MST and TRIC Technology to Reliably Study PROTAC Binary and Ternary Binding in Drug Development

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Cover Page

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“Running Head”: **MST and TRIC to Study PROTACs in Drug Development**

i MST and TRIC Technology to Reliably Study PROTACs in Drug Development

ii Abstract

PROTACs have shown promise as a new class of therapy, with a unique mechanism of action orthogonal to traditional small molecules that are used to regulate protein activity. Their novel MOA utilizing the body's natural protein degradation machinery degrades a protein of interest rather than inhibiting its function. This strategy has several advantages over conventional small molecule inhibitors, e.g. higher sensitivity, less off-target effects and greater target space. However, unlocking the potential of PROTACs necessitates drug discovery techniques that can support the complexity of the novel MOA. In this chapter we describe the application of MicroScale Thermophoresis and Temperature Related Intensity Change to characterize both the binary and ternary binding of PROTACs with target proteins and ubiquitin ligases along with an efficient determination of the cooperativity of the ternary complex formation. The assay development and experimental procedure to characterize the well-described BET PROTAC MZ1 shows how MST and TRIC can be applied as a fast and highly sensitive method for PROTAC discovery.

ii Key Words

MicroScale Thermophoresis, MST, Temperature-related intensity change, TRIC, PROTAC

1 Introduction

To date most drug targets under pharmacological intervention exhibit well-defined active sites suitable for the binding of small molecular structures that act by inhibiting their activity[1]. This strategy has proven successful and resulted in a plethora of effective drugs against a variety of diseases[2–4]. However, most of these research efforts are focusing on the enzymatic function of target proteins, thereby neglecting any function related to their scaffold structures[5]. In addition,

there are many proteins, such as transcription factors, scaffold proteins, and non-enzymatic proteins, without well-defined pockets or active sites, hindering their use as effective therapeutic targets[6,7]. Such proteins are considered undruggable targets and comprise approximately 80 percent of the human proteome[8]. Besides those challenges in small-molecule drug discovery, drug-resistance has become a major obstacle of small-molecular drugs that are being applied in the clinic[9]. A recently introduced class of molecules known as proteolysis targeting chimeras (PROTACs) has the potential to change the paradigm of small molecule drug development and to solve the problems listed above. Instead of modulating the activity of the drug target, PROTACs make use of the ubiquitin-protease system, leading to its degradation[1,6,10]. PROTACs are designed as hetero bifunctional molecules that consist of two covalently linked protein-binding molecules, whereby one part can bind an E3 ubiquitin ligase, and the other part binds to the target protein meant for degradation[1,11]. The PROTAC thereby functions to bring the target protein and E3 ligase in close proximity to one another, enabling the transfer of ubiquitin from an E2 to the target protein, targeting it for degradation by the 26S proteasome[11,12].

Since their introduction, PROTACs have shown great potential for a large variety of target proteins[5]. This tremendous increase in the diversity of potential targets arises from a PROTACs ability to affect its target protein without the need to bind a specific, well-defined active site[13]. Another advantage of protein degraders comes along with their MOA, that results in the complete depletion of a protein of interest (POI), including its enzymatic activity and non-enzymatic functions[5]. Depletion of the POI can also help to combat drug-resistance that is often faced by traditional small molecule-based treatments[1]. Additionally, like enzymes, PROTACs can perform their function multiple times. This molecular recycling enables lower drug doses to be used, which in turn will reduce the risk for any off-target toxicities[14,15].

Despite the huge potential of protein degraders, identification of suitable PROTACs remains challenging and requires special consideration of the protein degrader's unique MOA. In addition to characterizing the interactions between small molecules and drug targets (a core component of traditional small molecule drug development), PROTAC development requires characterizing interactions with ubiquitin ligases, ternary complex formation, and the efficiency of ubiquitin transfer to the drug target. Thus, successful PROTAC development depends on techniques that not only are well suited to the types of proteins formerly considered to be undruggable but also can disentangle the complexities of interactions in a multicomponent system, all while meeting the requirements of traditional drug discovery of low sample consumption and high throughput.

In this chapter we show how MicroScale Thermophoresis (MST), a method commercialized in 2008 to measure interactions with difficult protein targets and applied to both high affinity small-molecule and low-affinity fragment screening[16–22], can be applied to PROTAC characterization. The technique records changes in fluorescence as a result of an IR-laser induced temperature change. While the fluorescence detection can be performed using the intrinsic fluorescence from tryptophan, interactions involving more than one protein (as are required in PROTAC development) are best characterized with an extrinsic fluorophore. There are two physical phenomena that contribute to the measured fluorescence signal: the first is thermophoresis, which is the directed movement of molecules within a temperature gradient, and the second is temperature related intensity change (TRIC), which is the temperature dependence of a fluorophore's fluorescence intensity. While thermophoresis is a global property of the entire molecule being measured and any ligands bound to it, TRIC is highly sensitive to the local environment of the fluorophore, which can be strongly affected by the binding of a small molecule, protein, or PROTAC.

In addition to its suitability to the difficult drug targets that comprise the traditionally undruggable target space, MST is also well suited to PROTAC discovery and development through the quantification of affinities ranging from pM to mM[23–26]. This is important because while early targets degraded by PROTACs made of existing high-affinity inhibitors, recent work have shown that lower affinity drug target ligands can be used for successful PROTAC construction, benefitting from potency enhancement through cooperativity of ternary complex formation[27]. Bondeson et al. showed that a PROTAC could degrade its target p38α at nanomolar concentrations, despite its weak binding affinity (Kd 10 uM) as a result of forming high-affinity ternary complexes, as shown by cellular pulldown experiments[28]. The same holds true for weak binding affinity at the E3 ligase. Testa et al. showed that a PROTAC that lost >20-fold binding affinity at the E3 ligase VHL (Kd 3 uM) compared to MZ1[29], retained potent and selective degradation of Brd4 at concentrations well below the binary binding affinity (DC50 10 nM), as a result of the large cooperativity of the ternary complex[30]. More recently, Han et al. reported similar findings that PROTACs made of weak-affinity VHL ligands can still work as potent degraders[31]. Together these amongst other studies[32], have demonstrated feasibility of achieving potent degraders made of weak-affinity ligands. As PROTACs induce neo protein-protein interactions between the target protein and the E3 ligase, they can potentially target different surfaces of the partner proteins, and consequently cooperativity and stability of ternary complexes have been found to differ greatly between different ubiquitin ligases and different POIs[27,33]. This allows for enhanced target selectivity but also makes the rational design of PROTACs difficult at the present stage[5]. Addressing these unknowns is best accomplished with robust in vitro high-throughput screening approaches for both binary interactions and ternary complex formation.

