REAL-TIME CELL TARGET ENGAGEMENT

CASE STUDY: MAPK1

Case study

V1.0

ENGINEERING OF MICRO-TAG CONSTRUCT

The human Mitogen-activated protein kinase 1 (**MAPK1, Uniprot ID: P28482**) construct was engineered having the Micro-Tag at the C-terminus. The construct was transiently expressed in HEK293 cells. Expression was confirmed by Western blot analysis using an anti-Micro-Tag antibody. The construct expressed at the expected molecular weight (MW) of 42 kDa (**Figure 1A**). Cells expressing the construct were used for enzyme complementation assay. The MAPK1 construct demonstrated significant fluorescence signal in the assay (**Figure 1B**).

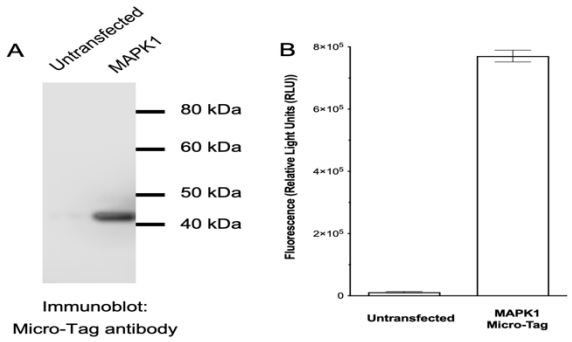


Figure 1: Expression and enzyme complementation assay of the MAPK1 Micro-Tag construct.

A) Immunoblot analysis, using Micro-Tag antibody, of HEK293 cells transfected with the MAPK1 Micro-Tag construct, along with a Negative Control (untransfected lysate).
B) Enzyme complementation assay using the transfected cells showing fluorescence signal generated.

REAL-TIME CONVENTIONAL TARGET ENGAGEMENT

Conventional cell target engagement is based on the concept of protein thermal shift whereby ligand binding to a protein imparts thermodynamic stabilization to the protein. This thermal stabilization can be detected when a heat challenge is applied to cells, forcing denaturation and aggregation of the protein target. As a prerequisite for testing ligand binding, the temperature of aggregation (Tagg) at which 50% of the protein is denatured and aggregated is first established. Ligands are subsequently interrogated for binding to the protein at the temperature of aggregation, T_{agg}.

Cells expressing MAPK1 were used to generate a thermal profile signature of the protein. Non-denaturing lysates were prepared, and a thermal gradient was applied to the lysates to identify a temperature of aggregation/denaturation (T_{agg}) at 50% protein denaturation/aggregation. Under a heat gradient, the fluorescence signal from enzyme complementation decreased and a T_{agg}(50%) was identified (**Figure 2**). A T_{agg}(50%) of 54°C was determined for MAPK1.

