Application Note:

Evaluation of CellarisBio MICRO-TAG® Cell Target Engagement Assay on Azure Cielo™ Real-Time PCR System

About MICRO-TAG® Cell Target Engagement:

MICRO-TAG® is a next-generation cellular target-engagement technology that combines enzymatic signal amplification with real-time thermal control to deliver high sensitivity and broad dynamic range. The platform integrates seamlessly with the Azure Cielo™ real-time PCR system, leveraging its precise thermal-cycler programming to enable step-gradient thermal target-engagement measurements.

MICRO-TAG® is based on enzyme complementation using a short, 15-amino acid, predominantly hydrophilic tag fused to the N- or C-terminus of a protein of interest. Unlike hydrophobic complementation tags that require spacer or linker regions, the MICRO-TAG® sequence minimizes structural perturbation of the target and is well suited for cellular target engagement assays across multiple target classes. Upon complementation with its large subunit, the enzyme forms an active RNase that cleaves a specific RNA FRET substrate to generate a fluorescence signal (**Figure 1**).

Signal generation through enzymatic cleavage provides intrinsic amplification, resulting in superior signal-to-noise, enhanced sensitivity, and a wide dynamic range. The modular FRET substrate design enables flexible donor–quencher pairing to support multiple detection wavelengths. Together, the MICRO-TAG® chemistry and Azure Cielo™ platform deliver a fast, sensitive, and scalable solution for cellular drug–target engagement, improving performance, workflow efficiency, and cost relative to existing approaches. Learn more on www.cellarisbio.com.

https://pubmed.ncbi.nlm.nih.gov/41285198/

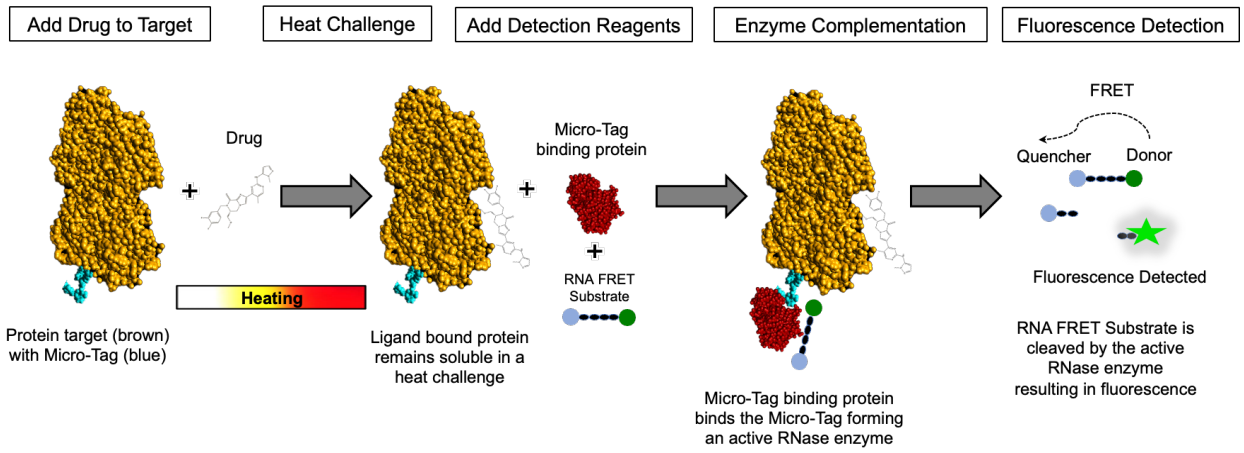


Figure 1. CellarisBio's MICRO-TAG enzyme complementation system for cell target engagement.

Introduction to Application Note:

The purpose of this application note was to test the Azure Cielo™ real-time PCR system for capability to perform cell thermal shift assay using CellarisBio MICRO-TAG® cell target engagement technology. The technology assesses drug engagement with its intended protein target in cells. As proof of concept, we employed the K-Ras G12D mutant MICRO-TAG® construct together with the Mirati Therapeutics compound MRTX1133. HEK293T cells were transfected with the K-Ras MICRO-TAG construct and tested for binding with compound 48hrs post-transfection.