Here we describe an experimental protocol to characterize the binary and ternary interactions of the MZ1 PROTAC with E3 ubiquitin ligase and a set of different BET bromodomain constructs using MST[29,33,34]. The entire experimental procedure, starting with assay

development, followed by the MST measurement, and ending with data interpretation, is described in detail. The results show that MST is a powerful method for PROTAC development and can be used to rank involved molecules according to their cooperativity. Although the measurements shown in this chapter are based on a small set of POIs, MST can be applied for high-throughput screening projects by integrating the instruments into any liquid handling system[22].

2 Materials

Prepare all solutions using ultrapure deionized water (dH_2O) and analytical grade reagents. Prepare and store all buffers and all reagents at room temperature, unless stated otherwise. Keep proteins on ice during the experiments.

2.1 Protein Preparation

2.1.1 Proteins and Constructs

1. VHL (UniProt accession number: P40337)

2. ElonginC (UniProt accession number: Q15369)

3. ElonginB (UniProt accession number: Q15370)

7. $Brd2^{BD2}$ (344-455)

8. $Brd3^{BD1}$ (24-146)

9. $Brd4^{BD1}$ (44-178)

10. $Brd4^{BD2}$ (333-460)

2.1.2 Protein Production and Purification

- *Escherichia coli* BL21(DE3)

- Isopropyl β-D-1-thiogalactopyranoside (IPTG)

- Pressure cell homogenizer (Stansted Fluid Power)

- HisTrap FF affinity column (GE Healthcare)

- Imidazole

- TEV protease

- MonoQ and Superdex-75 columns (GE Healthcare)

- Nickel Sepharose 6 fast flow beads (GE Healthcare)

- Sepharose 6 fast flow beads (GE Healthcare)

- Äkta FPLC purification systems (GE Healthcare)

- Glass econo-columns (Bio-Rad)

2.2 Protein Labeling

1. NHS labeling buffer (NanoTemper Technologies GmbH, Germany)

   130 mM $NaHCO_3$ pH 8.2, 50 mM NaCl

2. Monolith Protein Labeling Kit RED-NHS 2nd Generation (Amine Reactive) (NanoTemper Technologies GmbH, Germany)
3. DMSO (100 %)
4. Vortexer
5. Assay buffer (NanoTemper Technologies GmbH, Germany)

50 mM Tris-HCl pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM GSH, 0.05 % Tween-20

6. NanoDrop
7. Benchtop centrifuge

2.3 Affinity Measurements with MST

1. 384-well microtiter plate
2. BET PROTAC MZ 1 (obtained from opnMe) stock solution: 5,1 mg suspended in 1 mL DMSO to yield a 51 mM suspension
3. Monolith NT.115 Premium Coated Capillaries (NanoTemper Technologies GmbH, Germany)
4. Monolith™ NT.115pico (NanoTemper Technologies GmbH, Germany)
5. MO.Control Software (NanoTemper Technologies GmbH, Germany)
6. MO.Affinity Analysis Software (NanoTemper Technologies GmbH, Germany)

2.4 SD-Test

1. SD-mix (NanoTemper Technologies GmbH, Germany)

   4 % SDS, 40 mM DTT
2. Non-binding microcentrifuge tubes
3. Heating block

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Protein Production and Purification

Protein production and purification was done by Dr. Klaus Rumpel and Dr. Andreas Zoephel at Boehringer Ingelheim, according to Morgan S Gadd et al [27].

Briefly, wild-type and mutant versions of human proteins VHL (UniProt accession number: P40337), ElonginC (Q15369), ElonginB (Q15370), Brd2 (P25440), Brd3 (Q15059) and Brd4 (O60885) were used for all protein expression. For expression of VCB, N-terminally His6-tagged VHL (54–213), ElonginC (17–112) and ElonginB (1–104) were co-expressed in *Escherichia coli* BL21(DE3) at 24 °C for 16 hrs using 3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). *E. coli* cells were lysed using a pressure cell homogenizer (Stansted Fluid Power) and lysate clarified by centrifugation. His6-tagged VCB was purified on a HisTrap FF affinity column (GE Healthcare) by elution with an imidazole gradient. The His6 tag was removed using TEV protease and the untagged complex dialyzed into low-concentration imidazole buffer. VCB was then flowed through the HisTrap FF column a second time, allowing impurities to bind, as the complex eluted without binding. VCB was then additionally purified by anion exchange and size-exclusion chromatography using MonoQ and Superdex-75 columns (GE Healthcare), respectively. The final purified complex was stored in 20 mM HEPES 7.5, 100 mM NaCl, 1 mM TCEP $Brd2^{BD2}$ (344–455), $Brd3^{BD1}$ (24–146), $Brd4^{BD1}$ (44–178) and $Brd4^{BD2}$ (333–460) as well as equivalent mutant constructs were expressed with an N-terminal His6-tag in *E. coli* BL21(DE3) at 18 °C for 20 hrs using 0.2 mM IPTG. His6-tagged BDs were purified on nickel Sepharose 6 fast flow beads (GE Healthcare) by elution with increasing concentrations of imidazole. BDs were then additionally purified by size-exclusion chromatography using a Superdex-75 column. All chromatography purification steps were performed using Äkta FPLC purification

systems (GE Healthcare) or glass econo-columns (Bio-Rad) at room temperature. The final purified proteins were stored in the corresponding storage buffer (see table 1), at -80 °C.