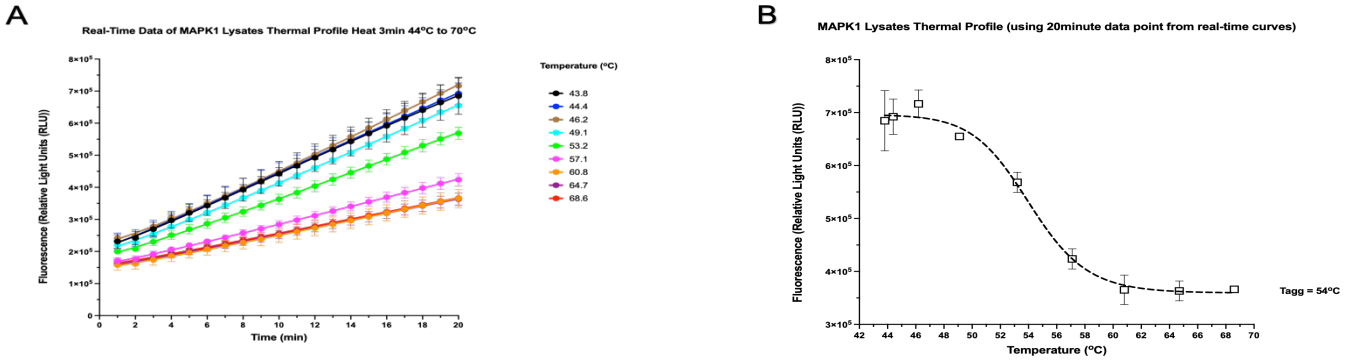


Figure 2: Micro-Tag fluorescence assay with thermal profile for MAPK1. A) Fluorescence signal detected in real-time from the enzyme complementation assay for the indicated temperatures. **B)** Non-linear regression analysis with Sigmoidal curve fitting with identified Tagg.

Serial dilutions of the compounds (**AZD0364 (Compound A)** and **Negative Control (Compound B)**) are prepared in DMSO to span a final concentration range from 10uM to 0.01nM. The compounds are incubated with non-denaturing lysates prepared from HEK293 overexpressing the MAPK1 Micro-Tag target. Lysates are incubated with the compounds for 1 hour on ice, then heated at 54°C for 3minutes. Fluorescence detected from the assay in real-time is shown in **Figure 3A**. Fluorescence data from the 20minute time point was plotted with dose of inhibitor on a semi-log scale, and non-linear regression analysis was used to fit a dose-response curve with variable slope to generate EC50 of target engagement (**Figure 3B**).

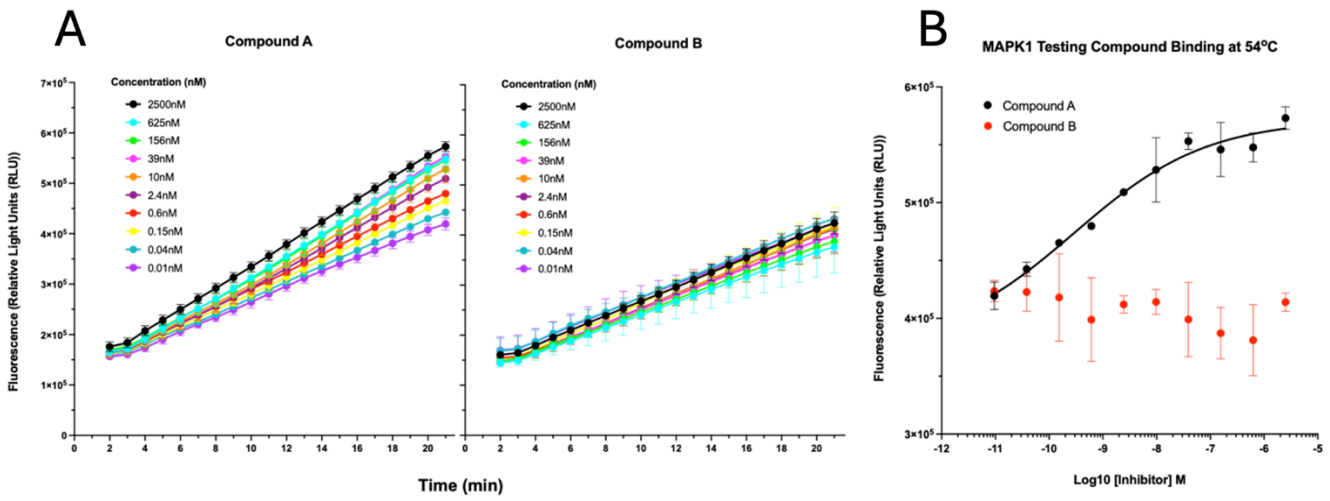
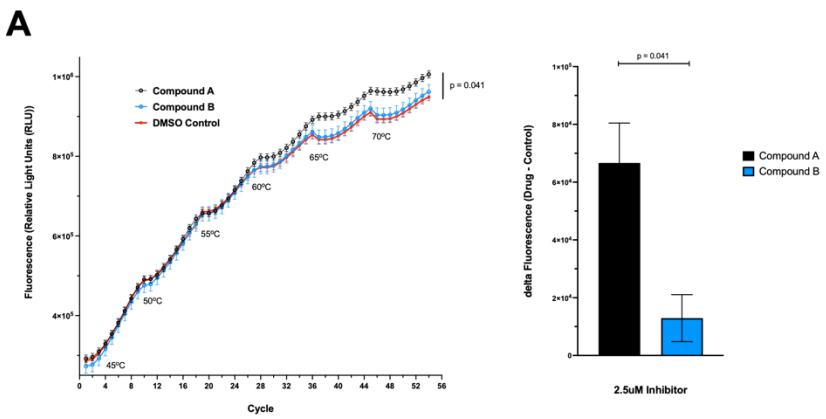


