PerkinElmer For the Better | TGR BioSciences | **Technical Data Sheet** | PELAGO BIOSCIENCE

Caution: Research use only.

Alpha *SureFire*® CETSA®
Raf-1 Assay Kit

Products: CETSA-RAF1-A500, CETSA-RAF1-A5K1, CETSA-RAF1-A5K2, CETSA-RAF1-A5K3, CETSA-RAF1-A5K4, CETSA-RAF1-A5K5

Kit-Specificity Information

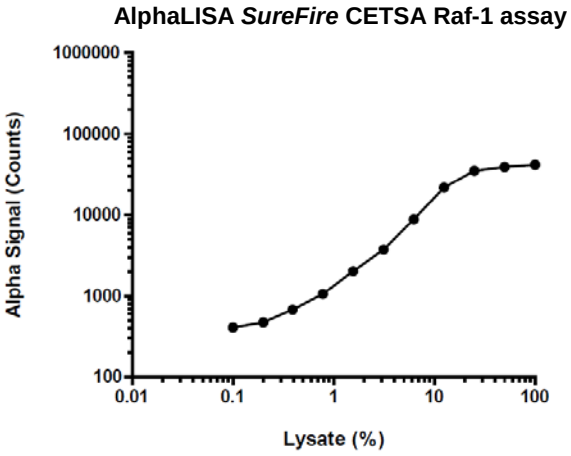
This assay kit contains antibodies which recognize invariant epitopes on Raf-1. These antibodies recognize Raf-1 of human, rat and mouse origin. Other species should be tested on a case-by-case basis. Raf-1 is also known as c-Raf. The human protein corresponds to Entrez Gene ID 5894.

Control Lysate Information

Positive Control Lysate: Prepared from A431 cells, cultured to 80% confluence in T175 flasks in 10% FBS containing medium, medium removed and lysed in 4mL of 1X *SureFire Ultra* Lysis buffer.

Representative Immunoassay data using the control lysate

Data obtained with a 2-plate, 2-incubation protocol with control Lysate was serially diluted in 1X CETSA Lysis Cell Buffer 2.



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 epithelial lung carcinoma cell line A549 (ATCC® CCL-185™) was cultured using the following conditions:

A549 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).

A549 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 A549 cells were harvested and resuspended to the desired density in HBSS. In a volume of 30 µL, the cells were serially diluted to: 5, 2.5, 1.25, 0.62, 0.31, 0.15 and 0.039 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 of each 2x Lysis Buffer (Resulting in a final top concentration of 2.5 million cells/mL) 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 25 000 to 195 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 A549 cell density titrations (195 to 25 000 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 4 was used for detection of Raf-1 using the Alpha *Surefire®* CETSA® assay kit, with a cell density of 0.625 million/mL (6 250 cells/well). These parameters yield a signal-to-background ratio of 180 (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.

