Drug discovery

# Quantify drug–target engagement in cells using a step gradient assay with real-time fluorescence detection

## Introduction

Analysis and confirmation of direct binding of a drug to its intended target is a critical part of drug discovery efforts. However, most proteins that are considered therapeutic targets are not amenable to gold-standard biophysical methods or co-crystallization strategies, due to the artificial acellular environment employed by these methods. Challenging drug targets require the complexities of the physiological cellular environment for proper folding and function. In-cell drug–target engagement techniques offer a means of validating and analyzing direct drug–target interactions in the context of living cells [1,2]. They are based on protein thermal shift assays, which rely on thermodynamic stabilization of a protein resulting from ligand binding [3]. This thermal stabilization can be detected when a heat challenge is applied to cells, forcing denaturation and aggregation of the protein target. Some of these drug–target engagement assays are limited by operational workflows that are laborious, time-consuming, and not scalable for high-throughput drug discovery [4]. Other methods for in-cell target engagement studies have improved on these limitations and offer capacity for high-throughput screening. These strategies involve enzyme complementation systems in which a small peptide tag is conjugated to the protein of interest [5,6]. Thermal challenge buries the tag in denatured and aggregated protein, making it inaccessible for enzyme complementation with its binding partner subunit. However, these methods are based on chemiluminescence or luminescence readouts that have sensitivity limits from high background noise, are not amenable to integration with standard scientific instruments used for monitoring live cells, lack versatility of detection chemistry, and have decreased instrument sensitivity and capability.

CellarisBio's MICRO-TAG™ fluorescence-based drug–target engagement technology improves on these techniques by integrating an enzymatic reaction for fluorescence development, supporting better signal-to-noise ratios, and enabling improved sensitivity and dynamic range by pairing with an Applied Biosystems™ QuantStudio™ instrument for real-time fluorescence detection. MICRO-TAG technology is based on enzyme complementation with a fluorescent readout. It involves a short 15–amino acid tag cloned to either the N or C terminus of a target protein of interest. This proprietary tag is predominantly hydrophilic, making it a better tag for use in thermal shift assays than other similar enzyme complementation tags that are predominantly hydrophobic and thus require addition of a spacer or linker region to extend the tag from the protein target. The tag is the small subunit of a two-subunit enzyme. Complementation of the tag with the large subunit forms an active RNase capable of cleaving a specific RNA sequence (Figure 1). In this target engagement method, the complemented RNase complex with its binding partner cleaves a specific RNA FRET substrate to generate a fluorescent signal [7]. The FRET substrate offers versatility through a choice of different donor/quencher FRET pairs to accommodate different detection wavelengths. Generating fluorescence by an enzymatic reaction amplifies the signal, making the system highly sensitive with a wide dynamic range. Drug–target engagement using MICRO-TAG technology is amenable to a step gradient method that utilizes the thermal cycler programming capability of highly sensitive QuantStudio instruments (Figure 2). This novel strategy helps improve upon previous drug–target engagement techniques through increased sensitivity and versatility of the detection chemistry, multiplexing capability, decreased time performing the experiments, and cost savings from the streamlined workflow.

This white paper features CellarisBio's MICRO-TAG drug–target engagement technology with the Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System. The step gradient method presents a novel approach with minimal sample handling, which employs the thermal cycling programming of the real-time PCR instrument. Programmed heating and cooling steps allow for simultaneous thermodynamic stabilization from ligand binding with detection of the fluorescent signal generated from FRET substrate cleavage. This step gradient method helps save time and costs of performing drug–target engagement studies by eliminating the requirement for identifying a melting temperature for the protein target of interest.

