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Caution: Research use only.

Alpha *SureFire*® CETSA®
MEK1 Assay Kit

Products: CETSA-MEK1-A500, CETSA-MEK1-A5K1, CETSA-MEK1-A5K2, CETSA-MEK1-A5K3, CETSA-MEK1-A5K4, CETSA-MEK1-A5K5

Kit-Specificity Information

This assay kit contains 2 antibodies which recognize invariant epitopes on MEK1. The protein detected by this kit corresponds to GenBank Accession NP_002746. MEK1 is also known as MKK1, MAPKK1, PRKMK1 and MAP2K1.

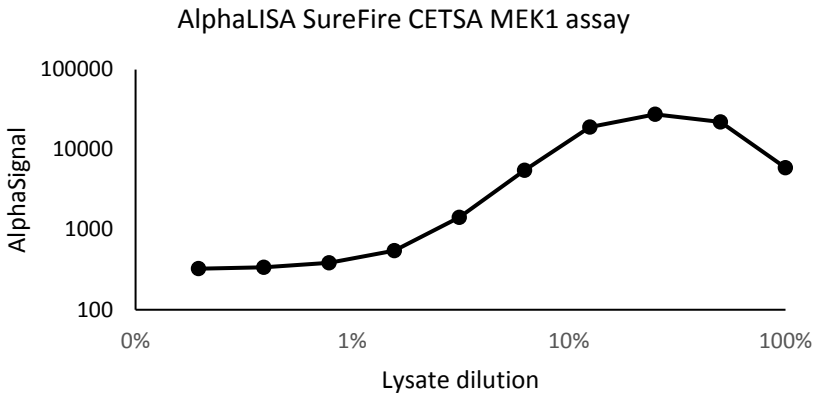
These antibodies recognize MEK1 of human and mouse origin. Other species should be tested on a case-by-case basis.

Control Lysate Information

Positive Control Lysate: Prepared from A431 cells, cultured to confluence in T175 flasks in 10% FBS containing medium, then treated with 200ng/mL EGF for 10min and lysed in 10mL of 1X *SureFire* Ultra Lysis buffer

Representative Immunoassay data using the control lysate

Data obtained with a 2-plate, 2-incubation protocol.



Representative CETSA® assay data

The Data in this Technical datasheet was obtained using the generic Alpha *SureFire®* CETSA® Assay Kits protocol (2-plate, 2-incubation protocol) with the following segments:

- Cell density titration and Lysis Buffer optimisation
- CETSA® assay melting and shift curve analysis
- CETSA® assay concentration-response analysis

For more information and detailed protocols, please refer to the generic Alpha *SureFire®*CETSA® Assay Kits Manual, available from http://www.perkinelmer.com/category/alpha-CETSA-kits

Cell line culture conditions

Human epidermis skin epithelial carcinoma cell line A431 (ATCC® CCL-1555 ™) was cultured using the following conditions:

A431 culture medium:

DMEM high glucose (Thermo Fisher, #11965092) supplemented with 10% FBS (Thermo Fisher, #10500064), 100 units/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher, #15140-122).

A431 culture conditions:

The cells were cultured in complete DMEM medium at 37°C, 5% CO_2, in a humified atmosphere to 70% confluency. For harvesting, the cells were first washed in Hanks Balanced Salt Solution (HBSS, Thermo Fisher, #14025-050) and then incubated with TrypLE (Thermo Fisher, #1256301) for 5 minutes. The cells were collected and washed twice in HBSS before resuspension to the desired density in HBSS.

