CellarisBio

MICRO-TAG Cell Target Engagement Platform

Decoding Challenging Drug Targets

WHITEPAPER
(v2.0)

Cell Target Engagement using MICRO-TAG Platform

Demonstrating engagement of drug candidates with therapeutic targets is a challenge in drug discovery. CellarisBio has developed a technology platform that revolutionizes early drug discovery. MICRO-TAG platform is based on the 3rd generation of cell target engagement technology that works on the basis of fluorescence. It enables interrogation of target engagement without interfering with folding, localization, and function of target proteins, all within the physiological milieu of the cell.

Workflow with MICRO-TAG Platform

The MICRO-TAG platform builds on previous developments in the field of cellular target engagement. It integrates seamlessly into the modern drug discovery practices by overall significantly augmenting the speed and cost effectiveness of preclinical programs. The workflow involves several simple steps, as shown in **Figure 1.**

Step 1: Select your drug target

We can work with one or multiple targets at once. You may have identified the targets from various rationales, such as literature review, proteomics analysis, synthetic lethality screen etc. Alternatively, you may choose to work with multiple versions of the same target, such as its isoforms or mutants. In order to start using the MICRO-TAG system, it is sufficient to have the UNIPROT IDs for these targets.

Step 2: Clone your target with MICRO-TAG

We will generate a DNA construct for your target in the MICRO-TAG system. This entails cloning of a 15-amino acid tag to either N- or C-terminus of the target protein. Unless biology of the target suggests otherwise, we will use C-terminus for its convenience. For an initial pilot test, a transient expression in HEK293 cells can be sufficient for mist targets. Alternatively, you can prefer generation of stable cells using lentiviral or CRISPR methods.

Step 3: Test your drug candidates

After the MICRO-TAG expression construct (either transient or stable) is ready, it can transfected / transduced to the cells of interest to generate a reporter cell line for your target. HEK293 cells serve as a fast a convenient vehicle for an initial feasibility test. Cells will be tested with your ligands in dose-dependent manner, usually for a short exposure of 5-30 min. Depending on the research question and biology of the target, you can choose to treat either intact cells or non-denaturing cell lysates. The cells will be challenged at T_{agg}, a temperature point at which your target is in a structurally fluid state.

Step 4: Detect signal

Once the target has been sufficiently exposed to the ligands, signal will be developed by addition of Micro-Tag-binding protein and FRET substrate. By virtue of enzyme complementation, the 15-amino acid tag cloned to the target will complement with the Micro-Tag-binding protein to form an active enzyme, which will cleave the FRET substrate to release fluorescent signal. The level of signal is directly proportional to the level of stabilized target. Potency of cell target engagement (EC50) can be calculated.