Methods:

1. Transfect HEK293 cells with the K-Ras G12D MICRO-TAG construct (2.5ug/well) by reverse transfection in a 6-well plate, using Lipofectamine 3000 transfection reagent according to manufacturer's instructions.
2. 48hrs post transfection, lift cells with pipetting up/down and transfer the 2ml to a 15ml tube, pellet cells 400g 3min, remove supernatant, wash with 3ml 1x TBS and pellet cells again. Remove TBS wash and resuspend cells in 1.4ml 1x TBS.
3. Dispense 49.5ul cells into 12 wells on PCR plate or strip.
4. Compounds are typically tested at 20uM down to 1nM final concentrations. Serial dilutions (1 in 3) of 100x compound (MRTX1133 positive control compound) in DMSO in a 12-well PCR tube strip.
5. Transfer 0.5ul of the 100x compounds to the cells. Final DMSO concentration will be 1% for each dose.

6. Incubate on ice 1hr.
7. Thaw the Binding Buffer in a room temperature water bath and keep at room temperature.
8. Thaw the FRET RNA Substrate quickly (2min) in a room temperature water bath and place on ice and keep vial in the dark.
9. Afte 1hr incubation of the cells with compound add 50ul of 0.5% Triton-X-100 (prepared in TBS) to the cells and place on ice 10min.
10. During this 10min incubation, thaw the Binding Protein on ice (takes about 10min).
11. Prepare Reaction Buffer by adding the FRET RNA Substrate and the Binding Protein to the Binding Buffer. Invert tube to mix (do not vortex) and dispense 50ul to the 96-well reaction plate compatible with the Cielo real-time qPCR system.
12. Spin the plate for 30sec at 400g to bring all the volume to the bottom of the wells.
13. Add 20ul of the Triton incubated cells to the 50ul Reaction Buffer being careful not to introduce air bubbles. Seal the plate and immediately place in the Cielo real-time qPCR system and run Temperature Series program: heat for 2min at 40°C then detect at 20°C for 10min, heat for 2min at 45°C then detect at 20°C for 10min, heat for 2min at 50°C then detect at 20°C for 10min, heat for 2min at 55°C then detect at 20°C for 10min, heat for 2min at 60°C then detect at 20°C for 10min.
14. Export raw fluorescence data from the real-time system. Normalize raw data to 0 by subtracting the first reading from all subsequent values. Normalize data to 0 after each heat step. Determine the slope (RLU/cycle) for each dose for each temperature tested and plot with inhibitor concentration on a semi-log scale to identify the EC50 of target engagement.

Results:

The binding curves generated with the Azure Cielo™ real-time PCR system are shown in **Figures 2-9**. The highest dose tested is 20uM and the lowest is 9nM. **Figures 2-9** also show the target engagement curves normalized to 0uM (DMSO control) after each heat step. **Figure 10** shows the EC50 of target engagement for each heat step calculated by determining the slope of fluorescence generated (RLU/cycle) from the curves.