[Table 1 here]

3.2 Protein Labeling

1. Dilute proteins in NHS labeling buffer to prepare 100 µL of protein to be labeled at a concentration of 10 µM (see **Note 1**).
2. Suspend 10 µg of RED-NHS 2nd Generation fluorophore in 25 µL DMSO and vortex to ensure proper dye suspension. The final concentration will be 600 µM.
3. Mix 7 µL of the RED-NHS 2nd Generation fluorophore suspension with 7 µL NHS labeling buffer to prepare 14 µL of a 300 µM dye solution in 50 % DMSO.
4. Initiate the protein labeling reaction by adding 10 µL of the 300 µM dye solution to 90 µL of the 10 µM protein solution to yield 100 µL of dye-protein solution with a 3-fold excess of dye. Ensure the dye-protein solution is well mixed by carefully pipetting up and down several times, followed by a 5-sec spin in a tabletop microfuge.
5. Incubate the labeling reaction for 30 min in the dark.
6. While the labeling reaction incubates, equilibrate the B-Column from the Monolith Protein Labeling Kit RED-NHS 2nd Generation (Amine Reactive) with the assay buffer. Remove the top cap from the B-Column and pour off the storage solution, then remove the bottom cap. Save both caps and set aside, then place the B-Column in a 15-mL centrifuge tube using an adapter. Fill the column with assay buffer and allow buffer to enter the packed resin bed completely by gravity flow. Discard the flow through.

Repeat filling the column with assay buffer and discarding the flow through until the column is equilibrated with 9 mL of assay buffer (see **Note 2**).

7. After the labeling reaction incubation is complete, transfer 100 µL of dye-protein solution to the equilibrated B-Column. Avoid contacting the inner walls of the column and load the sample directly onto the center of the resin bed. Let the sample enter the resin bed completely.
8. Add 550 µL of assay buffer to the B-column and allow buffer to enter the resin bed completely, discarding the flow through (see **Note 3**).
9. Elute the labeled protein into a fresh microcentrifuge tube placed under the column by adding 450 µL of assay buffer onto the column.
10. Determine the protein concentration and degree of labeling (DOL) to verify the success of the labeling reaction. Blank your nanodrop with 2 µL assay buffer, then record absorbance values at 280 nm and 650 nm with 2 µL labeled protein (or appropriate volume for your spectrophotometer). Use equation 1 to calculate the protein concentration and equation 2 to calculate the DOL (Table 2).

    1. $c_{prot} = \frac{A_{prot}}{\varepsilon_{prot} d} = \frac{A_{280} - A_{max} CF}{\varepsilon_{prot} d}$
    2. $DOL = \frac{c_{Dye}}{c_{Prot}} = \frac{A_{max}/\varepsilon_{max}}{A_{Prot}/\varepsilon_{Prot}} = \frac{A_{max}\varepsilon_{Prot}}{(A_{280} - A_{max} CF)\varepsilon_{max}}$

    $\varepsilon_{max}[\mathrm{M^{-1}cm^{-1}}] = 195\,000$

    $\mathrm{CF} = 0.04$

[Table 2 here]

3.3 Binary Interaction Analysis

3.3.1 Protein-PROTAC Affinity Measurements with MST

1. Prepare 1 mM solution of MZ 1 in DMSO, by adding 1 µL of MZ 1 stock solution to 50 µL DMSO.
2. Prepare 125 µL of MZ 1 at 40 µM in assay buffer with 4 % DMSO by diluting 5 µL of 1 mM MZ 1 solution into 120 µL assay buffer (see **Note 4**).
3. Prepare five copies of a serial two-fold dilution of MZ 1 in a 384-well plate, each serial dilution containing 16 MZ 1 concentrations. Add 10 µL assay buffer containing 4 % DMSO to each well of rows B-P in columns 1-5, then add 20 µL of MZ 1 at 40 µM to row A in columns 1-5. For all columns, transfer 10 µL from well A to B and mix by pipetting up and down three times. Proceed to transfer 10 µL from well B to C, from C to D, and so on until reaching row P. After the final transfer to well P, remove 10 µL from each well in row P to finish the serial dilution with each well containing 10 µL of sample (see **Note 4b**).
4. Dilute the labeled proteins to 10 nM in assay buffer ensuring to make at least 200 µL (see **Note 5**).
5. Mix the labeled proteins with the MZ 1 serial dilution. Add 10 µL of VCB to each well in column 1, using a fresh tip for each well and pipetting up and down three times to ensure good sample mixing. Repeat with the remaining proteins for columns 2-5.
6. Centrifuge the 384-well plate at room temperature for 1 min to remove any air bubbles (see **Note 6**).
7. Load samples from one column at a time by dipping premium coated capillaries into the micro-wells to aspirate the sample (see **Note 7**). Place the capillary with the highest concentration of MZ 1 from row A on position 1 of the capillary tray and the capillaries with decreasing concentrations of MZ 1 from rows B to P in positions 2 to 16 of the capillary tray. Alternatively, the 384-well plate can be loaded directly into the Dianthus NT.23 instrument to conduct higher throughput measurements (see **Note 8**).
8. Load the sample tray in the Monolith™ NT.115pico instrument.

9. Execute a binding affinity measurement in the MO.Control Software. Set the temperature control to 25 °C, the MST-power to medium, and the excitation power to achieve a fluorescence of approximately 8000 counts for all capillaries in the capillary scan. Complete the fields to record the information about the target protein, ligand MZ 1, buffer, and capillary type used in the assay.

