APPLICATION NOTE

Developing novel tetraubiquitin substrates and AlphaLISA technology to provide next generation deubiquitinase assays.

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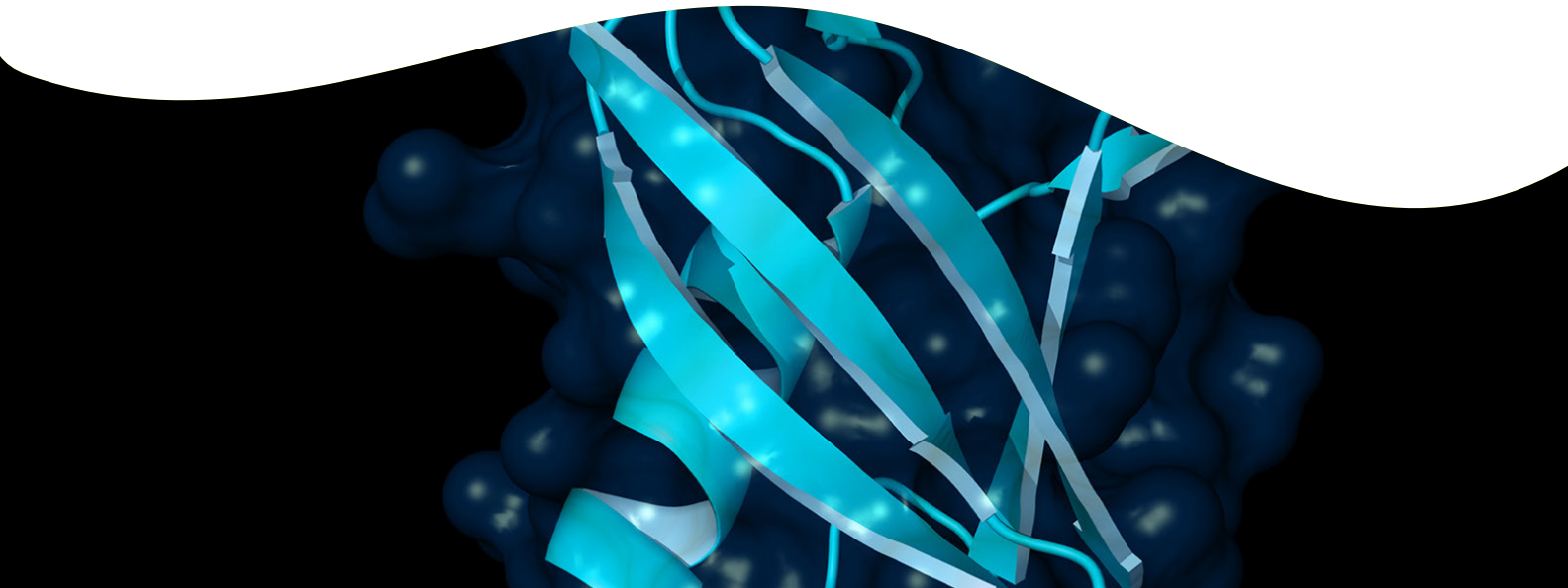
Introduction

The modification of cellular proteins with ubiquitin is an important physiological event that affects multiple aspects of protein stability and function. Ubiquitination is a dynamic, reversible process in that attached ubiquitin can be removed from proteins by deubiquitinating enzymes (DUBs).[1] DUBs are a highly specialized class of cysteine proteases that remove mono- and/or polyubiquitin chains from protein substrates. Ubiquitination (or deubiquination) may result in a variety of different fates for a protein substrate, including protein activation or deactivation, proteasome-mediated degradation, altered cellular localization, or modulation of protein-protein interactions.[2] Due to their importance in these physiological processes, abnormal DUB function has been implicated in cancer and a number of other disease states.[3]

Currently, most DUB assays utilize fluorescent substrates that are derivatives of monoubiquitin. It is possible that these may not sufficiently mimic the natural substrates that a DUB acts upon *in vivo*. In the search for superior lead compounds to enter into the clinic, more researchers are seeking to develop screening assays that better recapitulate the natural environment of the target molecule. One way to accomplish this is to interrogate polyubiquitinases and their interactions with DUB enzymes. In order to target polyubiquitinases, specific Tandem Ubiquitin Binding Entities (TUBEs) were developed for the isolation and identification of ubiquitinated proteins.[4]