Cell density titration and Lysis Buffer optimisation

Method

The A431 cells were harvested and resuspended to the desired density in HBSS. In a volume of 30 µL, the cells were serially diluted to: 20, 10, 5, 2.5, 1.25, 0.625 and 0.312 million cells/mL. A negative control of HBSS only was included. Ten dilution series were prepared. Five of these were heated to 37°C (corresponding to maximum protein levels) and five were heated to 70°C for 3 minutes (corresponding to background protein levels). One dilution series from each group was treated by the addition of 30 µL (Resulting in a final top concentration of 10 million cells/mL) of each 2x Lysis Buffer from the panel of five, creating

a maximum (37°C) and minimum (70°C) protein level dilution series for each Lysis Buffer. After the addition, plates were incubated on a plate shaking table for 30 minutes at RT (room temperature). 10 μL of the cell lysates (corresponding to 100 000 to 1 562 cells/well) was transferred to duplicate wells of the detection plate (Alpha plate, #6005350). The Alpha *SureFire®* CETSA® assay protocol was followed for Alpha signal detection.

Results

Typically, the type of Lysis Buffer and the cell density used to generate Alpha signals are critical in selecting conditions for a good CETSA® assay window. Figure 1 A-E show the Alpha signals generated in A431 cell density titrations (1 562 to 100 000 cells/well), heated to either 37°C or 70°C, and then treated with the panel of five Lysis Buffers. Overall, the 37°C heated samples (maximum protein level) have a strong Alpha signal in contrast to the 70°C treated group (minimal protein level), suggesting efficient lysis and a good soluble analyte concentration detected in several of the Lysis Buffers (Figure 1). It is preferable to use the lowest cell density with a high signal-to-background and optionally, if a hook exists, a cell density that yields less than the maximum alpha signal generated is optimal. In this case, a good signal was detected in several buffers. From these experiments, the CETSA® Cell Lysis Buffer 2 was used for detection of MEK1 using the Alpha *Surefire®* CETSA® assay kit, with a cell density of 0.625 million/mL (6250 cells/well). These parameters yield a signal-to-background ratio of 690 (Table 1).

N.B. The hook effect can occur through an excess of antigen present where saturation of the detection antibodies causes a reduced signal.

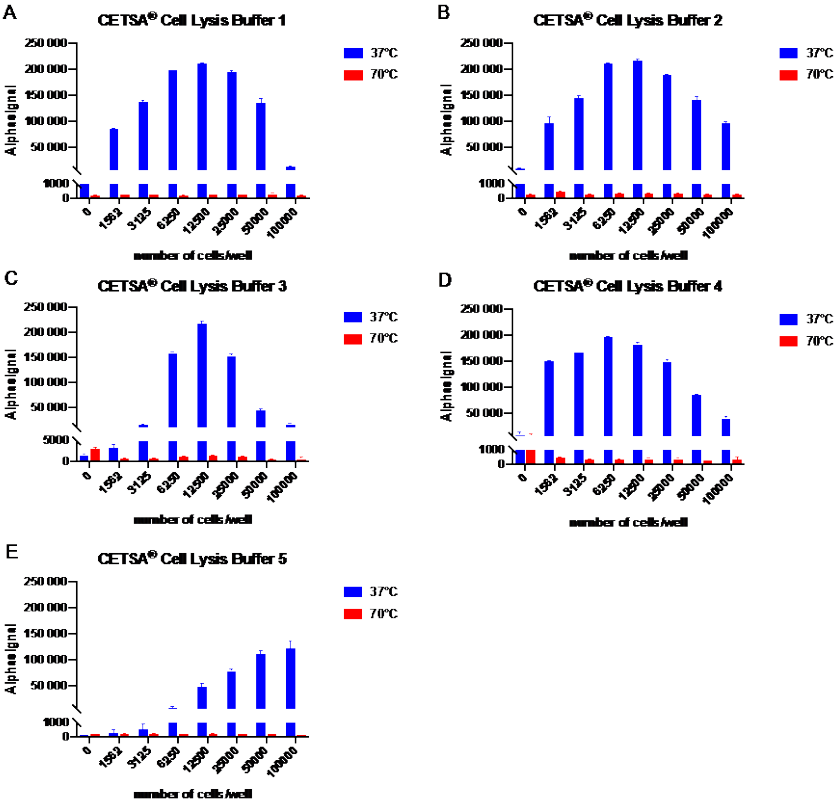


Figure 1. Cell density titration and Lysis Buffer optimization.
Panel A-E show dilution series of intact A431 cells ranging from 1 562 to 100 000 cells/well plus negative control (HBSS) treated with the respective CETSA® Cell Lysis Buffer. A. CETSA® Cell Lysis Buffer 1, B. CETSA® Cell Lysis Buffer 2, C. CETSA® Cell Lysis Buffer 3, D. CETSA® Cell Lysis Buffer 4, E. CETSA® Cell Lysis Buffer 5. Blue bars represent non-heated positive control samples and red bars, negative control samples heated to 70°C.