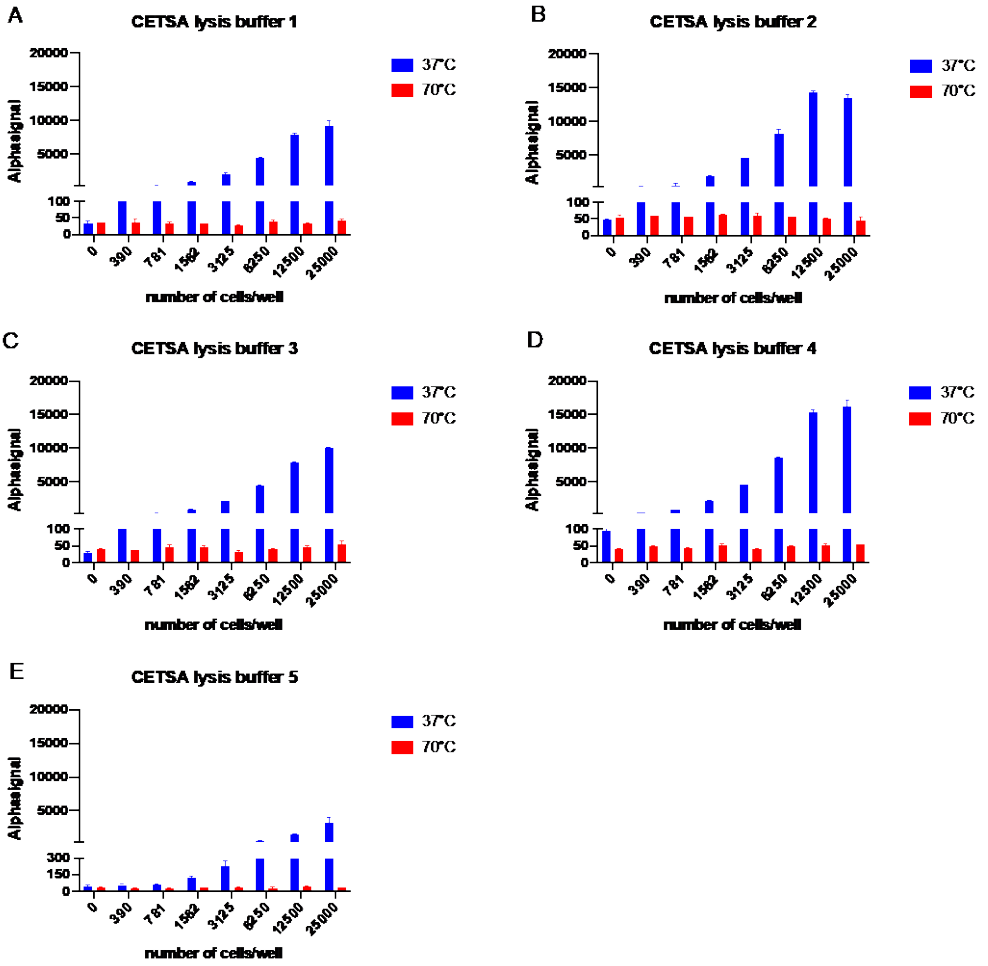


Figure 1. Cell density titration and Lysis Buffer optimization.
Panel A-E show dilution series of intact A549 cells ranging from 195 to 25 000 cells/well plus negative control (HBSS) treated with the respective CETSA® Cell Lysis Buffer. A. CETSA® Cell Lysis Buffer 1, B. CETSA® Cell Lysis Buffer 2, C. 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 Buffers 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
781	10	7	8	21	2
1562	27	30	20	43	4
3 125	77	79	72	117	6
6 250	113	144	117	180	19
12 500	236	281	174	300	40
25 000	216	312	186	304	100

CETSA® assay melting and shift curve analysis

Method

Melting and shift curves were generated of Raf-1 in intact A549 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 37-75°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 4. 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 Raf-1 in intact A549 cells were generated with four reference compounds (Table 2) at a final concentration of 10 μM in comparison to DMSO control. Due to the observation of a biphasic response of the Raf-1 stabilization in the presence of the compounds, and in order the get a better level of detail, three additional temperatures were added to the initially selected 12 temperature range and used for one of the biological

repeats, resulting in a total of 15 temperatures. Each technical repeat of the obtained 12/15 temperature melting curves were normalized separately to the largest obtained Alpha signal value for each curve. The largest value is defined to 100 % and the smallest value to 0%. The data was analyzed using either a non-linear regression curve fit with four parameters variable slope or a biphasic curve fit (GraphPad Prism 8.3.0, GraphPad Software) (Figure 2). Resulting T_m values, calculated from the GraphPad curve fitting, are listed in Table 2. These data indicate a biphasic stabilization of Raf-1 following treatment with three of the compounds. For the fourth compound a less prominent biphasic melting curve is observed. The shift allows for selection of a temperature with a significant amplitude between compound treated and control samples. The temperature of 49°C, marked by a dotted line (Figure 2A-D), was selected for isothermal concentration-response analysis of the compounds.

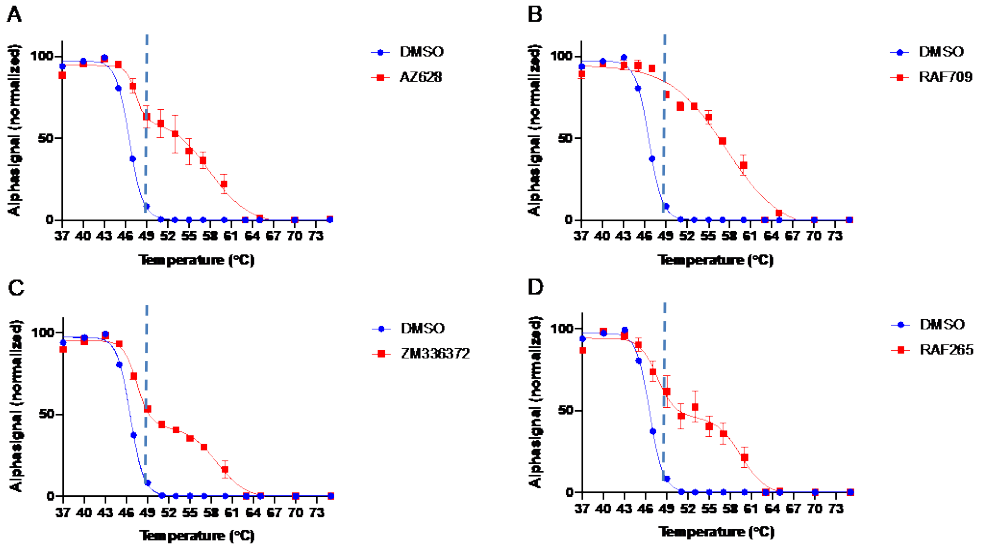


Figure 2. CETSA® assay melting and shift curve analysis of Raf-1 inhibitors in A549 intact cells. A549 cells incubated with 10 µM A. AZ628. B. RAF709., C. ZM336372., D. RAF265 or DMSO control. The dotted line indicates the temperature that has been selected to generate isothermal concentration-response 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 Raf-1 reference compounds in A549 intact cells. CI=confidence interval, N=Biological repeats, n=technical repeats. T_m1, T_m2, CI 1 and CI 2 represent the melting temperature an confidence interval for the biphasic curve fit.