Utilizing TUBEs aids in overcoming issues with the low binding affinity and stability of complexes using standard monomer ubiquitin binding domains (UBD). TUBEs have a 100 to 1000-fold increase in binding affinity for polyubiquitin chains compared to UBDs and form a stabilizing structure with ubiquitylated proteins. Here we demonstrate novel biochemical DUB assays that utilize TUBEs to bind to tetraubiquitin substrates of at least three natural linkages, including K48, K63, and linear. The TUBEs and tetraubiqutin reagents are combined with Revvity's AlphaLISA™ technology toolbox beads. Alpha (Amplified Luminescent Proximity Homogeneous Assay) is a highly flexible, homogeneous, no-wash assay ideal for the measurement of protein interactions and large protein complexes. Alpha assays utilize two bead types, Donor and Acceptor, each containing different chemical components key to Alpha technology. Donor beads contain phthalocyanine, a photosensitizer, which converts ambient oxygen to an excited and reactive form of O_2, singlet oxygen, upon excitation at a wavelength of 680 nM. During its 4 µsec half-life, the singlet oxygen can diffuse approximately 200 nM in solution. If the Acceptor bead is within this distance, the energy is transferred from the singlet oxygen to the thioxene derivatives within the Acceptor bead, resulting in the emission of light at 615 nM. If the Donor bead is not within 200 nM of the Acceptor bead, the singlet oxygen falls to its ground state and no signal is produced.

AlphaLISA has previously been coupled with TUBEs and used to detect ubiquitin activity and autoubiquitination.[5] A major benefit of using labeled beads is that the ubiquitin does not have to be tagged, reducing the possibility of an exogenous dye or tag interfering with the kinetics of the ubiquitination event. Using AlphaLISA Acceptor beads to capture the tetraubiquitin substrate and Streptavidin-coated Donor beads to capture the TUBEs, we were able to monitor signal decreases over time in the presence of different DUB enzymes that cleave the tetraubiquitin substrates under optimized assay conditions. FITC-labeled tetra-ubiquitin chains that are not cleaved by DUB enzyme bind to biotinylated-TUBEs. Anti-FITC Acceptor beads bind to teraubiquitin while streptavidin-coated Donor beads bind TUBEs. The introduction of different DUBs prior to adding TUBEs and beads results in cleavage of tetraubiquitins and a reduction in the Alpha signal (Figure 1). Here, we present data illustrating the utility of these assays and demonstrating the specificity of certain DUBs for tetraubiquitin substrates of different linkages. Our results demonstrate the power and versatility of Alpha technology as a tool in drug discovery programs for the identification and development of specific DUB inhibitors.

Materials and methods

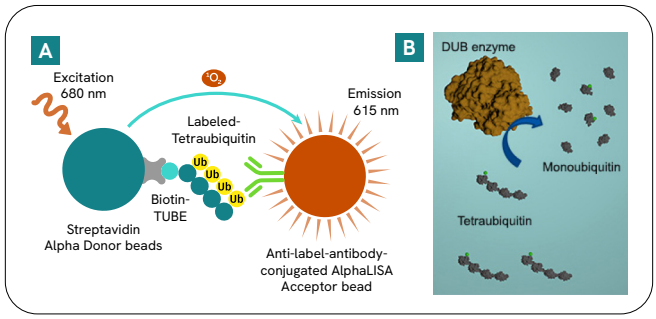


Figure 1: Assay principle. (A) AlphaLISA DUBs assay schematic. (B) Model of DUB enzyme breaking apart tetra-ubiquitin chains.

Tetraubiquitins, TUBEs, DUBs and inhibitors

All assays were performed using linear (also known as "Met1-linked"), K48- and K63-linked tetraubiquitin chains generated and purified at Boston Biochem using proprietary procedures. Biotinylated TUBEs were supplied by Boston Biochem. TUBEs have been developed for the isolation and identification of ubiquitinated proteins and display a protective effect on polyubiquitin proteins allowing for detection at low concentrations. Recombinant deubiquitinases were purified from either Sf21 insect cells (GST-AMSH, His_6-Cezanne) or bacteria (USP2 catalytic domain, His_6-Otubain 1, Otulin). A summary of Boston Biochem reagents is in Table 1. Revvity Alpha toolbox beads were used to develop these assays as screening tools.