Table 1. Signal-to-background ratios using the five Lysis Buffer with different cell densities.

Number of cells per well	CETSA® Cell Lysis Buffer #1	CETSA® Cell Lysis Buffer #2	CETSA® Cell Lysis Buffer #3	CETSA® Cell Lysis Buffer #4	CETSA® Cell Lysis Buffer #5
3 125	558	565	16	477	3
6 250	837	690	114	520	38
12 500	851	677	151	494	238
25 000	802	561	119	381	423
50 000	447	523	62	332	595
100 000	59	357	23	112	812

CETSA® assay melting and shift curve analysis

Method

Melting and shift curves were generated of MEK1 in intact A431 cells. The cells were harvested and diluted in HBSS to 2.5 million cells/mL. The reference compounds (Table 4) and the DMSO-control were diluted to 2x final concentration in HBSS. Equal volumes of the cell suspension and the 2x reference compounds or the 2x DMSO control were mixed, resulting in a cell density of 1.25 million cells/mL. The cell-compound suspensions were dispensed (20μL/well) into a 96 well PCR plate and incubated for 1h at 37°C with gentle continuous orbital shaking. A heat challenge at 12 temperatures between 37-63°C or 37-75°C was applied for 3 minutes using a PCR thermocycler with variable temperature zones. After cooling on ice for at least 1 minute, the samples were lysed by the addition of 20 μL of 2x CETSA Cell Lysis Buffer 2. An additional lysis step of flash freezing in liquid nitrogen and thawing in a RT water bath three times was implemented at this stage. The plate was then incubated on a plate shaking table for 30 minutes at RT. 10 μL of the cell lysates, (corresponding to 6 250 cells/well in the detection plate) were transferred to duplicate wells of the detection plate (Alpha plates, #6005350). The Alpha *SureFire®* CETSA® assay protocol was followed for Alpha signal detection.

Results

Melting and shift curves of MEK1 in intact A431 cells were generated with four reference compounds (Table 3) at a final concentration of 10 μM in comparison to DMSO control. The Alpha signals of each curve were normalized to the Alpha signal obtained for the same sample heated at 37°C. Each melting curve was normalized to the respective alpha signals obtained at 37°C (y= (x-min)/(Alphasignal$_{37°C}$ -min) where "min" is the background signal when no cells are added). The data was analyzed using a non-linear regression curve fit with four parameters variable slope (GraphPad Prism 8.1.1, GraphPad Software) (Figure 2). Resulting T_m values, calculated from the GraphPad curve fitting, are listed in Table 2. These data indicate a stabilization to MEK1 following treatment with all four compounds. The shift allows for selection of a temperature with a significant amplitude between compound treated and control samples. Such temperatures of 51°C (destabilizing compounds) and 55°C (stabilizing compounds), marked by a dotted line (Figure 2A-D), were selected for isothermal concentration-response analysis of the compounds.