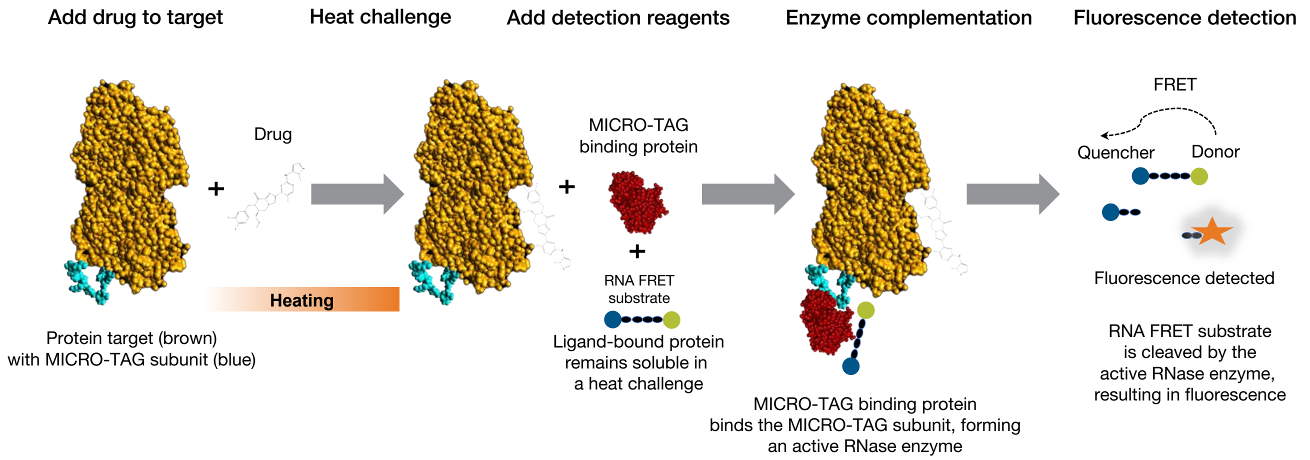

**Figure 1. CellarisBio's MICRO-TAG enzyme complementation system for drug–target engagement.** FRET: fluorescence resonance energy transfer.

**Figure 2. CellarisBio's step gradient drug–target engagement method employing a QuantStudio instrument for real-time fluorescence detection.**

## Materials and methods

### Materials

- Thermo Scientific™ Tris-Buffered Saline (TBS, 10X) (Thermo Fisher Scientific, Cat. No. J62938.K7), diluted to 1X (50 mM Tris-HCl, 150 mM NaCl, pH 7.4)
- Nondenaturing lysis buffer: 1% Triton™ X-100 surfactant in TBS containing cOmplete™ Mini EDTA-Free Protease Inhibitor Cocktail (Roche, Cat. No. 11836153001)
- MAPK1 reporter cells expressing protein target (CellarisBio)
- Binding buffer, binding protein, and FRET substrate (CellarisBio)
- Ligands for testing, prepared in DMSO (MilliporeSigma, Cat. No. D2650)
- Thermo Scientific™ PCR Plate, 96-well, clear (Thermo Fisher Scientific, Cat. No. AB0700)
- Applied Biosystems™ MicroAmp™ Optical Adhesive Film (Thermo Fisher Scientific, Cat. No. 4311971)
- Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate (Thermo Fisher Scientific, Cat. No. N8010560)
- Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific)
- Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes, 6-well (Thermo Fisher Scientific, Cat. No. 140675)
- Thermo Scientific™ Nunc™ 15 mL Conical Sterile Polypropylene Centrifuge Tubes (Thermo Fisher Scientific, Cat. No. 339650)
- Mastercycler™ X50s Thermocycler (Eppendorf)

### Lysate preparation

Cells expressing the tagged MAPK1 construct are grown in a 6-well tissue culture plate.

1. Lift cells from plate by pipetting up and down and then transferring to a 15 mL tube.
2. Pellet cells at 400 x *g* for 3 minutes.
3. Remove supernatant, wash cells with 3 mL of TBS, and pellet cells again.
4. Remove TBS wash, and lyse cells with 250 µL of nondenaturing lysis buffer for 1 hour at 4°C on a rotator.
5. Spin at 14,000 rpm for 1 minute in a microcentrifuge, and transfer the lysate to a new tube. Lysates may be stored at –80°C.

### Step gradient drug–target engagement assay

1. Prepare ligands for testing at 100X in DMSO.
2. Dilute nondenaturing lysates 1:20 with cold TBS on ice. Aliquot 49.5 µL/well to 12 wells of a 96-well PCR plate and add 0.5 µL of the 100X ligands. Include a DMSO control in adjacent wells as a vehicle control.
3. Prepare reaction buffer by adding the FRET substrate and binding protein to binding buffer. Invert tube to mix (do not vortex), and dispense 80 µL to a MicroAmp Optical 96-Well Reaction Plate.
4. Add 20 µL of lysate plus ligand (2 tests can be performed from the 50 µL available) to the 80 µL reaction buffer. Seal the plate and immediately place in a real-time PCR system, and detect FAM™ dye fluorescence (excitation 493 nm, emission 517 nm) with step programming as shown in Figure 3**.**