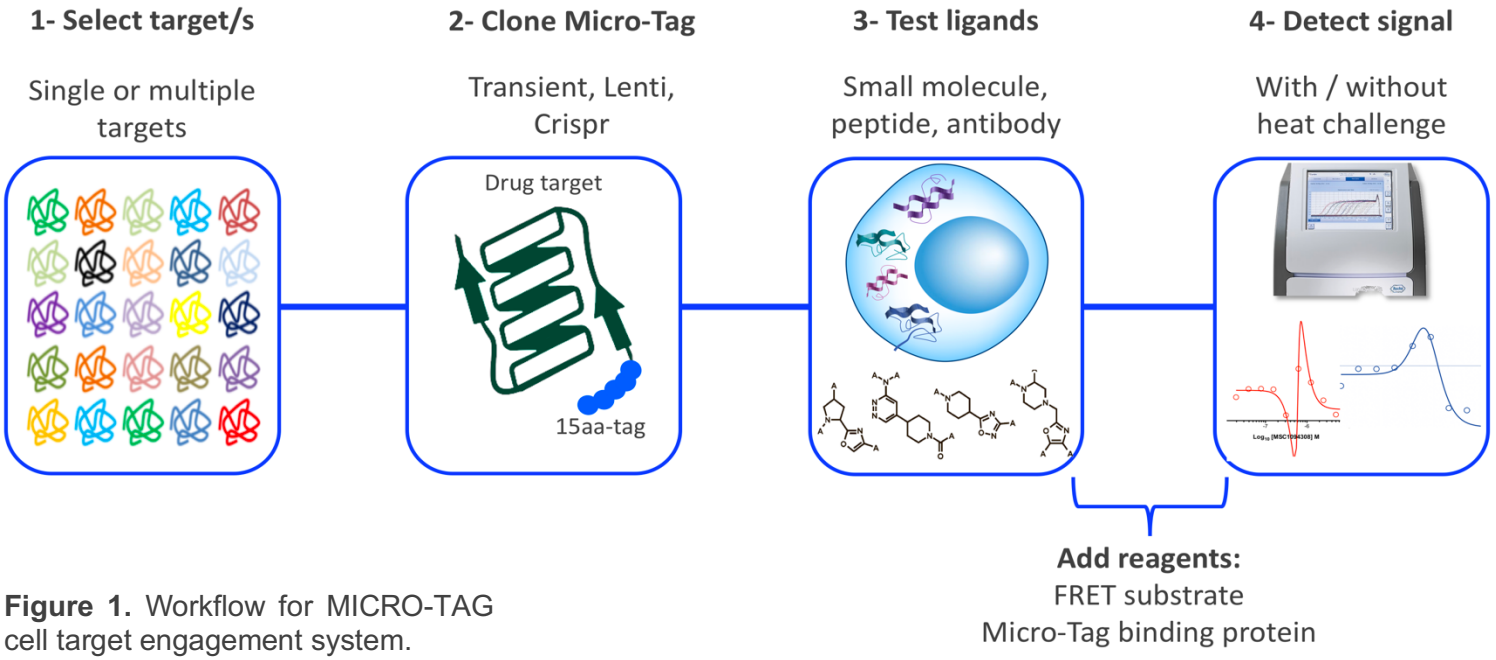


Figure 1. Workflow for MICRO-TAG cell target engagement system.

Design of MICRO-TAG Cell System

The MICRO-TAG platform represents the next-gen of cellular target engagement technology and relies on novel chemistry. It is built on the principle of *enzyme complementation* technology. It The technology does not require use of antibodies or any western blot analysis.

A short 15-amino acid tag is recombinantly engineered to N- or C-terminus of the target protein. The tag is small enough and structurally inert not to interfere with folding, localization, protein interactions, or function of the target protein.

The application of MICRO-TAG to cell target engagement relies on the principle of *protein thermal melting*. Basically, cells expressing a MICRO-TAG target construct are put through a thermal gradient to identify a temperature of aggregation (T_{agg}), a temperature at which half of the target protein is in structurally unstable state. The target protein can be rescued from thermal denaturation (i.e. re-stabilized) by direct engagement of a ligand in dose-dependent manner.

For detection of signal, a MICRO-TAG-binding protein is added to the reaction. It complements with the 15-amino acid tag, which is part of the target protein, to form an active enzyme. The active complemented enzyme cleaves the *FRET substrate* to release fluorescent signal. The level of signal is directly proportional to that of the *ligand-induced thermal stabilization* of the target protein.

The MICRO-TAG platform is built to improve the scalability and sensitivity of cellular target engagement for drug discovery. The strength and dynamic range of the signal enables detection of low expressed proteins while providing an ability to use potentially any cell type of interest.

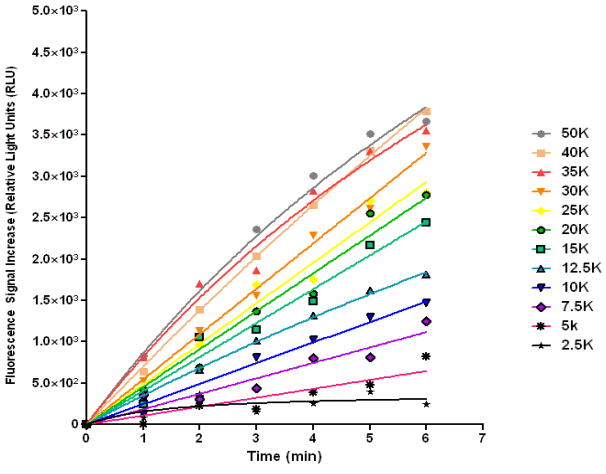


Figure 2. Level of fluorescence is sensitive to the amount of MICRO-TAG target protein.

