagents.
- Binding Buffer produced the highest signal-to-background ratios and offered the best performance (Fig 2).
- Universal Buffer and PPI Buffer had low S/B ratios that did not allow for discrimination of the ternary complex.

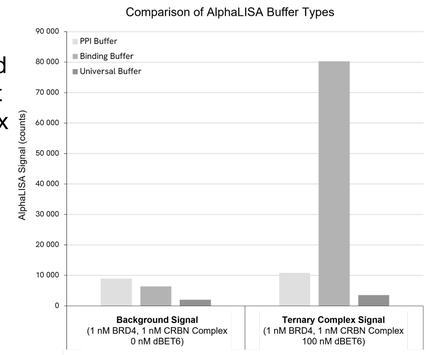
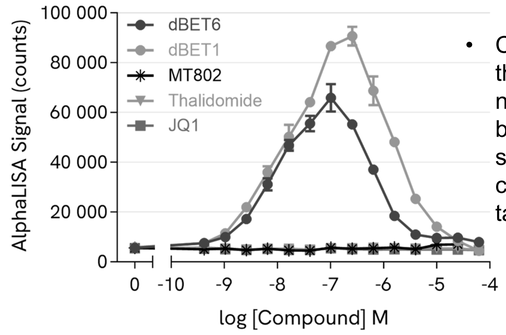


Figure 2. Comparison of AlphaLISA signal based on assay buffer type (yellow bars: PPI Buffer; turquoise: Binding Buffer; orange: Universal Buffer). AlphaLISA signal for the ternary complex is shown compared to background signal.

Titration of PROTACs in a Ternary Complex:

- A full titration of the PROTAC dBET6 demonstrated a bell-shaped curve, with a hook point observed at ~100 nM (Fig 3).
- A related PROTAC, dBET1, also produced a ternary complex signal, however, the hook point was slightly increased (~250 nM), suggesting dBET1 to be less efficient at forming ternary complexes.



- Control molecules: MT802, thalidomide, & JQ1 showed no signal above. background, demonstrating specificity of the ternary complex assay for the target compounds.

Figure 3. Titration of PROTAC and control compounds in the ternary complex assay allows for comparison of PROTAC efficiency at forming a ternary complex, as well as demonstrates specificity of the ternary complex assay for the selected targets.

5 Conclusions

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. With Toolbox assays it is important to test different bead combinations to ensure the ternary complex signal can be distinguished from background. In addition, the buffer matrix plays a critical role in the ternary complex assay.

Further optimizations, including testing additional tag-types, concentrations of Donor and Acceptor beads, modified incubation times, or altering the order of reagent additions, may offer additional improvements to the ternary complex assay.