In Vitro Proximity and Ubiquitylation Assays for CRBN, VHL and Emerging Ligases of Interest in TPD

Zachary Stiffler [1] , Daniel Banas [1] , Jean Qiu [2] , Peter Li [1] , Polly Mitchell [2] , Shahedul Islam [3] , Bradley Brasher [1]

[1]Trilogie Bioscience, [2]Conifer Life Sciences Group, [3]NanoTemper Technologies

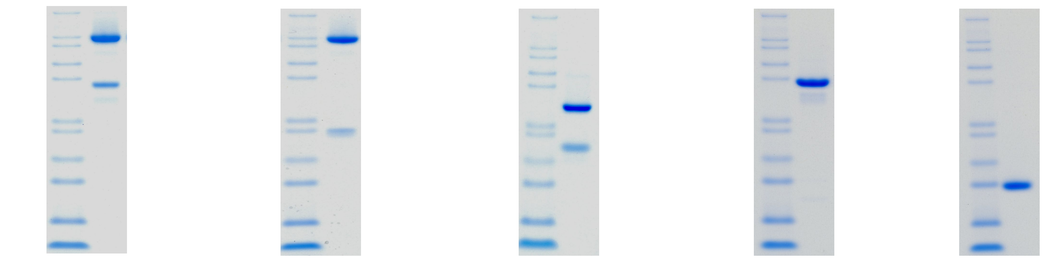
Introduction

Trilogy offers top-quality products and CRO services. Our innovative *in vitro* assays complement cell-based methods, providing valuable insights in TPD workflows. We feature HTS-compatible reagents and assays for screening and mechanistic studies, including highly active enzymes such as E3 ligases **DCAF16**, **KLHDC2**, **FBXO22**, **FBXO31**, and **FEM1B**. Our approaches incorporate HTRF, second-generation Alpha, spectral shift readouts, and multi-component substrate ubiquitylation assays, ensuring comprehensive and reliable data for your research needs.

In vitro proximity assays are valuable in drug discovery workflows because they help identify and characterize the interactions between small molecules and their biological targets. These assays can detect whether a compound binds to or influences the proximity of two or more proteins, which is useful for understanding mechanisms of action and optimizing lead compounds. Orthogonal to cell-based assays, which frequently supply a “yes or no” readout with little mechanistic insight, *in vitro* assays allow precise control over experimental variables such as compound concentration, environment, and assay components. These assays are highly specific, sensitive, and rapid, making them ideal for high-throughput screening campaigns and mechanistic studies. A wide variety of HTS-amenable assay formats are available for *in vitro* assays, such as AlphaLISA (Amplified Luminescent Proximity Homogeneous Assay), HTRF (Homogeneous Time-Resolved Fluorescence), FRET (Förster Resonance Energy Transfer) and FP (Fluorescence Polarization), complementing other biophysical techniques such as SPR (Surface Plasmon Resonance), ITC (Isothermal Titration Calorimetry), TSA (Thermal Shift Assay) and Spectral Shift.

Reagents

Every reagent from Trilogie is designed with specific applications in mind. Our enzymes, proteins and reagents are not just pure—they are functionally tested in meaningful ways including ternary proximity assays, *in vitro* ubiquitylation assays and others.



CRBN/DDB1 Complex | DCAF16/DDB1 Complex | FBXO22/SKP1 Complex | BRD4 (49-463), AVI-tag | FKBP12, AVI-tag

Ternary Complex Binding Assays

Binding assays exist in a variety of forms, each having its strengths and limitations. For example, SPR and BLI are excellent for analyzing binding kinetics, but these are low throughput techniques. ITC provides highly accurate protein-ligand thermodynamics data, but the technique requires a significant amount of protein. For HTS-compatible screening campaigns, two commonly used binding assays are HTRF and AlphaLISA. These assays rely on protein pairs bringing together dyes (HTRF) or beads (Alpha) in response to a proximity-inducing ligand. Successful ternary complex formation (protein-ligand-protein) generates a fluorescent signal that is monitored by a sensitive platereader. In contrast to HTRF and AlphaScreen, the Spectral Shift technology requires only one environmentally sensitive fluorophore attached to the target protein. It is a direct target engagement method that allows monitoring of both binary and ternary complex formation.

A common trait of proximity assays involving bivalent ligands is the "hook point" effect, a phenomenon where assay signal unexpectedly decreases at very high concentrations of target analyte. At optimal concentrations, ligands bring the proteins into proximity, so a detectable signal is generated. However, at very high analyte concentrations, each detection probe binds separately to different target molecules rather than both binding to the same target. This prevents formation of the required probe–target–probe complex, reducing proximity-dependent signals, as shown in Figures 1 and 2.