One key component of the MICRO-TAG system is thermal challenge, which is essential for separation of ligand-engaged from non-engaged target population.

Thermal challenge is exerted at T_{agg}, which is an empirically determined unique fingerprint of every target protein. At T_{agg}, the target protein exists at half-unwound and thermally vulnerable state. At higher temperatures, the MICRO-TAG target unfolds and aggregates. The aggregated target is hence not able to form an active enzyme with the MICRO-TAG-binding protein.

Non-denaturing lysis of the cells together with enzyme complementation yields fluorescent signal. The reaction is fast and reproducible, and is dependent on the remaining amount of the target in the MICRO-TAG system, as shown in (**Figure 2**).

There is a linear increase in signal immediately upon addition of substrate. By keeping the binding protein and substrate concentration in excess, the rate of the reaction becomes solely dependent on the level of the MICRO-TAG target. This is the basis for the quantitation of the MICRO-TAG target that becomes stabilized by ligand binding. A decrease in fluorescence signal at higher temperatures is due to the target protein denaturation (**Figure 3A**).

Denaturation and loss of the target protein is prevented when a ligand specifically binds directly to the target. Binding to the target alters the conformation of the protein, stabilizing it under thermal challenge. Samples with drug target engagement will yield higher fluorescence signal relative to vehicle control (**Figure 3B**).

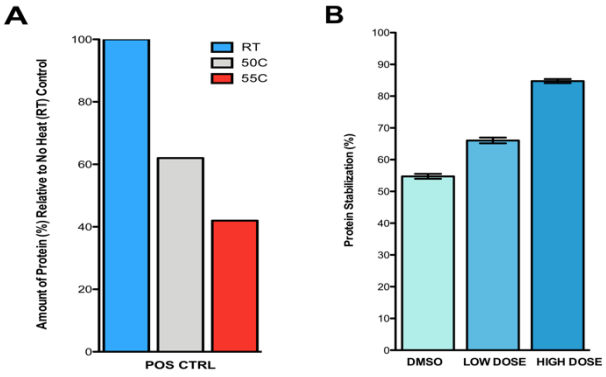


Figure 3. (A) Amount of the positive control protein after heating at the indicated temperatures. (B) Cells treated with a drug that binds the control protein at low dose and high dose for 1hr followed by heating to 50°C, shows increase in amount of protein stabilization with increasing drug concentration.

Power of MICRO-TAG Platform

MICRO-TAG platform supports 1) Drug discovery, 2) Validation & ranking, 3) Analysis of drug candidates.

Physiological environment of the cell

The MICRO-TAG system uses a 15-amino acid-long tag; it is significantly smaller than those used by the other enzyme complementation technologies. It does not interfere with localization, protein-protein interactions, or function of the tagged target protein. Additionally, the inert nature of the tag means it will not impact the folding of the protein that is critical when assessing drug-target engagement within the physiological environment of the cell.

Speed and sensitivity

MICRO-TAG platform is more robust than luminescence-based target engagement technologies. This enables fast signal and assay development for low- to high-throughput drug discovery.

It is important to note that the fluorescence signal generated during the enzyme complementation reaction is not due to a fluorophore attached to the target protein, but rather enzymatic cleavage of the FRET substrate catalyzed by the MICRO-TAG target. This unique chemistry makes the system highly sensitive with a wide dynamic range and adjustable assay time window. As shown in time-course kinetic analysis in **Figure 4**, the signal develops within seconds, proceeds through a linear log phase before reaching saturation. These unique time-course and speed-adjustability features enable real-time analysis of cell target engagement.