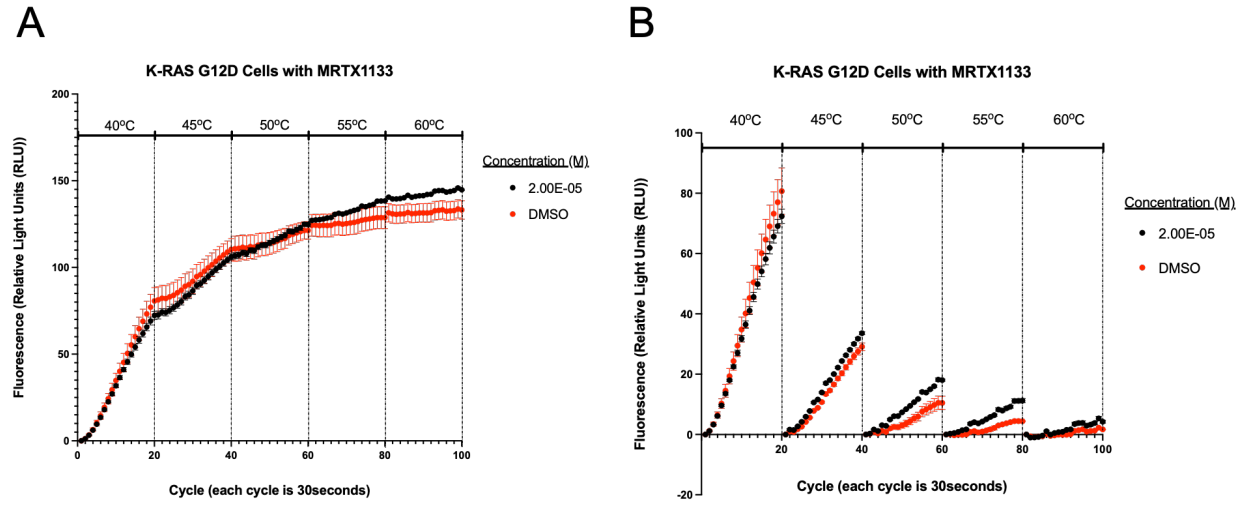


Figure 2: Cell target engagement curves for 20uM dose of MRTX1133 binding to K-Ras G12D Micro-Tag mutant.

(A) Real-time for MRTX1133 binding at 20uM compared to DMSO; (B) Real-time curves normlized to 0 fluorescence after every temperature step in the programmed series.

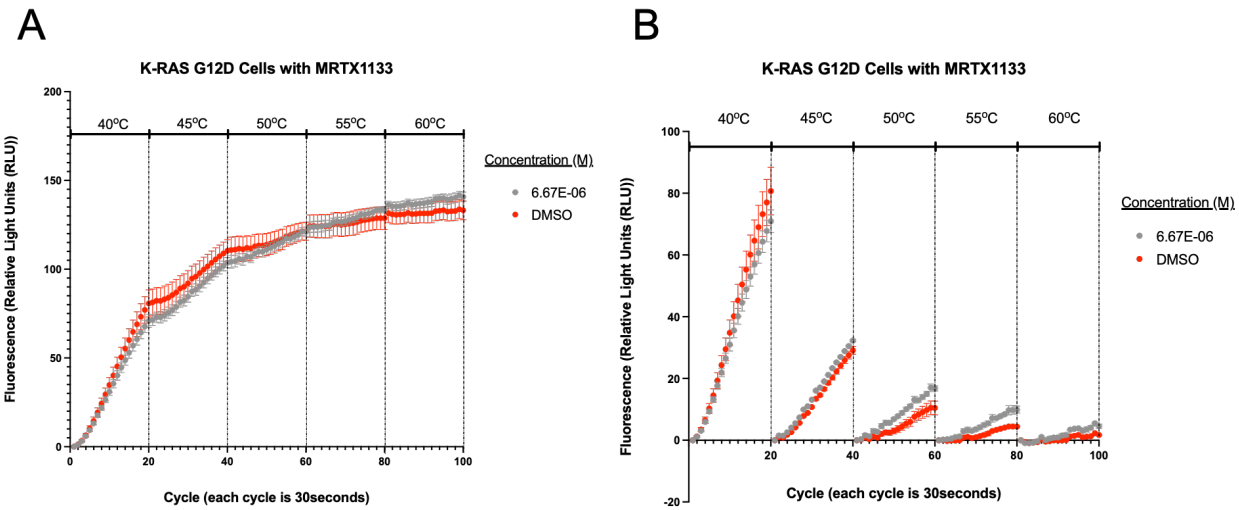


Figure 3: Cell target engagement curves for 6.7uM dose of MRTX1133 binding to K-Ras G12D Micro-Tag mutant.

(A) Real-time for MRTX1133 binding at 6.7uM compared to DMSO; (B) Real-time curves normlized to 0 fluorescence after every temperature step in the programmed series.