Table 1: Reagents from Boston Biochem.

Reagents from Boston Biochem	Catalog #
rh USP2-His_6 (catalytic domain)	E-506
rh His_6-Otubain-1, isoform 1	E-522B
rh GST-AMSH/STAMBP	E-549
rh STAM-1	E-550
rh His_6-OTUD7B/Cezanne (full-length)	E-562
rh OTUD7B /Cezanne (catalytic domain)	E-563
rh FAM105B/OTULIN	E-558
rh Biotin-hHR23A Tandem UBA (TUBE1)	UBE-215
rh Biotin-Ubiquilin-1Tandem UBA (TUBE2)	UBE-115
rh Ubiquitin Aldehyde	U-201

Additional reagents

The reaction buffer consisted of: 50 mM HEPES at pH 7.3; 150 mM NaCl; 0.01% Triton X-100; 0.1% BSA; 2 mM TCEP. DTT was added to the enzyme buffer at a concentration of 0.45 mM in reaction (0.13 mM final concentration in well).

AlphaLISA enzymatic assay protocol

1. Add 5 µL of inhibitor (at 4X final concentration) in 5% DMSO.
2. Add 5 µL of DUB enzymes and 5 uL of tetraubiquitin (at 4X final concentration).
3. Incubate for 60 minutes at room temperature with gentle shaking.
4. Add 5 µL of TUBE1 (at 4X final concentration) and incubate for 10-15 minutes.
5. Add 10 µL of Acceptor beads (20 µg/mL final).
6. Incubate for 60 minutes at room temperature in the dark.
7. Add 10 µL of Donor beads (20 µg/mL final).
8. Read after 30 minutes incubation at room temperature in the dark.

Instrumentation

For the assays, plates were mixed slowly during incubation steps on a rotating shaker (Revvity DELFIA™ PlateShake). All DUB assays were measured on a Revvity EnVision™ multilabel plate reader using standard settings for Alpha detection (Figure 2). The EnVision system offers fast, sensitive Alpha detection technology, in addition to fluorescence, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence measurements, The incorporation of unique temperature controls within the system ensures accurate, reproducible results for Alpha and AlphaPlex™ assays.

Figure 2: EnVision multilabel plate reader for Alpha detection.

Data analysis

Inhibition curves were plotted and IC_{50} values calculated using GraphPad Prism (version 6.0) according to a non-linear regression using the four-parameter logistic equation sigmoidal dose-response curve with variable slope. All data points shown represent an average of at least three wells.

Results and discussion

Optimizing concentrations

When designing AlphaLISA protein-protein interaction assays such as this, the first step is to titrate several concentrations of the two proteins to ensure the assay is within the linear range, the signal-to-background ratio is high, and to ensure the concentrations chosen for subsequent enzymatic assays are below the hook point for each binding partner. Additionally, it is important to test various buffers to optimize the signal-to-background ratio for the assay. To determine concentrations of each tetraubiquitin and TUBE to be used, each component was titrated by halves from 100 nM through eight points and tested with all eight concentrations of the other component. Three tetraubiquitins (K48, K63 and linear) were crossed with both TUBE1 (data not shown) and TUBE2. Representative data for the K48 x TUBE2 titration are shown in Figure 3a. The final concentration of 3 nM was chosen for all tetraubiquitin linkages and TUBE2 (at 3 nM) was used for all subsequent assays.

To assess the tolerance of this assay for DMSO, a common diluent used for compound testing, the assay buffer was spiked with DMSO and titrated down 16 times by halves and tested. The data from this experiment indicate no significant effect of DMSO on binding or signal strength for concentrations ≤ 8% (Figure 3b).