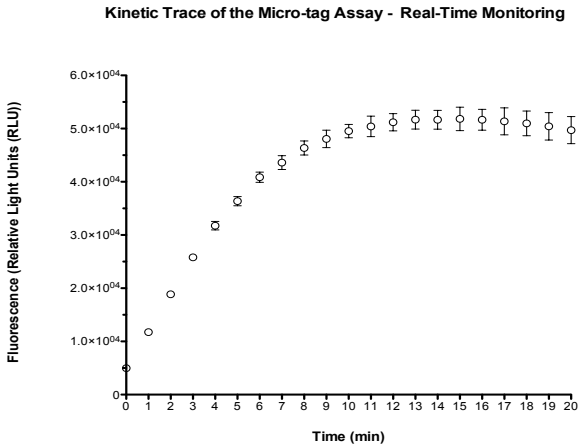


Figure 4. A kinetic trace of the development of the Micro-tag fluorescence signal. The kinetics of the signal is a result of the enzyme-catalyzed reaction of cleavage of the FRET substrate.

Various therapeutic modalities

The MICRO-TAG platform integrates well with discovery, validation and analysis of various therapeutic modalities:

- Small-molecules (allosteric and non-allosteric)
- PROTACS
- Peptides
- Antibodies

Target versatility

MICRO-TAG is a target-agnostic system that works well for a variety of drug target families. We tackle various target families:

- Enzymes
- Transcription factors
- Regulatory proteins
- Membrane proteins
- Other challenging targets

The small 15-aa tag allows for the testing of challenging protein targets such as membrane bound proteins, transcription factors or organelle proteins. Depending on the modality and target biology, the setup of the system may need to be optimized accordingly. Some points to consider include degree of thermal challenge, cell lysis vs no lysis etc.

Establishing a reporter system using the MICRO-TAG platform enables discovery and validation of drug candidates with unprecedented speed and sensitivity.

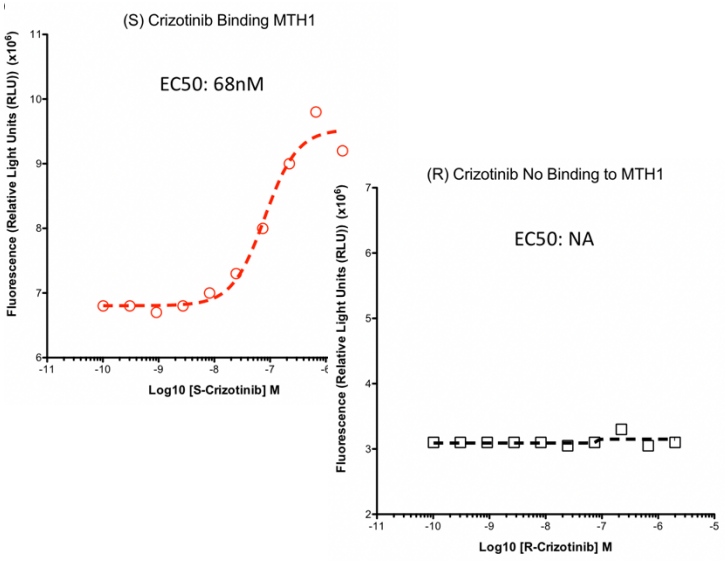


Figure 5. MICRO-TAG cell target engagement signal of MTH1 with (S) Crizotinib and (R) Crizotinib. The kinetics of the signal is a result of the enzyme-catalyzed reaction of cleavage of the FRET substrate.

Quantitation of cellular target engagement

We have tested MTH1 as a target in the MICRO-TAG system. We generated a construct with the 15-aa tag at its C-terminus. The target transiently expressed in HEK293 cells was exposed to thermal challenge and its temperature of aggregation (T_{agg}) was established as 55C.

Using the MICRO-TAG system, we were able to quantitate cellular engagement of MTH1 inhibitors. S-Crizotinib is an inhibitor of MTH1 that is well described in the literature. S-Crizotinib engaged MTH1 with EC50 of 68nM as determined. However, its inactive enantiomer, R-Crizotinib, engaged the target at much higher EC50 of 800nM. See **Figure 5**.