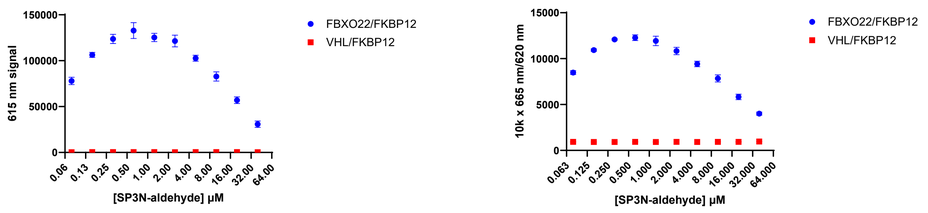


Figure 1. SP3N-aldehyde forms a ternary complex with FBXO22 and FKBP12
Binding reactions were run in 384w plates. Wells contained 25 nM N-terminal labeled (biotinylated AVI-tag) FKBP12, 25 nM VHL/ELOB/ELOC complex or FBXO22/SKP1 complex, and various concentrations of SP3N-aldehyde (a PROTAC molecule specific for FBXO22 and FKBP12).[1] SP3N-aldehyde mediated ternary complex formation between FBXO22 and FKBP12, but not the negative control VHL, was observed using both AlphaLISA (left panel) and HTRF (right panel) assay formats.

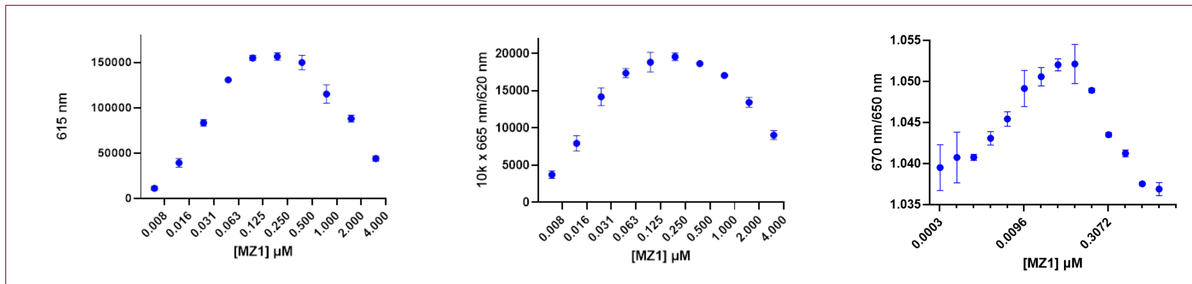


Figure 2. MZ1 forms a ternary complex with VHL and BRD4
Binding reactions were run in 384w plates. For AlphaLISA and HTRF assays, wells contained 50 nM N-terminal labeled (biotinylated AVI-tag) BRD4, 50 nM VHL/ELOB/ELOC complex and various concentrations of MZ1 (a PROTAC molecule for VHL and BRD4). For Spectral Shift assays, wells contained 50 nM VHL/ELOB/ELOC labeled with 2nd Generation maleimide-Red (Nanotemper), BRD4, and various concentrations of MZ1. MZ1-induced VHL-BRD4 binding was observed using AlphaLISA (left panel) and HTRF (middle panel) and Spectral-Shift (right panel) assay formats.

Once hook point analysis has been used to determine the optimal ligand concentration for ternary complex formation, assays may be designed to identify molecules that interfere with some aspect of the binding event. This approach can be useful in screening compounds for their ability to bind one or the other proteins in the complex, without having to generate the complete bivalent ligand. An example of this is shown in Figure 3 below.