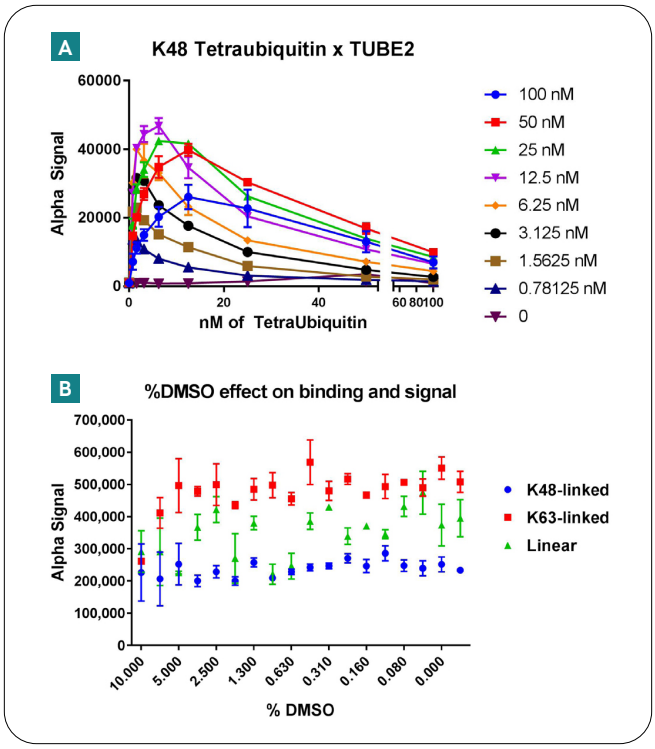


Figure 3: (A) 2D titration of K48, K63 and linear tetraubiquitins and TUBE2 (B) Effect of DMSO on the AlphaLISA DUB assay.

Non-linkage specific DUB

A non-linkage specific DUB, $USP2_{cd}$, was titrated and tested for its ability to cleave all three tetraubiquitins. The three tetraubiquitins tested were cleaved by $USP2_{cd}$ as seen in Figure 4. The maximal Alpha signal varies depending on the specific tetraubiquitin used in the assay (Figure 4a), so it is necessary to normalize these signals to background (i.e. no enzyme) for proper comparison (Figure 4b).

Linkage-specific DUB

One important feature of DUB enzymes is their specificity and ability to discriminate between different ubiquitin chain linkages. DUBs known to prefer specific linkages were assayed for their ability to cleave three different tetraubiquitins (Figure 5). Otubain 1 demonstrated specificity for K48-linked tetraubiquitin, whereas Otulin preferred the linear chain. AMSH demonstrated K63-linked specificity and activity was enhanced by STAM1. Cezanne, known to prefer K-11 linkages, was shown to also cleave K63-linked chains since both are full length and contain the catalytic domain.

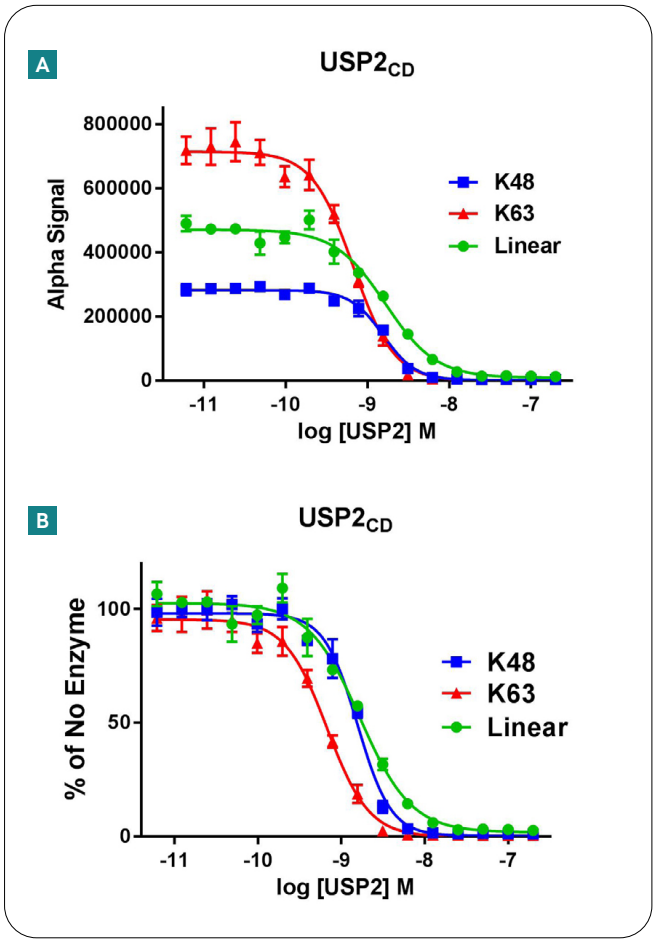


Figure 4: (A) Alpha signal versus concentration of the non-specific DUB, $USP2_{cd}$ showing cleavage of the three tetraubiquitins tested (B) Alpha signal normalized to no enzyme versus concentration of non-specific DUB, $USP2_{cd}$.