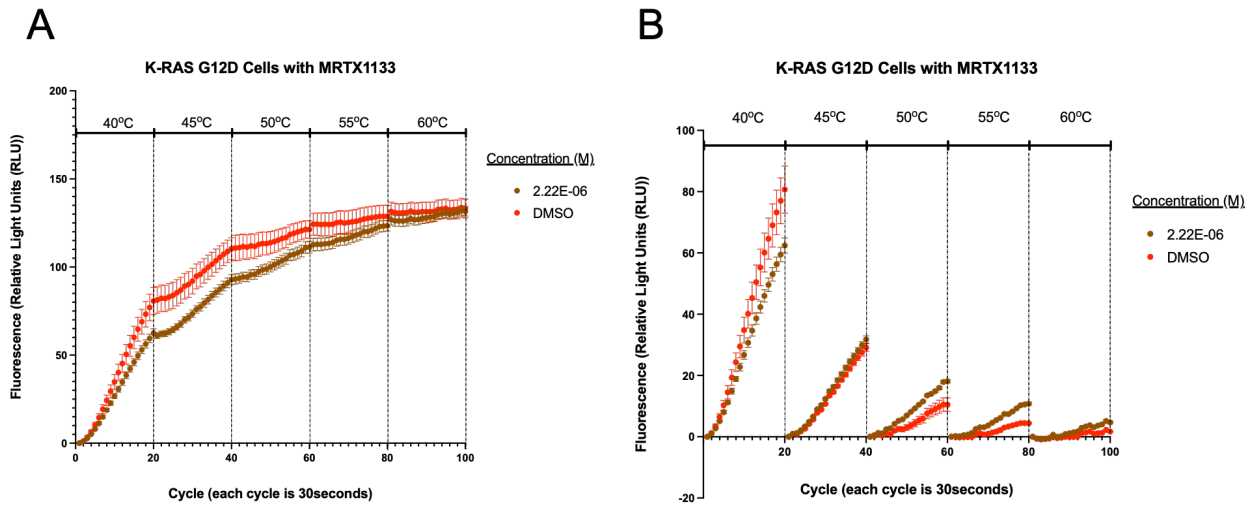


Figure 4: Cell target engagement curves for 2.2uM dose of MRTX1133 binding to K-Ras G12D Micro-Tag mutant.

(A) Real-time for MRTX1133 binding at 2.2uM compared to DMSO; (B) Real-time curves normlized to 0 fluorescence after every temperature step in the programmed series.

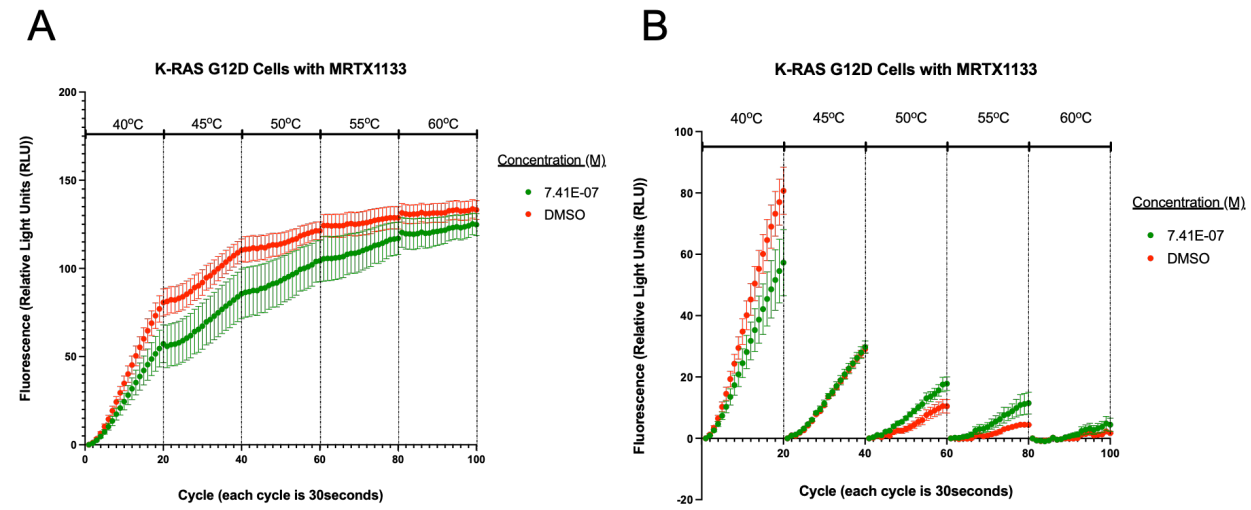


Figure 5: Cell target engagement curves for 0.74uM dose of MRTX1133 binding to K-Ras G12D Micro-Tag mutant.