3.3.2 Data Analysis

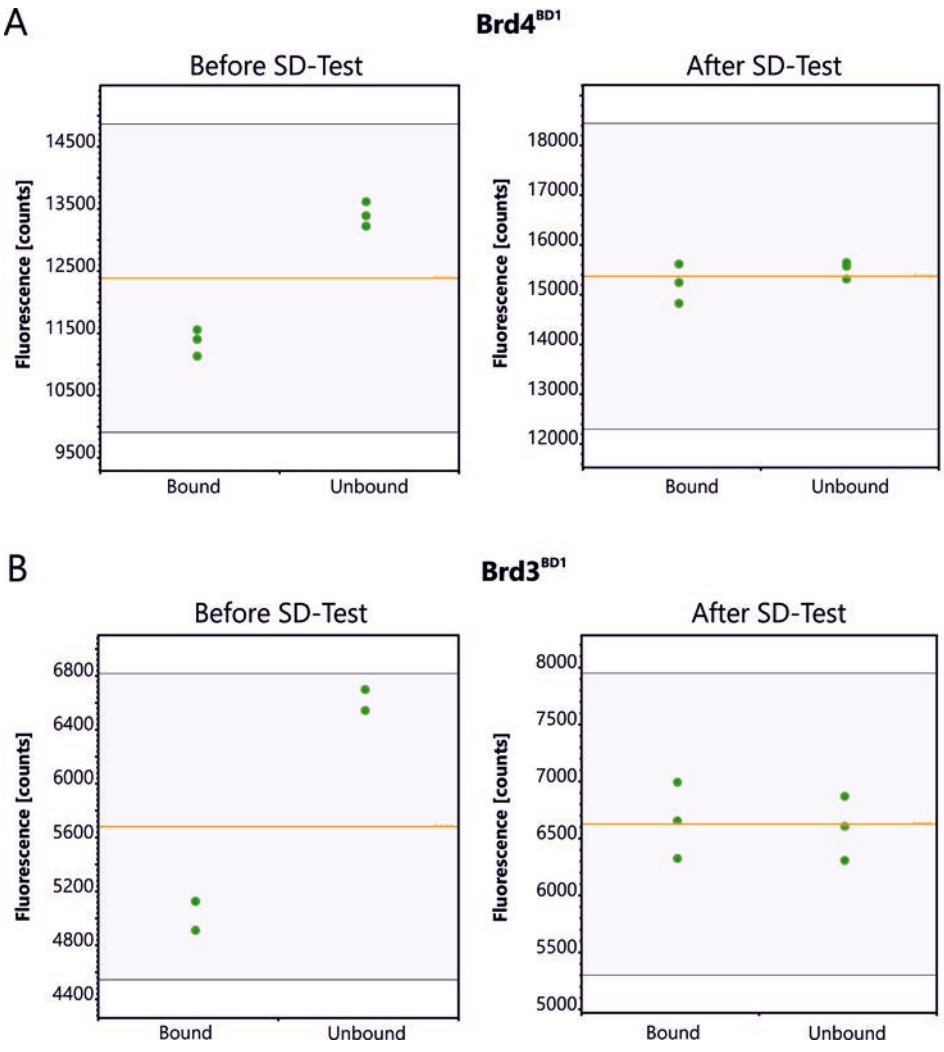
1. The MO.Control Software automatically performs a number of checks as the MST measurement is executed and data recorded. These checks include variations in fluorescence intensity, adsorption to surfaces, sample aggregation, and photobleaching rate changes. If the data pass all of these quality checks, the MO.Control Software performs an initial analysis on the data to determine the K_d of the interaction. The interaction of MZ 1 with all five proteins passes all the quality checks. However, the interaction of MZ 1 with $Brd3^{BD1}$ and $Brd4^{BD1}$ results in a biphasic dose response curve along with a ligand dependent fluorescence change that deserves deeper analysis (see **Note 9**).
2. To perform a more thorough analysis of the data and compare the interaction of MZ 1 with the five proteins, analyze the acquired data in the MO.Affinity Analysis Software. Begin by creating a new *MST Analysis* and adding the data for the interaction of MZ 1 with VCB, $Brd2^{BD2}$, and $Brd4^{BD2}$. Any replicates can be combined by dragging the data to the appropriate *Merge Set* (see **Note 10**). On the dose response fit tab, switch the analysis set to expert mode and change the MST evaluation strategy to manual mode and set the on-time to 1.5 sec. Fit the data with a 1:1 K_d model and obtain dose-response curves that can be normalized to ΔFNorm on the *Compare Results* tab (see **Figure 1a**).

3. To perform a deeper analysis of the biphasic dose response seen in the *MST Analysis* of MZ 1 interacting with $Brd3^{BD1}$ and $Brd4^{BD1}$, create a new *Initial Fluorescence Analysis* and add the data. On the dose response fit tab, set the *Fluorescence Evaluation Strategy* to *Manual: Initial Fluorescence* and compare the ligand dependent fluorescence change at the beginning of the measurement to different time points by manually adjusting the analysis window. As the ligand dependent fluorescence change shifts over the course of the measurement, this is most likely the cause of the biphasic dose response observed in the MST analysis of the interaction of MZ 1 with $Brd3^{BD1}$ and $Brd4^{BD1}$. The interaction of MZ 1 with $Brd3^{BD1}$ and $Brd4^{BD1}$ thus needs to be analyzed based on the ligand dependent initial fluorescence change (see **Figure 1b**) if the fluorescence change is shown to be caused by a specific interaction between MZ 1 with $Brd3^{BD1}$ and $Brd4^{BD1}$ through a SD-test (see **Note 11**).