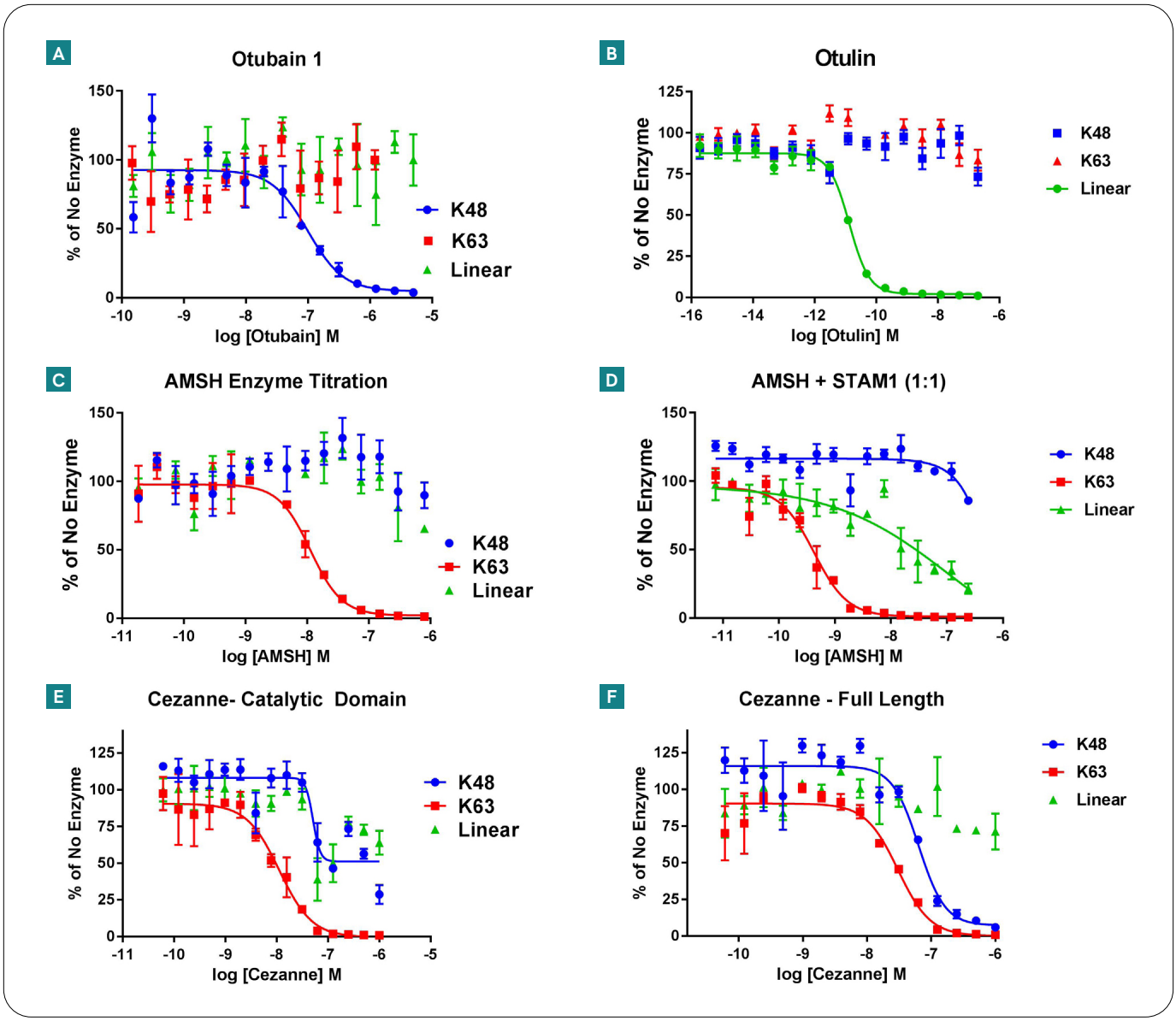


Figure 5: Alpha signal versus concentration of the specific DUBs showing cleavage that is linkage-specific.