(A) Real-time for MRTX1133 binding at 0.74uM compared to DMSO; (B) Real-time curves normlized to 0 fluorescence after every temperature step in the programmed series.

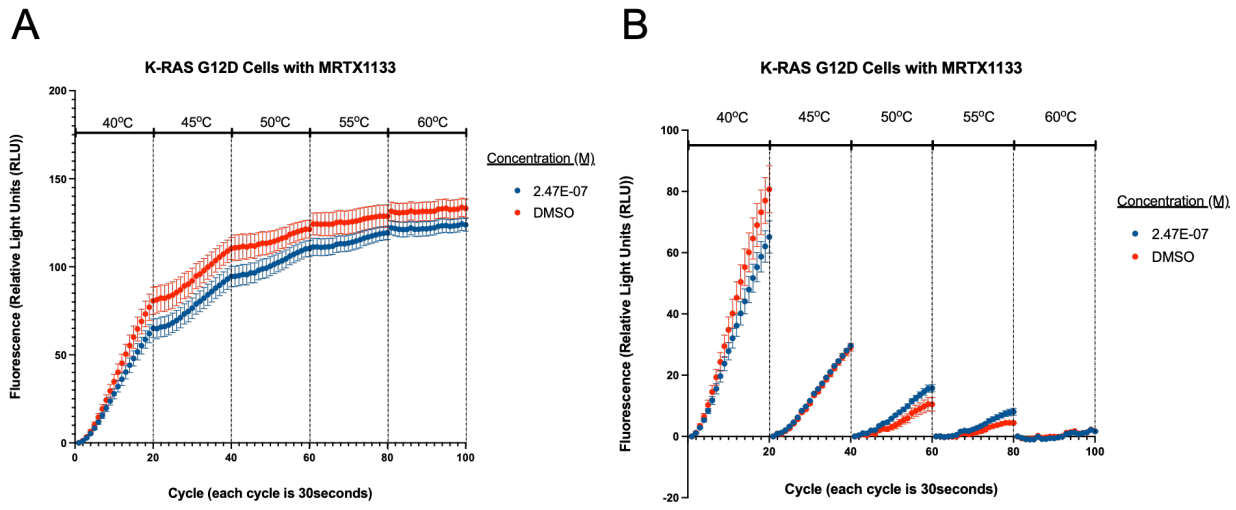


Figure 6: Cell target engagement curves for 0.25uM dose of MRTX1133 binding to K-Ras G12D Micro-Tag mutant.

(A) Real-time for MRTX1133 binding at 0.25uM compared to DMSO; (B) Real-time curves normlized to 0 fluorescence after every temperature step in the programmed series.