[Figure 1 here]

Figure 1 Binary complex formation. A) Binary interaction of VCB (black), $Brd4^{BD2}$ (dark grey) and $Brd2^{BD2}$ (light grey) against PROTAC. Measurement was evaluated after 1.5 sec MST on-time at medium MST-power. K_d values and standard deviations of triplicate measurements are shown in the figures. B) Binary interaction of $Brd3^{BD1}$ (grey) and $Brd4^{BD1}$ (black) against PROTAC. Here the initial fluorescence was evaluated, after confirmation of binding specific ligand induced fluorescence change using the SD-test (C) (see **Note 11**). K_d values and standard deviations (n=3) are illustrated in the graphs.

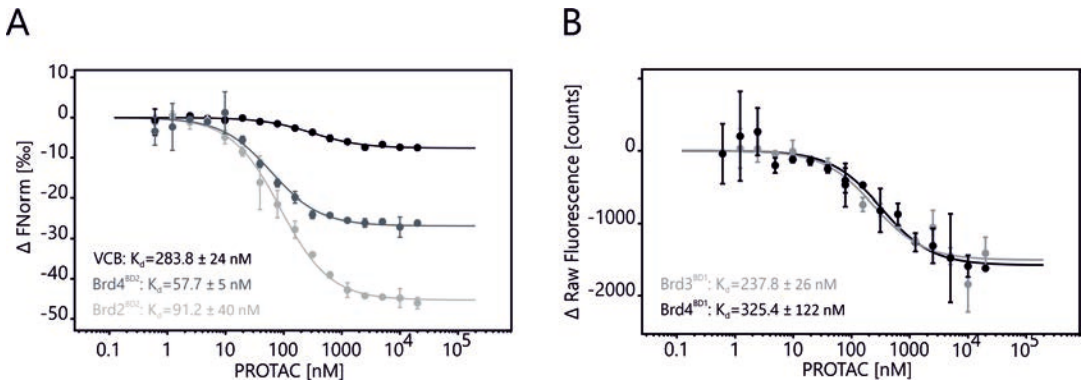
3.3.3 SD-Test


A
Brd4BD1
Before SD-Test
After SD-Test
Fluorescence [counts]
14500
13500
12500
11500
10500
9500
18000
17000
16000
15000
14000
13000
12000
Bound
Unbound
B
Brd3BD1
Before SD-Test
After SD-Test
Fluorescence [counts]
6800
6400
6000
5600
5200
4800
4400
8000
7500
7000
6500
6000
5500
5000
Bound
Unbound

1. To verify that the initial fluorescence change observed with the interaction of MZ 1 with $Brd3^{BD1}$ and $Brd4^{BD1}$ are caused by an interaction and not non-specific effects, transfer the remaining 10 µL of rows A-C and N-P (the highest three concentrations of MZ 1 and lowest three concentrations of MZ 1, respectively) of the $Brd3^{BD1}$ and $Brd4^{BD1}$ samples to non-binding microcentrifuge tubes and centrifuge at 15 000 g for 10 min at 4 °C.
2. Transfer 7 µL from the centrifuged tubes to fresh microcentrifuge tubes taking care not to disturb or transfer any pellet or aggregated sample at the bottom of the centrifuged tubes (see **Note 12**).
3. Add 7 µL of SD-mix to each microcentrifuge tube and mix by pipetting up and down.
4. Incubate samples for 5 min in a 95 °C hot plate to ensure the proteins are fully denatured and no specific interaction between MZ 1 and the protein's structure is possible.
5. Spin samples quickly in a tabletop microfuge to ensure the samples are at the bottom of the microcentrifuge tubes.
6. Load samples from the microcentrifuge tubes by dipping premium coated capillaries into the sample at the bottom of the tubes (see **Note 7**). Place the capillaries in the capillary tray with the MZ 1 decreasing from position 1 to position 6.
7. Execute a SD-test measurement in the MO.Control Software. Set the temperature control to 25 °C and the excitation power to achieve a fluorescence of approximately 8000 counts for all capillaries in the capillary scan (see **Figure 2**).

[Figure 2 here]

Figure 2 SD-test of the binary interactions $Brd4^{BD1}$ and $Brd3^{BD1}$. A) The left graph shows the initial fluorescence counts of capillary 1 – 3 and 14 – 16 of the MZ 1-$Brd4^{BD1}$ titration series, while the right graph represents the same samples after addition of SD-


A
B
Δ FNorm [‰]
PROTAC [nM]
VCB: Kd=283.8 ± 24 nM
Brd4BD2: Kd=57.7 ± 5 nM
Brd2BD2: Kd=91.2 ± 40 nM
Δ Raw Fluorescence [counts]
Brd3BD1: Kd=237.8 ± 26 nM
Brd4BD1: Kd=325.4 ± 122 nM

mix. B) The left graph shows the initial fluorescence counts of capillaries 1 -3 and 14 – 16 of the $Brd3^{BD1}$-MZ 1 titration series, while the right graph represents the same samples after the SD-test was carried out.

3.4 Ternary Interaction Analysis

3.4.1 Ternary Complex Affinity Measurements with MST

The described assay design is one of several possible approaches to analyze ternary complex formation with MST. This assay design is ideal when the ternary interaction of different PROTACs or target proteins with a defined ubiquitin ligase is studied (see **Note 13**).

1. Prepare a 50 µM solution of MZ 1 in DMSO through a two-step dilution by adding 1 µL of the 51 mM stock solution into 9 µL of DMSO to make a 5.1 mM solution and then adding 1 µL of this 5.1 mM solution to 101 µL of DMSO.
2. Prepare a 4 µM solution of MZ 1 in assay buffer (with 8 % DMSO) by adding 4 µL of the 50 µM MZ 1 solution into 46 µL of assay buffer (see **Note 4b**).
3. Prepare 500 µL of assay buffer, supplemented with 8 % DMSO, by adding 40 µL of DMSO to 460 µL of assay buffer.
4. Create a 16-point serial two-fold dilution of MZ 1 in microcentrifuge tubes (see **Note 4** and **Note 14**). Transfer 50 µL of MZ 1 at 4 µM to tube 1, then add 25 µL of the assay buffer supplemented with 8 % DMSO to tubes 2 to 16. Begin the serial dilution by transferring 25 µL of solution from tube 1 to tube 2 and mix by pipetting up and down three times. Proceed to transfer and mix 25 µL from well B1 to C1, from C1 to D1, and so on until reaching well P1. After mixing the contents of well P1 by pipetting, finish the serial dilution by discarding 25 µL from well P1 to finish the serial dilution with 25 µL of sample in each well of column 1.