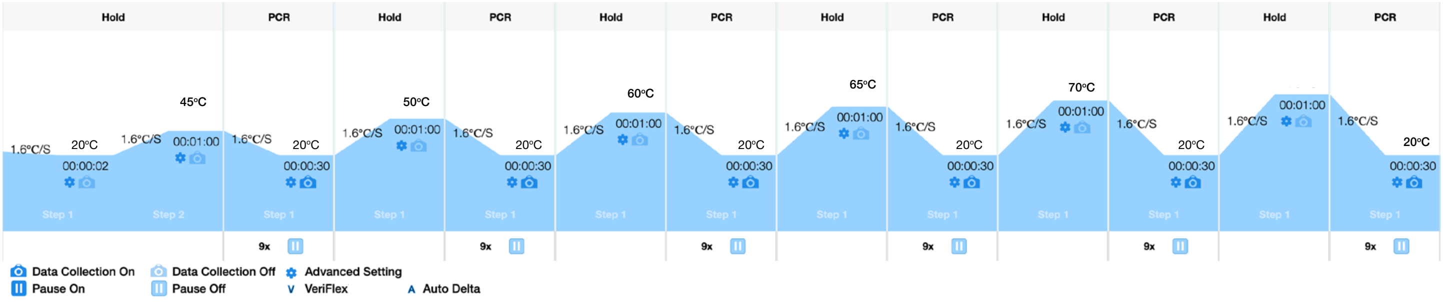

**Figure 3. Real-time step gradient programming for detection of ligand binding.**

## Results

Binding of a ligand to its target results in thermal stabilization, yielding an increase in the fluorescence signal. The fluorescence real-time curve for bound ligand increases above the DMSO control (Figure 4A). The real-time curves from the analysis of the step gradient show that a separation of the drug-treated sample from the DMSO control takes place at the 55°C heat step. Above 55°C the curves diverge, indicating that the protein target remains soluble because of thermodynamic stabilization imparted by ligand binding. Below 55°C there is no difference between drug-treated and control samples. This separation of signal at the 55°C heat step correlates with the reported temperature of melting and aggregation for MAPK1 of 54°C. The experimental setup can include testing at a single concentration, such as 2.5 µM in this study (Figure 4A). This type of setup is typical for a high-throughput screening assay and allows for quick identification of binding compounds. Alternatively, a dose-response experiment can be used to identify the $EC_{50}$ 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 a 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 the DMSO control from the fluorescence for the ligand test. The delta fluorescence was plotted against the ligand concentration, and an $EC_{50}$ of target engagement of approximately 2 nM was determined (Figure 4D).