BCL6 is transcription factor, which is also known as a challenging drug target. We have followed a similar path to interrogate cell target engagement of its inhibitors. The well-characterized BCL6 inhibitor, BI-3812, interrogated at T_{agg} of 43.8C revealed EC50 of 3nM. However, its close structural analog, BI-5273, did not engage the target at high micro-molar doses. See **Figure 6**.

Mechanism of action of target engagement

The MICRO-TAG can be used to revel valuable insight into mechanism of action and binding stoichiometry between drug-target pairs. The MICRO-TAG method can be used to identify the dose of a drug at which the protein target is saturated by the drug to give maximum fluorescence (E_{max}). The target saturation dose can be identified by this method as shown in **Figure 7**.

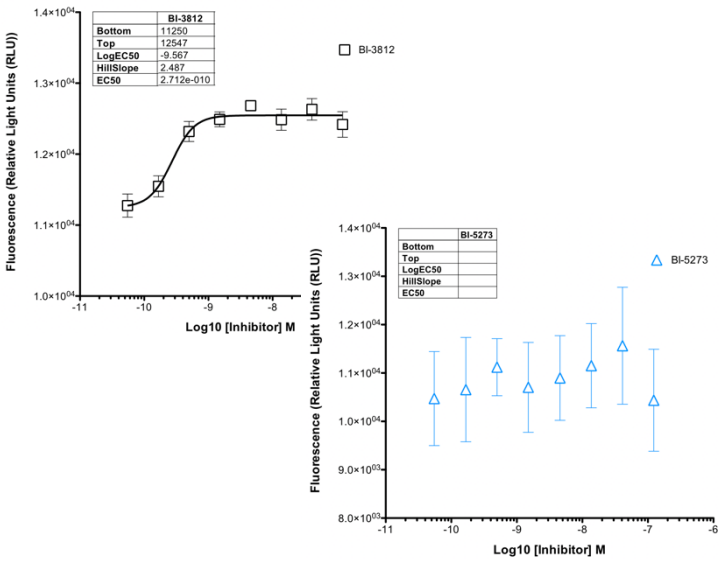


Figure 6. Testing BCL6-Micro-tag with BI-3812 and BI-5273 inhibitors. Micro-tagged wild-type BCL6 was expressed in HEK293 cells and lysates from these cells were treated with BI-3812 and BI-5273. No sigmoidal dose-response curve could fit the data for the BI-5273 compound.

E_{max} is identified by an inflection point (**Figure 7A-C**) at which the fluorescence signal begins to decrease over time rather than increase. MICRO-TAG enables time-resolved monitoring of the target engagement at various doses of inhibitor in order to accurately calculate E_{max}. It is an important parameter for drug discovery as it is used for defining target occupancy.

EC50, potency cell target engagement, is determined from the sigmoidal dose-response curve of the early time points. **Figure 7D**). Non-linear regression analysis fitting a sigmoidal dose-response curve can be used to calculate EC50. EC50 can be used as a readout for ranking and prioritizing drug candidates.

Furthermore, apparent equilibrium dissociation constant (apparent K_D) can be calculated by fitting the observable fluorescence signal data to a Saturation Binding Equation (**Figure 7E**). K_D of a drug-target pair can be confidently calculated using the log phase of the curve where the amount of signal is directly proportionality with target stabilization.

Such valuable quantitative parameters can be used for rigorous and efficient discovery of novel therapeutics in the physiological environment of the cell.