5. Prepare the four Brd samples ($Brd3^{BD1}$, $Brd4^{BD1}$, $Brd4^{BD2}$, and $Brd2^{BD2}$) in a 384-well plate to form binary complexes with the MZ 1 serial dilution (see **Note 15**). Transfer 10 µL of 100 µM solutions of $Brd3^{BD1}$, $Brd4^{BD1}$, $Brd4^{BD2}$, and $Brd2^{BD2}$ in assay buffer to wells A2 – A5, respectively. Add 5 µL assay buffer to wells B2 – D5 and serially dilute the Brd proteins three times. In each of columns 2 – 5, transfer 5 µL from row A to row B and mix by pipetting up and down three times. Repeat for row B to C and for row C to D, mixing the solution after each transfer and changing pipette tips when changing columns. Discard 5 µL of solution from wells D2 – D5, leaving 5 µL of Brd sample in wells A2 – D5. Finally, prepare 70 µL of each Brd protein at 8 µM and transfer 5 µL of the $Brd3^{BD1}$, $Brd4^{BD1}$, $Brd4^{BD2}$, and $Brd2^{BD2}$ solutions to the remaining wells of columns 2 – 5, respectively.
6. Combine the MZ 1 serial dilution with the Brd proteins to form binary complexes of the MZ 1 with the four Brd proteins, separately. Transfer 5 µL of the MZ 1 solution in tube 1 to each of wells A2 – A5 and mix by pipetting up and down three times, taking care to avoid air bubbles (see **Note 6**). Use a fresh pipette tip for each well. Proceed to transfer 5 µL of the MZ 1 serial dilution to wells B2 – B5 from tube 2 to C2 – C5 from tube 3, continuing down the plate to P2 – P5 from tube 16.
7. Incubate the interaction for 10 min at room temperature. Seal the plate to avoid any evaporation.
8. While the plate incubates, prepare 800 µL of a 10 nM solution of the RED-NHS labeled VCB prepared in section 3.2 using assay buffer as the diluent.
9. After the 10 min incubation, add 10 µL of the 10 nM RED-NHS labeled VCB solution to each well containing the MZ 1-Brd mixture (wells A2 – P5) and mix by pipetting up and down three times.
10. Centrifuge the plate for 1 min at room temperature to remove any air bubbles.

11. Load samples from one column at a time, starting with column 2, by dipping one premium coated glass capillary per well to aspirate the sample (see **Note 7**). For each Brd, place the capillary with the highest concentration of MZ 1 from row A on position 1 of the capillary tray and the capillaries with decreasing concentrations of MZ 1 from rows B to P in positions 2 to 16 of the capillary tray. Alternatively, the 384-well plate can be loaded directly into the Dianthus NT.23 instrument to conduct higher throughput measurements (see **Note 8**).
12. Load the sample tray in the Monolith™ NT.115pico instrument.
13. Execute a binding affinity measurement in the MO.Control Software. Set the temperature control to 25 °C, the MST-power to medium, and the excitation power to 5 % (see **Note 16**). Complete the fields to record the information about the target protein, ligand MZ 1-Brd complex, buffer, and capillary type used in the assay.

3.4.2 Data Analysis

4. The MO.Control Software automatically checks for variations in fluorescence intensity, sample adsorption to capillary walls, sample aggregation, and photobleaching rate changes. If the data pass all of these quality checks, the MO.Control Software determines the K_d of the interaction. All four ternary complex formation measurements pass the quality checks performed by the MO.Control Software (see **Note 17**).
5. Open the acquired data in the MO.Affinity Analysis Software for data processing and analysis. Begin by creating a new *MST Analysis* and adding the data for the interaction of VCB with the $Brd3^{BD1}$-MZ 1 complex. Any replicates can be combined by dragging the data to the appropriate *Merge Set* (see **Note 10**). On the *Dose Response Fit* tab, switch the analysis set to expert mode and change the MST evaluation strategy to manual mode and set the on-time to 1.5 sec (see **Note 18**). Fit the data with a 1:1 K_d

model and obtain a dose response curve that can be normalized to FNorm on the *Compare Results* tab (see **Figure 3**). Repeat with the data for the interaction of VCB with the Brd4^{BD1}-MZ 1, Brd4^{BD2}-MZ 1, and Brd2^{BD2}-MZ 1 complexes, setting the on-time to 1.5 sec for the Brd4^{BD1}-MZ 1 complex and 20 sec for the Brd4^{BD2}-MZ 1 and Brd2^{BD2}-MZ 1 complexes.

[Figure 3 here]

Figure 3 Determination of binding affinity of ternary interactions of VCB with MZ 1-Brd complexes. Measurements with Brd3^{BD1} (A) and Brd4^{BD1} (C) are evaluated after 1.5 sec MST on-time. Brd4^{BD2} (B) and Brd2^{BD2} (D) are evaluated after 20 sec MST on-time. K_d values are shown in each figure. Error bars indicate the standard deviation of three independent measurements (n=3).

3.4.2 Cooperativity Assessment of Ternary Complex

1. Calculate the cooperativity (α) of the ternary complex formation, an important metric related to the successful ubiquitinylation of the target protein by the ubiquitin ligase[33]. Divide the K_d of the binary interaction between MZ 1 and VCB determined in section 3.3 by the K_d of the ternary interaction between MZ 1 bound to the Brd proteins and VCB determined in sections 3.4-3.5 (see **Table 3**). Higher cooperativity values indicate an attractive interaction between the ubiquitin ligase and target protein catalyzed by the PROTAC MZ 1.