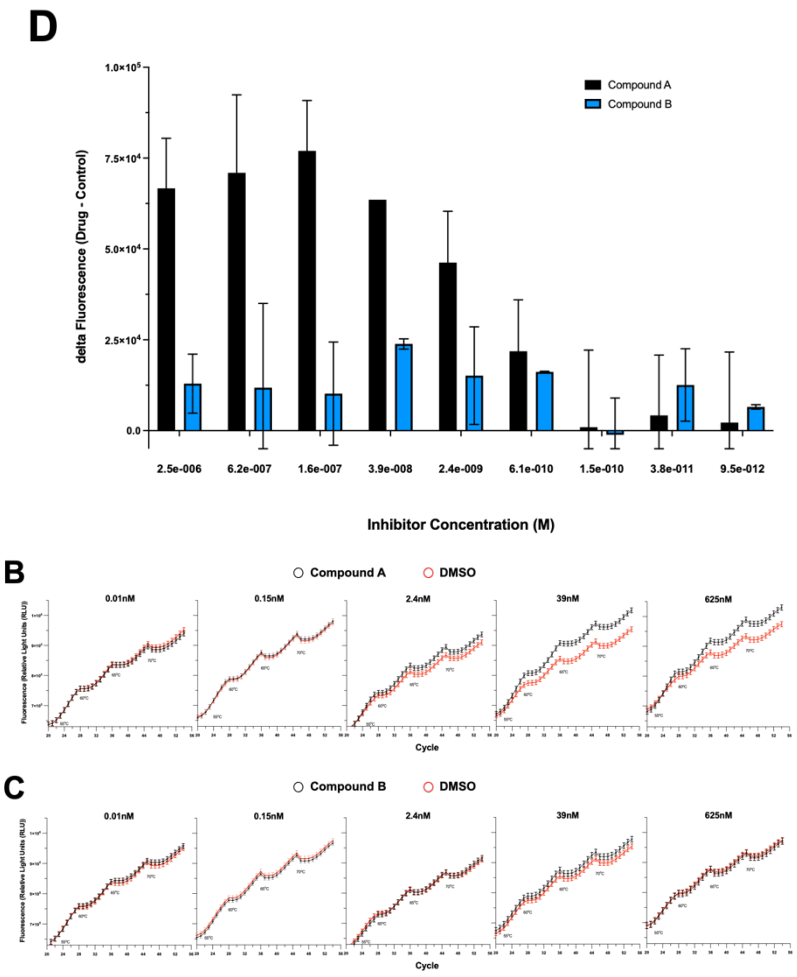
Figure 3. Testing ligand binding to MAPK1 Micro-Tag construct at 54°C.

A) Real-time curves for binding of Compound A and B to MAPK1 shows stabilization of the target for Compound A but not Compound B. **B)** Fluorescence plotted with ligand concentration on a semi-log scale. Non-linear regression analysis fits a sigmoidal dose-response curve.