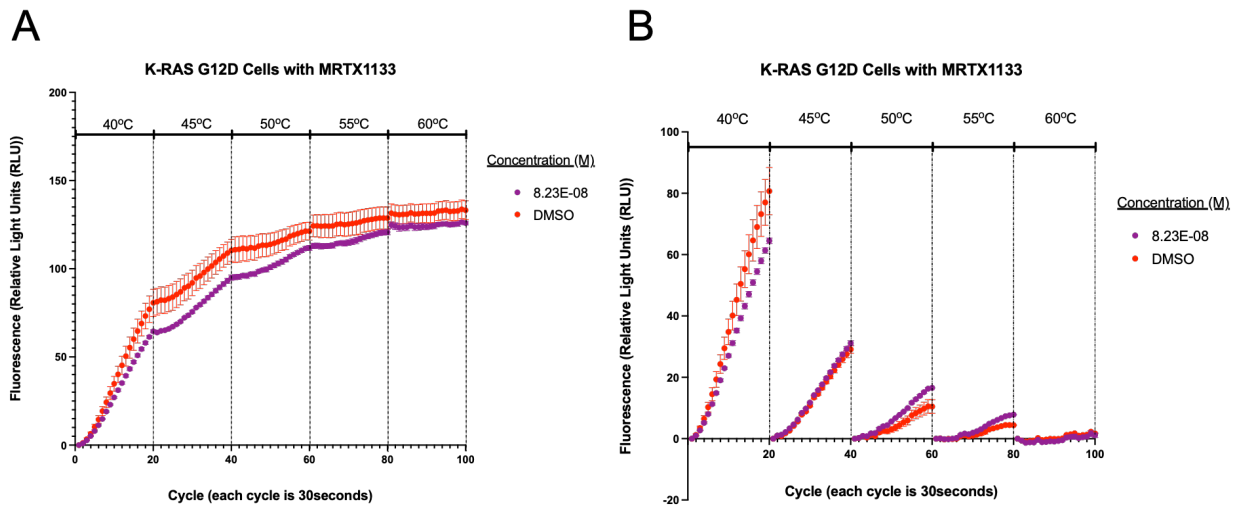


Figure 7: Cell target engagement curves for 82nM dose of MRTX1133 binding to K-Ras G12D Micro-Tag mutant.

(A) Real-time for MRTX1133 binding at 82nM compared to DMSO; (B) Real-time curves normlized to 0 fluorescence after every temperature step in the programmed series.

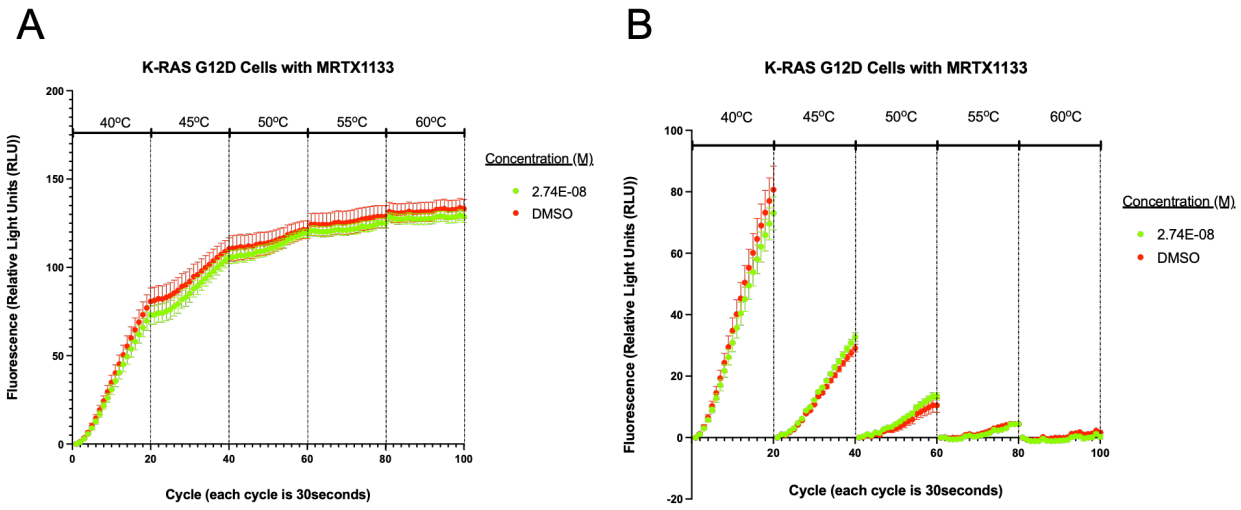


Figure 8: Cell target engagement curves for 27nM dose of MRTX1133 binding to K-Ras G12D Micro-Tag mutant.

(A) Real-time for MRTX1133 binding at 27nM compared to DMSO; (B) Real-time curves normlized to 0 fluorescence after every temperature step in the programmed series.