Cell Type	Compound	T_m 1 (°C)	95% CI 1	T_m 2 (°C)	95% CI 2	N; n
A549	DMSO control	46.6	46.0-47.1			3;6
A549	AZ628	47.4	46.3-51.6	58.1	55.3-61.6	3;6
A549	RAF709	58.5	56.1-61.6			3;6
A549	ZM336372	47.5	47.0-48.1	58.9	57.6-60.1	3;6
A549	RAF265	47.6	46.2-49.6	59.6	56.8-61.6	3;6

CETSA® assay concentration-response analysis

Method

CETSA® assay concentration-response (C-R) curves were generated of Raf-1 in intact A549 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 49°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 4. 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 (y = x/Alphasignal100µM) 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 Raf-1 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.

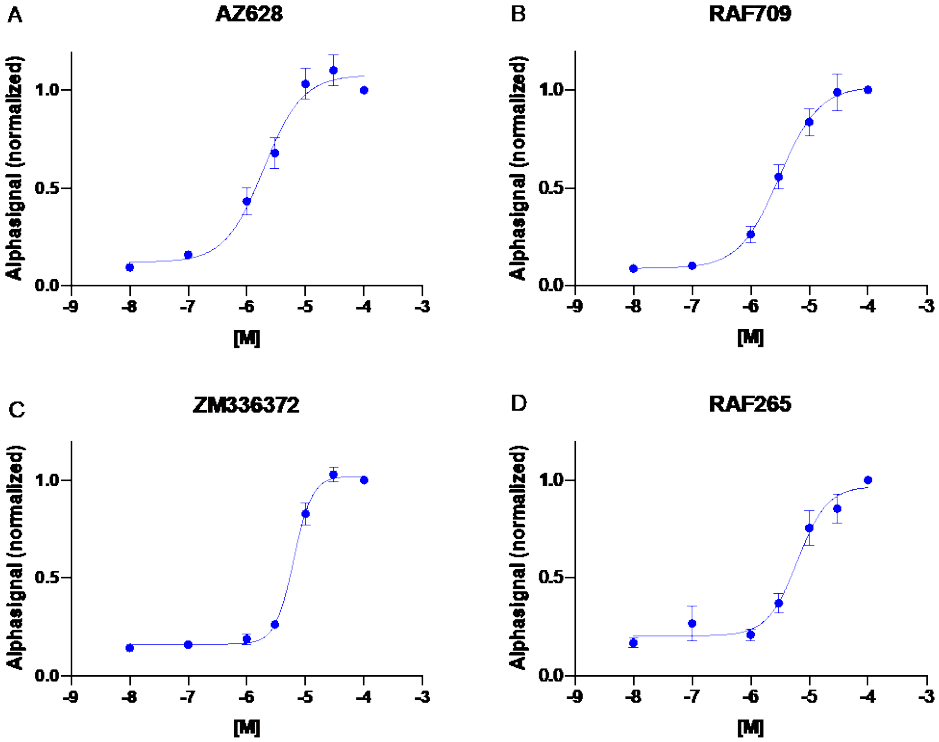


Figure 3. CETSA® assay isothermal concentration-response curves for Raf-1 in A549 intact cells. The solid line represents the logistic curve fit to the data. The concentration-response curves represent Raf-1 in intact A549 cells incubated with a serial dilution of A. AZ628., B. RAF709., C.ZM336372., D.RAF265. The plotted values are normalized to the obtained Alpha signals at 100 µM.

Table 3. CETSA® assay EC_{50} values for Raf-1 reference compounds in A549 cells. CI= confidence interval, N= biological repeat, n= technical repeat, n.a= not applicable.

Compound	CETSA® EC_{50}* (µM)	95% CI (µM)	N; n
AZ628	1.9	1.3-2.9	3;9
RAF709	3.1	2.1-4.7	3;9
ZM336372	6.3	5.4-7.3	3;9
RAF265	6.3	4.0-12	3;9

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

Table 4. Reference compounds used in the Raf-1 CETSA® assays.

Compound	Provider	Cat #	Stock Solution
AZ628	Selleckchem	S2746	10 mM in 100% DMSO
RAF709	Selleckchem	S8690	10 mM in 100% DMSO
ZM336372	Selleckchem	S2720	10 mM in 100% DMSO
RAF265	Selleckchem	S2161	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