Inhibitor titration

The AlphaLISA assay utilizing novel tetraubiquitin substrates is amenable to generating IC_{50} curves for specific inhibitors allowing this assay to be used for better understanding DUB enzyme function as well as screening for new inhibitor compounds. Figure 6 shows data from a titration of the cysteine protease inhibitor, ubiquitin aldehyde. Ubiquitin aldehyde is a potent, specific inhibitor of DUBs. This protein prevents the hydrolysis of polyubiquitin chains, enhancing ubiquitin chain accumulation.

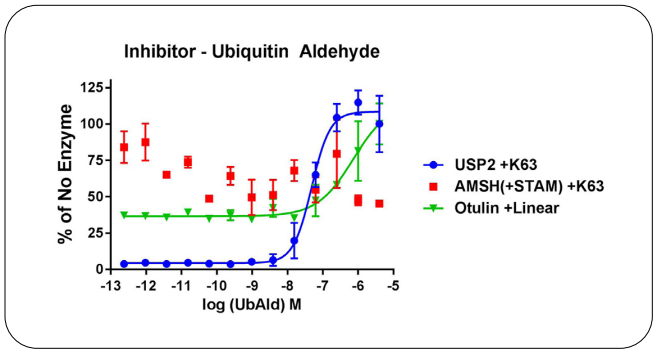


Figure 6: Alpha signal versus concentration of ubiquitin aldehyde in the presence of the DUB enzymes USPS-2, AMSH/STAMBP, and Otulin (linear).

Multiplexing DUB assays

The utility of AlphaLISA can be further extended to allow multiplexing for the detection of two assays in one well. By using both Europium and Terbium Acceptor beads to associate with different labels, two tetraubiquitin linkages can be assayed in the same well. Figure 7 shows the effects of $USP2_{cd}$ on the K63 and K48 linkages and the K63 and linear linkages.

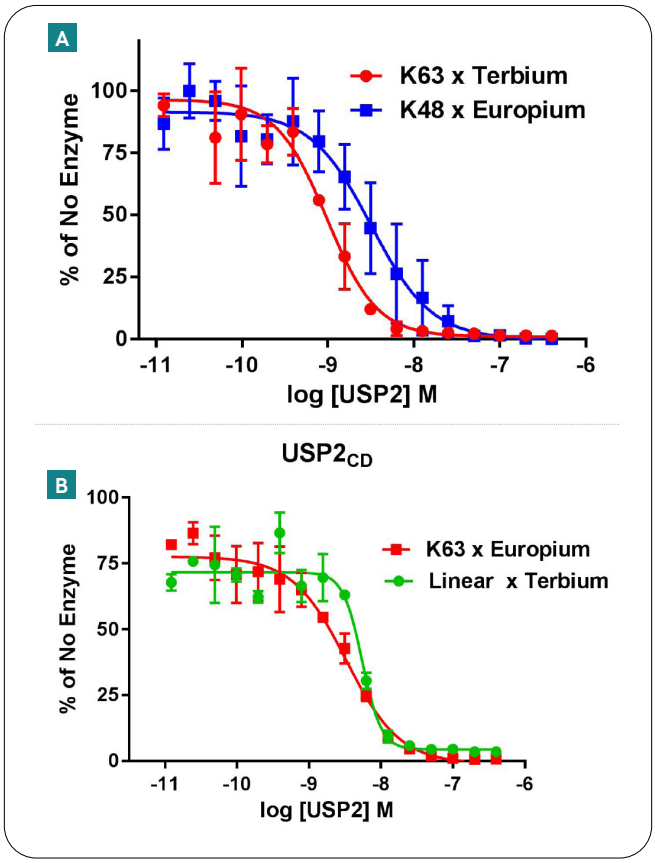


Figure 7: AlphaPlex multiplexing using both Europium and Terbium Acceptor beads targeted to different labels.

Conclusions

The AlphaLISA assays described here can be used in a multifaceted approach to better screen for compounds to modulate DUB activity on more physiologically-relevant ubiquitin chains. By combining linkage-specific tetraubiquitins and TUBEs developed by Boston Biochem with Revvity's AlphaLISA technology, we have demonstrated a robust, physiologically-relevant DUB assay. Here we show this AlphaLISA DUB assay can differentiate between linkage-specific DUBs, measure assay inhibition, generate IC_{50} curves, and can be multiplexed within a single well. This technology can be extended to understand more linkages (K6, K11, K27, K29 for example) to test more DUB enzymes and specific inhibitors.

References

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