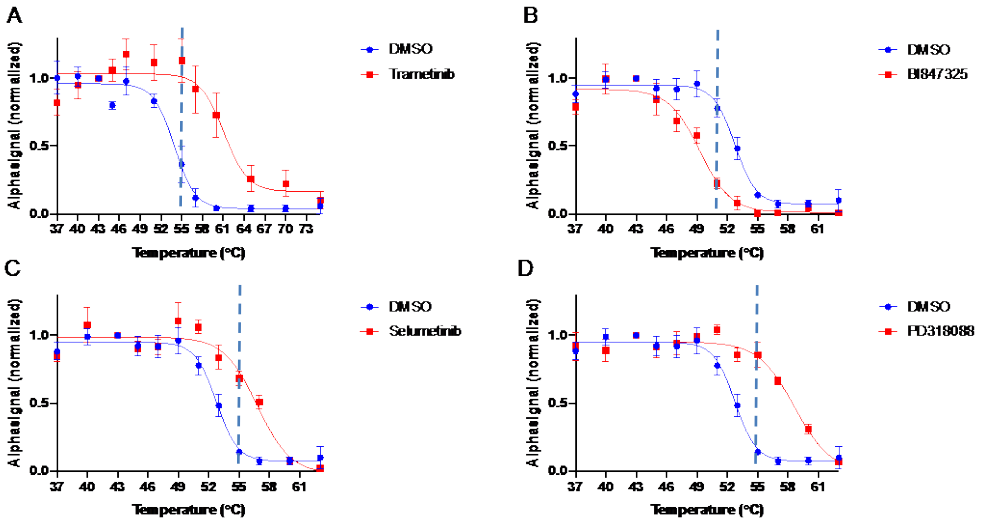


Figure 2. CETSA® assay melting and shift curve analysis of MEK1 inhibitors in A431 intact cells. A431 cells incubated with 10 µM A.Trametinib. B. BI847325., C.Selumetinib., D. PD318088 or DMSO control**.** The data is normalized to the corresponding value at 37°C. The dotted line indicates the temperature that has been selected to generate isothermal concentration-response curves. Data are shown as mean values with error bars indicating ± SEM. No error bars are shown visible if SEM is smaller than the symbol.

Table 2. CETSA® T_m values of MEK1 reference compounds in A431 intact cells. CI=confidence interval, N=Biological repeats, n=technical repeats

Cell Type	Compound	T_m (°C)	95% CI	N; n
A431	DMSO control	53.9	52.8-55.0	3;6
A431	Trametinib	61.1	59.2-64.1	3;6
A431	BI847325	49.3	48.3-50.1	3;6
A431	Selumetinib	56.8	55.6-58.5	3;6
A431	PD318088	58.6	57.4-61.3	3;6

CETSA® assay concentration-response analysis

Method

CETSA® assay concentration-response (C-R) curves were generated of MEK1 in intact A431 cells. The cells were harvested and diluted in HBSS to 2.5 million cells/mL. The reference compounds (Table 4) were serially diluted at a 2x final concentration in the range 200 µM – 0.2 nM (corresponding to a final concentration series of 100 µM – 0.1 nM) in HBSS. A DMSO control was included. Equal volumes of the cell suspensions were mixed with the serially diluted reference compounds or the DMSO control, giving a final density of 1.25 million cells/mL. The cell-compound suspensions were dispensed into PCR-plates (20 µL/well) and incubated for 1h at 37°C with gentle continuous orbital shaking. The cell-compound suspensions were heated for 3 min at 51°C or 55°C. After cooling on ice for at least 1 minute, the samples were lysed by the addition of 20 µL 2x CETSA Cell Lysis Buffer 2. An additional lysis step of flash freezing in liquid nitrogen and thawing in a RT water bath three times was implemented at this stage. The plate was then incubated on a plate shaking table for 30 minutes at RT. 10 µL of the cell lysates (corresponding to a density of 6 250 cells/well) was transferred to duplicate wells of the detection plate (Alpha plate, # 6005350). The Alpha *SureFire®* CETSA® assay protocol was followed for Alpha signal detection.

Results

For each compound, the Alpha signal was normalized to the Alpha signal obtained at 100 µM compound treatment ($y = x/Alphasignal_{100\mu M}$ for stabilizing compounds or $y = x/Alphasignal_{10nM}$ for destabilizing compounds) and analyzed using a non-linear regression curve fit with four parameters variable slope (GraphPad Prism 8.1.1, GraphPad Software). The normalized Alpha signals for the MEK1 C-R curves are shown in Figure 3 and the CETSA® assay EC_{50} -values are presented in Table 3. The CETSA® assay EC_{50} values were obtained from the curve fitting and allowed the determination of the potency of the compounds tested.