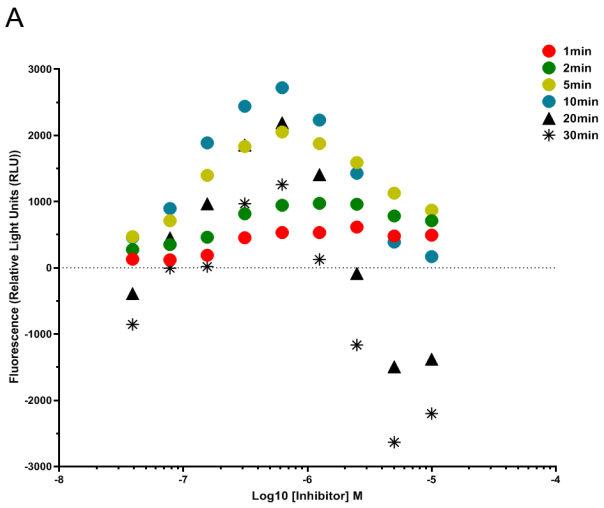


Figure 7. Identification of target saturation dose, E_{max}, EC50 of target engagement, and apparent K_D. (A) Kinetic trace showing fluorescence over time for each concentration of inhibitor tested.

Figure 7, continued.

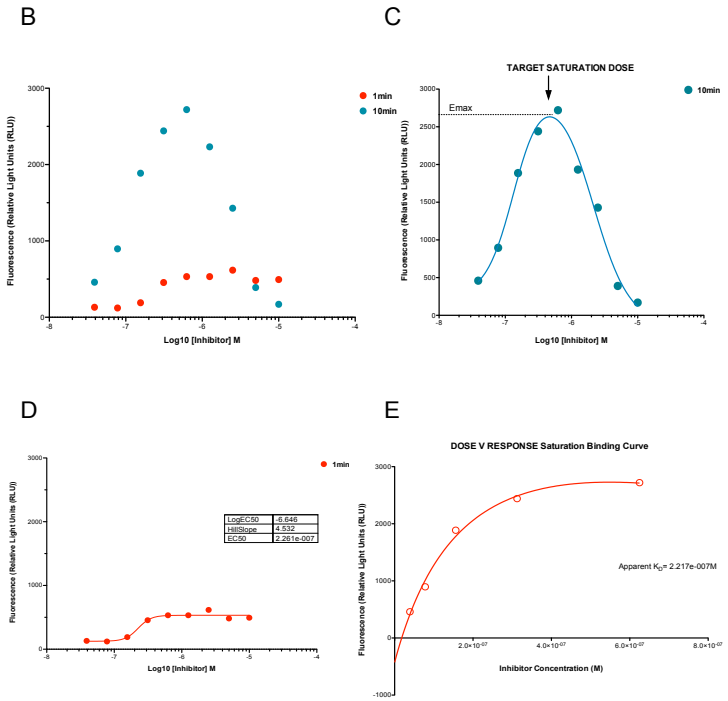
(B) At higher drug doses the later time point (10min) shows lower signal than the early time point (1min). This decreased signal at higher doses of drug results in a bell-shaped curve.

(C) A bell-shaped curve identifies the saturating concentration of drug (target saturation dose) that yields maximum target stabilitty (E_{max}).

(D) EC50 of target engagement can be determined from fitting a sigmoidal dose-response curve to the early time point data.

(E) From the identification of the target saturation dose a saturation binding curve can be generated, and nonlinear regression analysis of curve fitting can identify an apparent equilibrium dissociation constant, K_D.

The EC50 of Target Engagement (2.6e-7M) and Apparent K_D (2.2e-7M) are identical demonstrating that the fluorescence readout is directly proportional to drug binding and can be used to determine apparent affinity binding constants.



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Accelerate and De-risk Drug Discovery with CellarisBio

CellarisBio is a drug discovery platform company, based in San Diego, California. Our MICRO-TAG cell target engagement platform is built to tackle challenging drug targets and de-risk drug discovery programs.

We work across various therapeutic targets classes such as:

- Enzymes
- Membrane proteins
- Transcription factors
- Other challenging proteins

We work with multiple therapeutic modalities such as:

- Small molecules
- Peptide
- PROTACS
- Antibodies

Accelerate your drug discovery with CellarisBio:

- Discover & validate novel drug candidates;
- Validate drug candidates for potency and selectivity;
- Analyze mechanism of action of novel drug candidates.

Some of the drug targets we worked with:

What drug targets are you interested in?

Get in touch

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