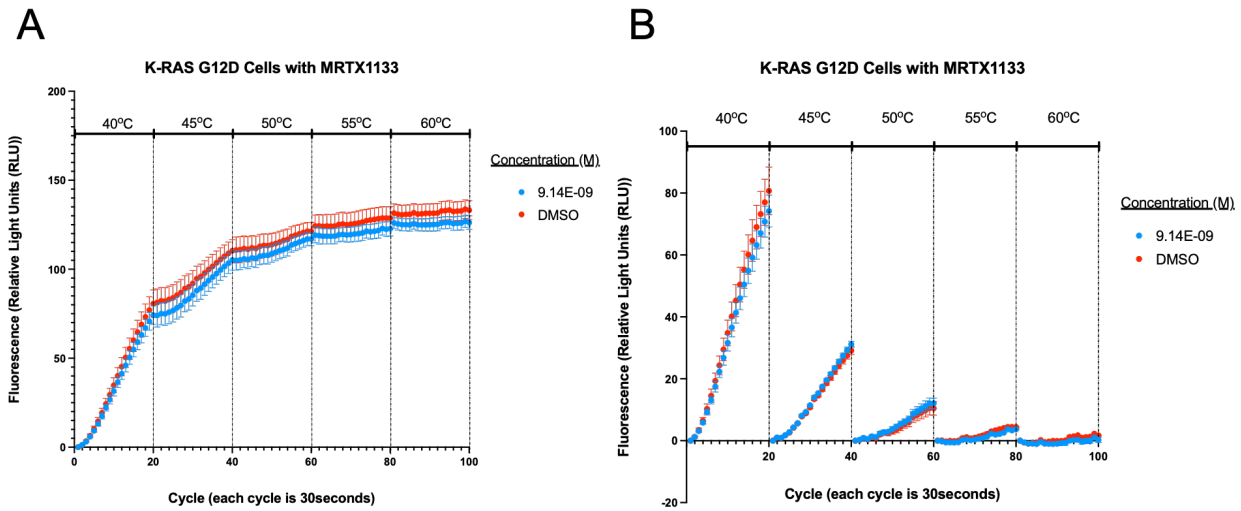
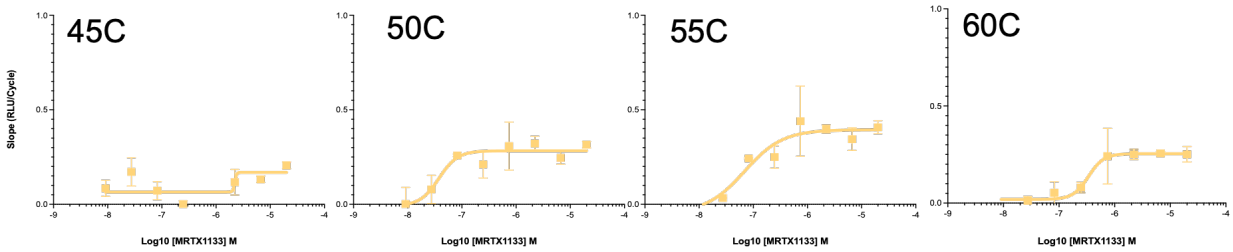


Figure 9: Cell target engagement curves for 9nM dose of MRTX1133 binding to K-Ras G12D Micro-Tag mutant.

(A) Real-time for MRTX1133 binding at 9nM compared to DMSO; (B) Real-time curves normlized to 0 fluorescence after every temperature step in the programmed series.



EC50:	2.2uM	37nM	69nM	337nM

Figure 10: Per-temperature EC50 determined from slope of signal generation (RLU/ cycle) for each temperature tested.

Conclusions:

Cellular thermal shift assay employing CellarisBio's MICRO-TAG® technology was successfully performed using the Azure Cielo™ real-time PCR system. The data generated is consistent with previous data generated using Applied Biosystems QuantStudio 3 real-time instrument. The results demonstrate the Azure Cielo™ real-time PCR system can be used successfully to perform drug-target engagement studies with intact cells using CellarisBio's MICRO-TAG® technology.

More about MICRO-TAG® cell target engagement:

https://pubmed.ncbi.nlm.nih.gov/41285198/

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