MICRO-TAG STEP GRADIENT FOR CELL TARGET ENGAGEMENT

The Step Gradient Method by CellarisBio presents a completely novel technique with minimal sample processing which employs the thermal cycling programming of real-time instruments. Programmed heating/cooling steps allows for simultaneous thermodynamic stabilization from ligand binding with detection of fluorescent signal generated from FRET substrate cleavage. This Step gradient method significantly saves on time and cost of performing cell target engagement studies by abrogating the T_{agg} determination step in the workflow. In addition, this “mix and detect” strategy provides direct real-time measures of drug target interaction before an equilibrium is established, all within the context of the cellular environment.

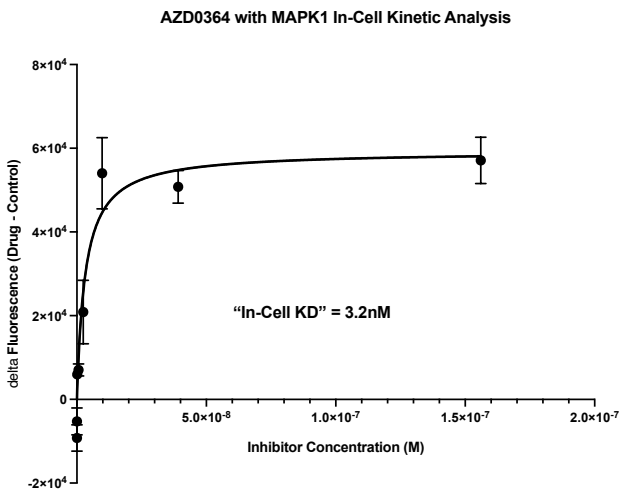




The experimental setup for the Step gradient involves combining the non-denaturing lysates with compound to be tested along with the detection reagents. The sample is mixed and immediately monitored for binding at several temperatures. Binding of a ligand to the target results in thermal stabilization resulting in an increase in the fluorescence signal. The fluorescence real-time curves for bound ligand increases above the DMSO control (**Figure 4A**). The experimental setup can include testing at a single high concentration such as 2.5uM in this study (**Figure 4A**). This type of setup is typical of a high throughput screening assay and allows for quick identification of binding compounds. Alternatively, a dose-response can be used to identify the EC50 of target engagement (**Figure 4B - D**). Real-time curves show the fluorescence for each dose of ligand tested. A binding ligand will stabilize the target in the step gradient resulting in higher fluorescence signal compared to the DMSO control (**Figure 4B**). A ligand that does not bind does not generate signal above the DMSO control resulting in real-time curves overlapping with the DMSO control curves (**Figure 4C**). The delta fluorescence can then be determined by subtracting the final fluorescence reading of DMSO control from the fluorescence for the ligand test. The delta fluorescence can be plotted with ligand concentration (**Figure 4D**).

Figure 4. Real-time Step gradient results for testing ligand binding to MAPK1 Micro-Tag target.

A) Real-time data (left panel) from testing compound A and compound B at 2.5uM along with DMSO control shows binding of compound A, and the difference in fluorescence (delta fluorescence) between drug treated and DMSO treated samples (right panel).
B) Real-time curves from the Step gradient analysis for increasing doses of compound A.
C) Real-time curves from the Step gradient analysis for increasing doses of compound B.
D) Plot of the fluorescence difference (delta fluorescence) between drug treated and DMSO treated samples at the end of the Step program.



From the real-time data an "in-cell equilibrium dissociation constant (Kd)" measure can be determined by fitting the data to saturation binding equation with Graphpad prism software (Equation: one site-specific). An in-cell Kd of 3.2nM was determined for AZD0364 binding to MAPK1 (**Figure 5**).

Figure 4. In-Cell equilibrium constant (Kd) for AZD0364 determined for MAPK1 Micro-Tag target.

Some of the drug targets we worked with:

cMyc	BCL6	UBE2N
b-Catenin	EGFR	PI3K
DHFR	MTH1	PLK1/2

What drug targets are you interested in?

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