[Table 3 here]

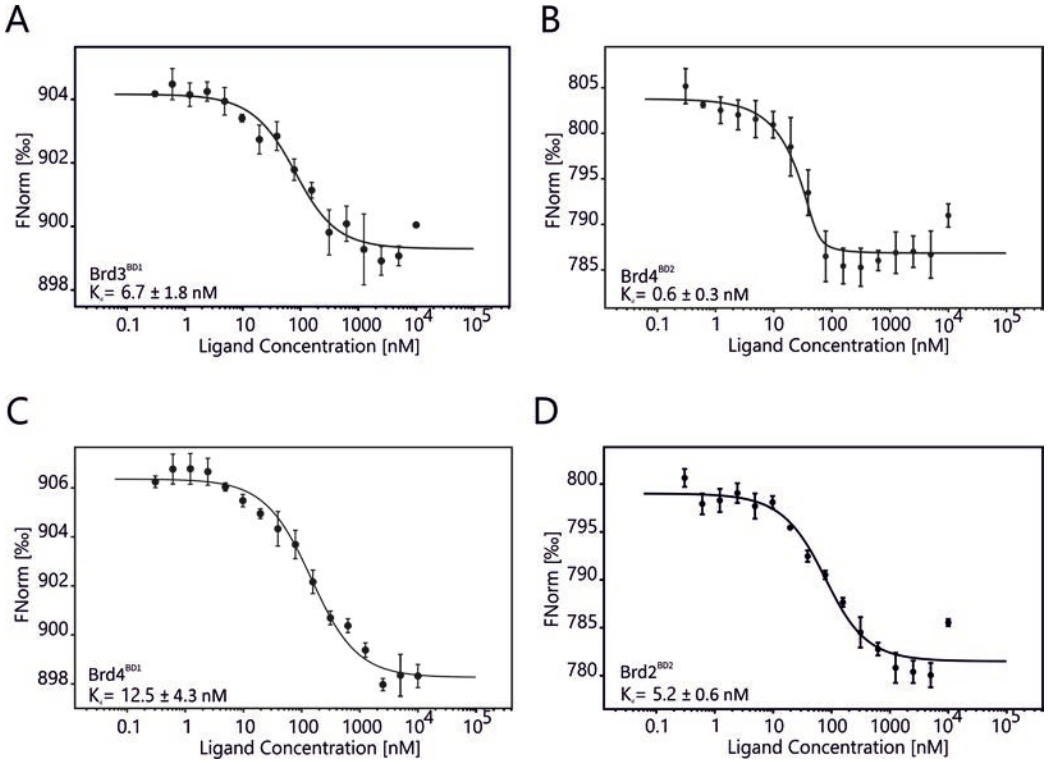

A
FNorm [‰]
904
902
900
898
Brd3BD1
Kd= 6.7 ± 1.8 nM
0.1
1
10
100
1000
10^4
10^5
Ligand Concentration [nM]
B
FNorm [‰]
805
800
795
790
785
Brd4BD2
Kd= 0.6 ± 0.3 nM
0.1
1
10
100
1000
10^4
10^5
Ligand Concentration [nM]
C
FNorm [‰]
906
904
902
900
898
Brd4BD1
Kd= 12.5 ± 4.3 nM
0.1
1
10
100
1000
10^4
10^5
Ligand Concentration [nM]
D
FNorm [‰]
800
795
790
785
780
Brd2BD2
Kd= 5.2 ± 0.6 nM
0.1
1
10
100
1000
10^4
10^5
Ligand Concentration [nM]

Table 3 lists the cooperativity values α for each Brd. The values are calculated by dividing the K_d of the binary interaction between MZ 1 and VCB by the K_ds for the ternary interaction between MZ 1 bound to a Brd and VCB.

4 Notes

1. For proteins with a concentration of less than 100 µM or proteins stored in Tris buffers it is best to perform a buffer exchange into the NHS labeling buffer using desalting A-Column in the Monolith Protein Labeling Kit RED-NHS 2nd Generation as dilution into the NHS labeling buffer will leave too much of the original buffer components that can have a negative effect on the protein labeling efficiency.
2. If the equilibration of the B-column is finished before the 30 min labeling incubation is completed, place the caps you set aside back on the column to prevent the resin bed from drying out.
3. Use care when adding the 550 µL of assay buffer to the B-column to avoid disturbing the resin bed and disturbing the separation of the labeled protein from the unreacted dye.
4. Serial dilution of the ligand should begin at a concentration approximately 50-fold the estimated K_d of the protein-ligand interaction to ensure saturation of the protein target is achieved and the full dose response curve is captured.

   4b. The final DMSO concentration should always be kept as low as possible (best below 5 %) to ensure that the protein is not affected in its activity. In general, measurements at very high additive/solvent concentrations (> 2 % DMSO, > 5 % glycerol, > 100 mM sucrose) are possible without limitations when applied at constant concentrations throughout the dilution series.

5. For proper dissociation constant determination, the target protein concentration must be below the K_d (e.g. for an interaction with a K_d of 100 nM, the concentration of the target protein should be no greater than 100 nM).
6. Failure to remove air bubbles and aggregated sample from solution can affect the quality of the results. Both can be removed by centrifugation of the samples at 15000 g for 10 min at 4 °C.
7. Aspiration of samples into capillaries is best performed horizontally to eliminate the force of gravity from counteracting the capillary force. One of the easier approaches is to hold the 384-well plate vertically or place the plate in the NanoTemper plate holder and then dip the capillaries into the plate column with the samples to be measured.
8. Dianthus instruments leverage the Temperature Related Intensity Change (TRIC) component of the MST signal to enable measurements in industry standard microwell plates. TRIC is based on the generation of a rapid and highly precise temperature change in a sample well by infrared (IR) laser light. Changes in sample fluorescence upon activation of the IR laser are monitored to characterize interactions and derive affinity constants.
9. Any measurement that results in a biphasic dose response curve from an MST analysis and also has a ligand dependent change in initial fluorescence needs further analysis as the biphasic dose response from the MST analysis can be an artifact from a time or temperature related shift to the initial fluorescence change. Best practice is to perform an initial fluorescence analysis in MO.Affinity Analysis Software with the *Fluorescence Evaluation Strategy* set to *Manual: Initial Fluorescence* and to compare the ligand dependent fluorescence change at various points in the measurement by manually adjusting the analysis window. If a time dependent shift

is observed for the initial fluorescence change, this is most likely the cause of the biphasic dose response seen in the MST analysis.