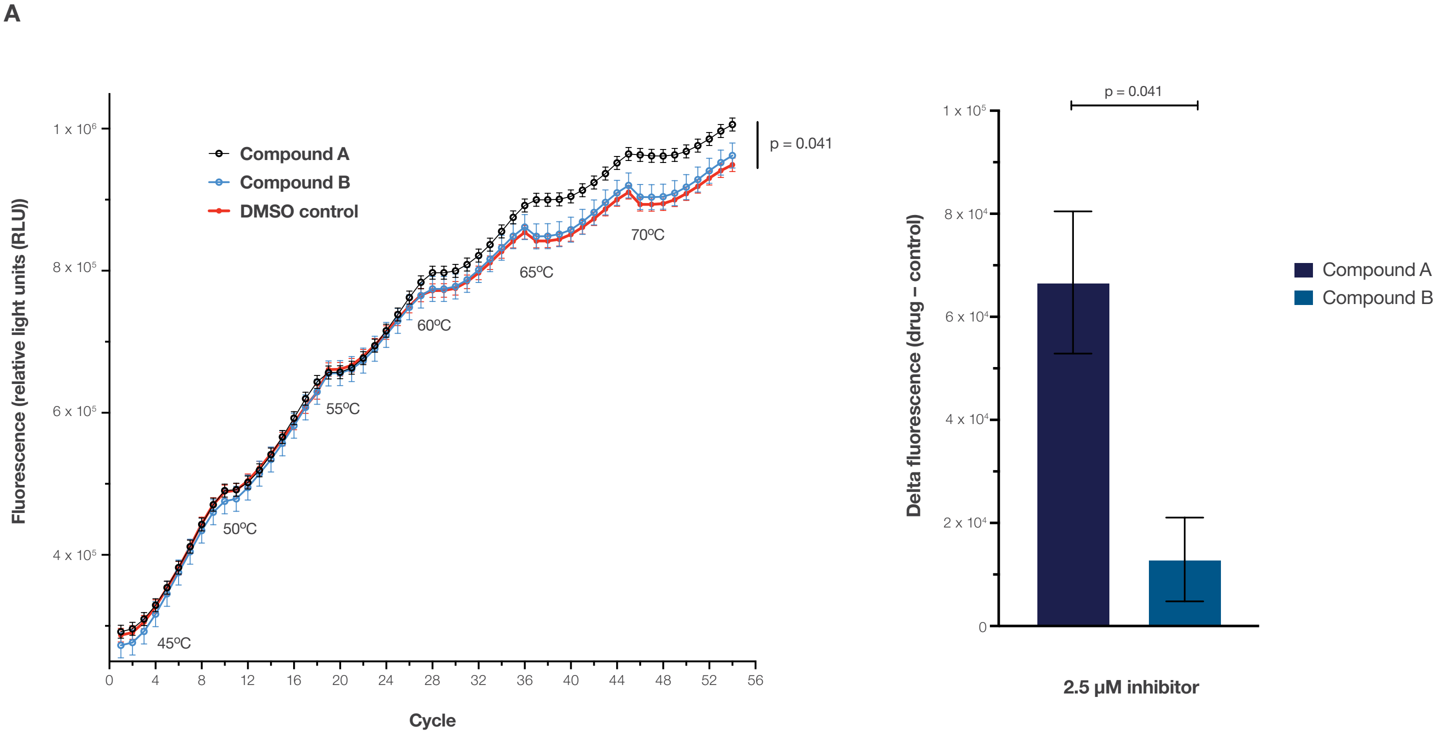

**Figure 4. Real-time step gradient results for testing ligand binding to tagged MAPK1 target.** (A) Real-time data (left panel) from testing compound A and compound B at 2.5 µM along with DMSO control show binding of compound A, and the difference in fluorescence (delta fluorescence) between drug-treated and DMSO-treated samples (right panel).

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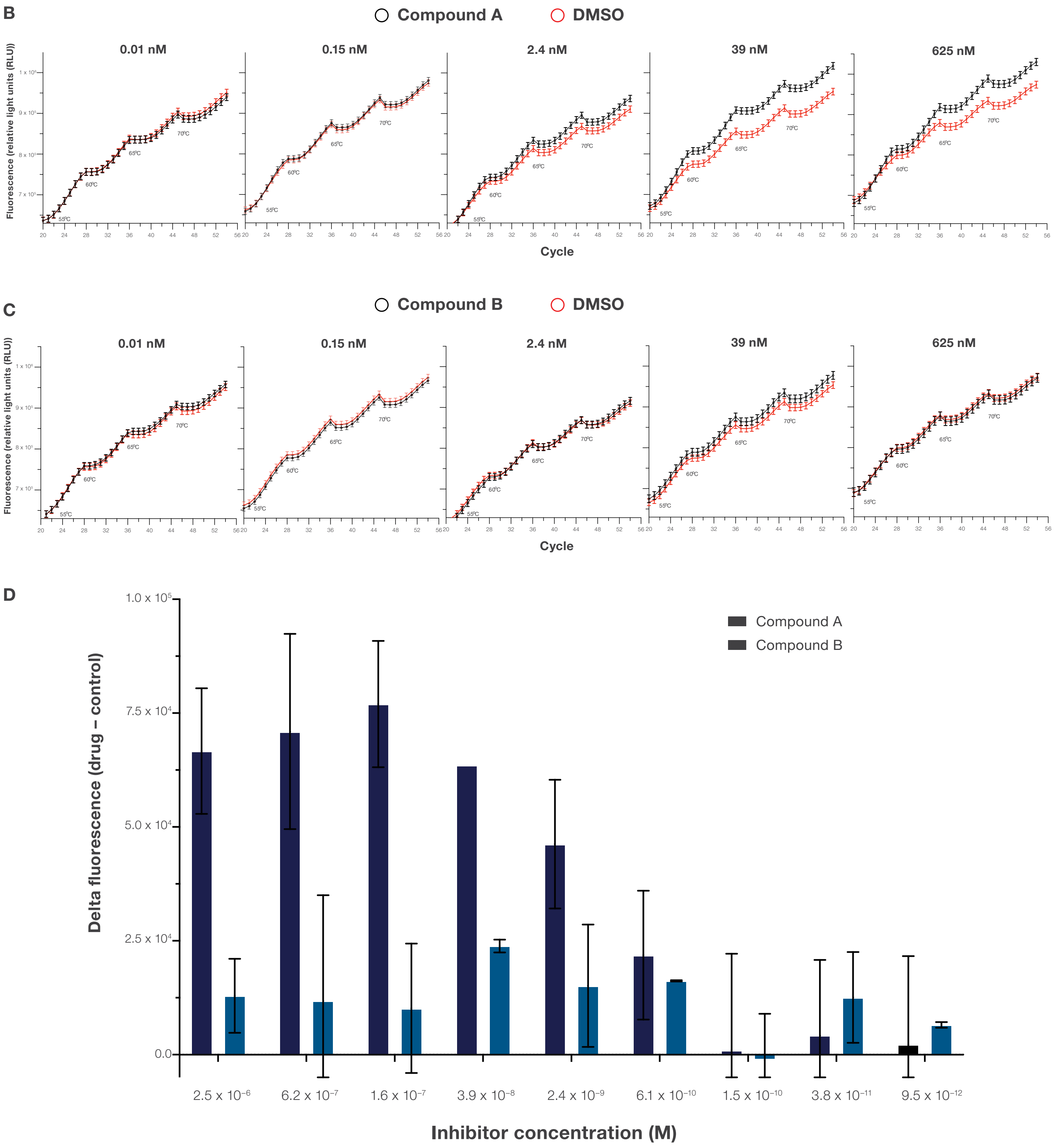