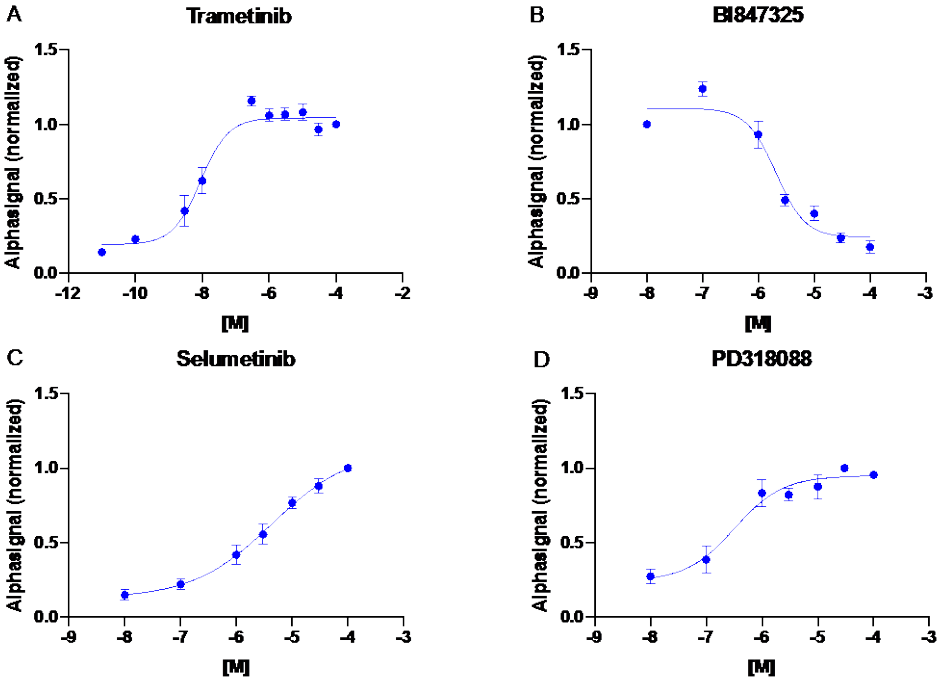


Figure 3. CETSA® assay isothermal concentration-response curves for MEK1 in A431 intact cells. The solid line represents the logistic curve fit to the data. The concentration-response curves represent MEK1 in intact A431 cells incubated with a serial dilution of A. Trametinib.,B. BI847325., C.Selumetinib., D. PD318088. The plotted values are normalized to the obtained Alpha signals at 100 µM (stabilizing compounds) and 10nM (destabilizing compounds).

Table 3. CETSA® assay EC_{50} values for MEK1 reference compounds in A431 cells. CI= confidence interval, N= biological repeat, n= technical repeat, n.a= not applicable.

Compound	CETSA® EC_{50}* (µM)	95% CI (µM)	N; n
Trametinib	0.008	0.005-0.018	3;9
BI847325	2.1	1.5-3.5	3;9
Selumetinib	4.3	2.0-130	3;9
PD318088	0.32	0.12-0.88	3;9

* please refer to the generic manual for an explanation of CETSA® EC_{50}.

Table 4. Reference compounds used in the MEK1 CETSA® assays.

Compound	Provider	Cat #	Stock Solution
Trametinib	Selleckchem	S2673	10 mM in 100% DMSO
BI847325	Selleckchem	S7843	10 mM in 100% DMSO
Selumetinib	Selleckchem	S1008	10 mM in 100% DMSO
PD318088	Selleckchem	S1568	10 mM in 100% DMSO

For more information and detailed protocols, please refer to the generic Alpha CETSA® *SureFire*® Assay Kits Manual, available from http://www.perkinelmer.com/category/alpha-CETSA-kits