10. We selected to perform our assays in triplicate. Like all experiments, repeating the assay increases the statistical significance of the results.
11. The SD-test is not suitable if the target is a fluorescent fusion protein like GFP or YFP. These fluorescent proteins will be denatured as well, and no fluorescence will be left for analysis. If potassium salts are used in the assay buffer, SDS should be avoided due to precipitation of the salt. Instead, a final concentration of 4 M urea should be used to denature the proteins to execute the SD-test. For samples containing RED-tris-NTA labeled protein, please perform an ECP-Test instead of an SD-test [35].
12. It is essential to ensure that none of the pellet after centrifuging is transferred to the new tubes. If the pellet is disturbed, centrifuge again for at least 10 min at ≥15,000 g.
13. While the described assay design is ideal to study the ternary interaction of different PROTACs and target proteins with a defined ubiquitin ligase while in addition eliminating the hook effect (the hook effect describes how high concentrations of a bifunctional molecule can prevent ternary complex formation), alternative assay designs are possible that offer different advantages. By fluorescently labeling the target protein instead of the ubiquitin ligase, the assay can be easily modified to study the ternary interaction of different PROTACs and ubiquitin ligases with a single target protein while still avoiding the hook effect. Pushing the assay design further, unpublished results have shown assays utilizing excess PROTAC pre-incubated with the fluorescently labeled protein to study ternary interactions are able to recapitulate the cooperativity ranking as the assay design described in this chapter despite the hook effect. When designing an assay using an excess of PROTAC pre-incubated with the fluorescently labeled protein, care must be taken to strike a

balance between having enough but not too much PROTAC in solution. While sufficient PROTAC is necessary to ensure saturation of the fluorescently labeled protein to enable ternary complex formation, a too large excess of PROTAC will lead to the hook effect, precluding the target protein and ubiquitin ligase finding the same PROTAC to form a ternary complex.

14. The use of low-binding plastics (multiwell plates, microcentrifuge tubes, and pipette tips) is recommended to prevent loss of sample through sticking to plastics. Losses of small amounts of sample that go unnoticed when working at higher concentrations are more pronounced and can have a larger effect when working at the low concentrations common in MST measurements.
15. It is important that the concentration of Brd protein is high enough to ensure ≥ 95 % of the MZ 1 in solution is bound to the Brd. Any MZ 1 not bound to Brd will interact with VCB without any cooperativity effects. Having two different ligands in solution (MZ 1 in complex with Brd and MZ 1 alone) can mask the effects of cooperativity on the ternary complex formation. Given the K_ds of the binary interactions of MZ 1 with the Brd proteins, the concentration of Brds are set so ≥ 95 % of the MZ 1 is bound by a Brd at all concentrations of the MZ 1 serial dilution.
16. In the MO.Control Software the LED power can be adjusted automatically by selecting the “auto” function on the plan page of the software.
17. Of all the quality checks performed by the MO.Control Software, the initial fluorescence (the fluorescence of the samples before the IR laser is switched on) is one of the most important. Several of the most frequently encountered complications that impact MST assays can be identified and diagnosed via an analysis of the initial fluorescence. As the concentration of the fluorescent target is constant in an MST assay, the initial fluorescence should be homogenous for all ligand concentrations. While random fluctuations in the initial fluorescence typically signify protein

aggregation that can be addressed through alterations to buffer composition or pH, ligand-dependent changes require a SD-Test (for covalently fluorescently labeled targets) or ECP-Test (for his-tag fluorescently labeled targets) to distinguish between interaction-based effects and artifacts such as adsorption to labware or ligand autofluorescence.

18. It is best to study interactions using the lowest MST-power and shortest analysis time that resolves a good amplitude (typically 5 or more Fnorm units) and signal to noise ratio (typically 13 or more). For most interactions this is the medium MST-power.

Table 1: Mutant constructs and storage buffers

Protein	Storage buffer
Ubiquitin ligase (VCB)	20 mM HEPES 7.5, 100 mM NaCl, 1 mM TCEP
$Brd2^{BD2}$ (344-455)	10 mM HEPES 7.5, 500 mM NaCl, 5 % Glycerol
$Brd3^{BD1}$ (24-146)	25 mM HEPES 7.5, 150 mM NaCl, 10 mM DTT
$Brd4^{BD1}$ (44-178)	10 mM HEPES 7.5, 100 mM NaCl, 1 mM DTT
$Brd4^{BD2}$ (333-460)	10 mM HEPES 7.5, 100 mM NaCl, 10 mM DTT

Table 2: Protein concentration and DOL

Protein	A_{280}	A_{650}	Concentration	DOL
VCB	0.06	0.38	1.3 µM	1.45
$Brd2^{BD2}$	0.04	0.27	1.8 µM	0.8
$Brd3^{BD1}$	0.08	0.54	2.1 µM	1.3
$Brd4^{BD1}$	0.08	0.33	2.5 µM	0.7
$Brd4^{BD2}$	0.06	0.14	3.9 µM	0.19

Table 3: Cooperativity values

BRD	$Brd4^{BD2}$	$Brd2^{BD2}$	$Brd3^{BD1}$	$Brd4^{BD1}$
α	473	54.6	42.4	22.7

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