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**Figure 4. Real-time step gradient results for testing ligand binding to tagged MAPK1 target. (B)** Real-time curves from the step gradient analysis of increasing doses of compound A. **(C)** Real-time curves from the step gradient analysis of increasing doses of compound B. **(D)** Delta fluorescence between drug-treated and DMSO-treated samples at the end of the step program.

## Discussion

Conventional drug–target engagement strategies require exact determination of a protein melting and aggregating temperature, followed by interrogation of ligand binding at that specific temperature after a prolonged incubation with ligands. However, this temperature of melting and aggregation may vary for each experiment depending on expression levels of the target, numbers and health status of the cells expressing the target, volume of cells or lysates, and accurate heating of samples at the identified temperature. To overcome these variables, it is advantageous to remove the requirement to precisely measure the melting and aggregating temperature and to decrease processing steps. The integration of CellarisBio's MICRO-TAG fluorescence-based drug–target engagement technology with the thermal programming and real-time fluorescence detection capabilities of QuantStudio instruments eliminates the necessity for melting temperature determination prior to testing ligand binding.

Testing ligand binding using the step gradient thermal cycling program offers convenience by decreasing the number of processing steps involved in setting up the reaction. In addition, the "mix and detect" feature of the protocol without any preincubation allows for faster turnaround time and provides real-time analysis of drug–target interaction within a cellular context, prior to equilibrium being reached between drug and target. This provides additional layers of information regarding rate of drug association with the target. Combining a QuantStudio instrument with CellarisBio's MICRO-TAG fluorescence drug–target engagement technology allows for simultaneous determination of aggregation temperature with ligand binding analysis. The technique is amenable to the 384-well QuantStudio instrument for increased throughput and ease of automation.

Advantages of MICRO-TAG drug–target engagement technology combined with a QuantStudio instrument for drug discovery:

- **Helps save time and cost**—No need to identify the protein melting and aggregation temperature prior to testing ligand binding. Therefore, fewer experimental steps streamline the process, use less reagents, and enable faster time from setup to analysis of binding.
- **Increased sensitivity with greater dynamic range**—Real-time PCR instruments are designed for sensitive, broad detection of fluorescence.
- **Increased flexibility of the assay**—The assay is not limited to just one substrate. Choice of multiple FRET pairs and substrates for modified readout (available through Thermo Fisher Scientific) offers versatility in experimental setup.
- **Increased throughput and ease of automation**—Assays can be adapted to high-throughput drug discovery applications.
- **Less troubleshooting from fewer procedural steps**—Melting temperature of some targets can be a technical challenge. However, the step gradient technique provides detailed information of binding across all temperatures in one run.*

## Conclusions

CellarisBio's step gradient assay for in-cell drug–target engagement integrated with a QuantStudio instrument for real-time fluorescence detection offers a versatile, fast, cost-efficient, and robust strategy for analyzing the binding of a drug to its target. This technology is applicable to challenging protein targets that cannot be expressed outside the cellular context. The real-time strategy empowers the assay setup and analysis through increased sensitivity and flexibility of instrumentation programming and choices in donor/quencher FRET pairs offered with the substrate. This methodology streamlines the drug discovery process, offering savings on time and cost. It allows increased throughput and ease of automation while enabling discovery of therapeutics for challenging drug targets.

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* Technical support with assay optimization: CellarisBio supports assay optimization and technology transfer/licensing.

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