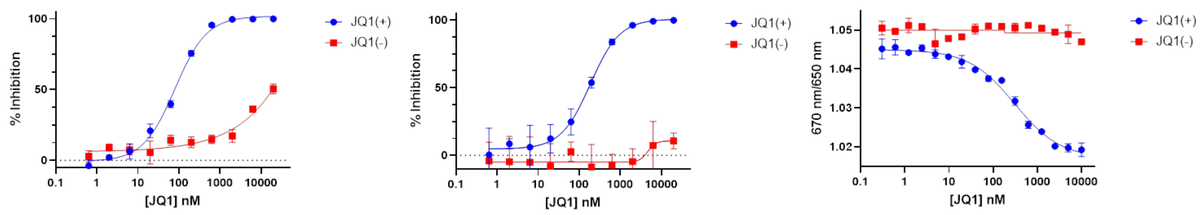


Figure 3. JQ1(+) inhibits VHL-MZ1-BRD4 Ternary Complex Formation
Binding reactions were run in 384w plates. For AlphaLISA and HTRF formats, wells contained 25 nM N-terminal labeled (biotinylated AVI-tag) BRD4, 25 nM VHL/ELOB/ELOC complex, and 100 nM MZ1 (a PROTAC molecule for VHL and BRD4) For Spectral Shift assays, wells contained 25 nM VHL/ELOB/ELOC labeled with 2nd Generation maleimide-Red (Nanotemper), 25 nM BRD4, and 100 nM MZ1. JQ1(+), a competitive inhibitor of MZ1, was added to wells at concentrations from 1 nM to 20 µM. Inhibition of VHL-MZ1-BRD4 complex formation was measured using AlphaLISA readout (left panel) or HTRF readout (right panel). Observed JQ1(+) IC_{50} values (80 nM, AlphaLISA; 190 nM, HTRF; 300 nM, Spectral Shift) were in good agreement with literature reports.[2] Dose-response curves were also generated using JQ1(-), an inactive stereoisomer of JQ1(+), providing a negative control. IC50 values for JQ1(-) were greater than 10 µM in all assay formats, also consistent with literature reports.

In Vitro Ubiquitylation Assays

In vitro polyubiquitylation assays are utilized to confirm results from ternary complex assays, cell-based assays and other formats. Proximity assays (ternary complex) may indicate that a PROTAC or molecular glue induces an interaction between and E3 ligase and protein of interest, but no data is provided regarding the ligase’s ability to ubiquitylate the bound neosubstrate. Further, *in vitro* assays can provide mechanistic insight that supplements findings from cell-based assays

In vitro ubiquitylation assays consist of (minimally) a functional E3 ligase, an appropriate E2 (or E2’s), ubiquitin, recombinant (or IP’d) target of interest, and Mg-ATP solution. Readouts for the assays include Western Blot, Simple Western, and in some cases, a proximity assay based on TUBE reagents.

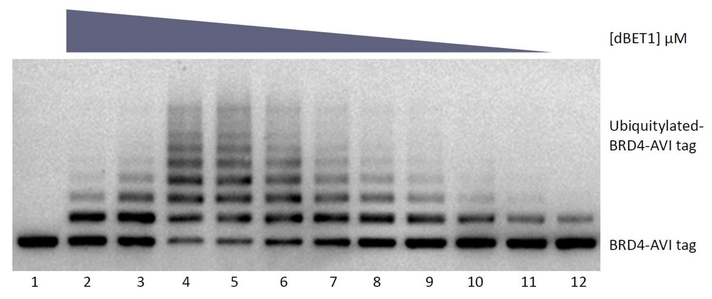


Figure 4. dBET1-dependent, *in vitro* polyubiquitylation of BRD4 by CRL4CRBN
All *in vitro* reactions contained CRBN/DDB1/CUL4A/RBX1, UBE2D1, UBA1, ubiquitin and N-terminal labeled (biotinylated AVI-tag) BRD4. Reaction time was 90 minutes at 37°C. Reactions were run out on reducing, 4-12% SDS-PAGE gels, transferred to nitrocellulose, then developed with streptavidin-HRP to visual BRD4.

Lane 1: no ATP in reaction (negative control)
Lanes 2-12: ATP in reactions; decreasing concentrations of dBET1 from 30 µM (Lane 2) to 30 nM (Lane 11)
Lane 12: ATP in reaction; no dBET1

About Triologie Bioscience

Trilogie Bioscience is a new company providing CRO services and catalog reagents in the UPS (broader) and TPD (focused) fields. We bring decades of combined experience in the field of ubiquitin research.

Hardware and Additional Reagents

- HTRF reagents including anti-His6-terbium cryptate and streptavidin-d2 were from Revvity
- 2nd generation AlphaLISA beads (“Omega” beads) including streptavidin-Sensibeads and anti-His6-Chemibeads were from Biosignal2.
- HTRF and AlphaLISA assays were measured using a BMG Labtech ClarioSTAR Plus platereader
- Spectral Shift assays were measured using a NanoTemper Dianthus system

References: 1. Kagiou, C., et al. (2024)Nature Communications 15: 5409 PMID: 38926334, **2.** Filippakopoulos, P., et al. (2010) Nature 468: 1067–1073 PMID: 20871596,. **3** Zengerle, M., et al. (2015) ACS Chem Biol 10(8):1770-1777 PMID: 26035625, **4**. Edmonds, A.K., et al. (2025) J Med Chem 68(9):9638-9660 PMID: 40244695