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Caution: Research use only.

Alpha *SureFire*® CETSA®
Akt1 Assay Kit

Products: CETSA-AKT1-A500, CETSA-AKT1-A5K1, CETSA-AKT1-A5K2, CETSA-AKT1-A5K3, CETSA-AKT1-A5K4, CETSA-AKT1-A5K5

Kit-Specificity Information

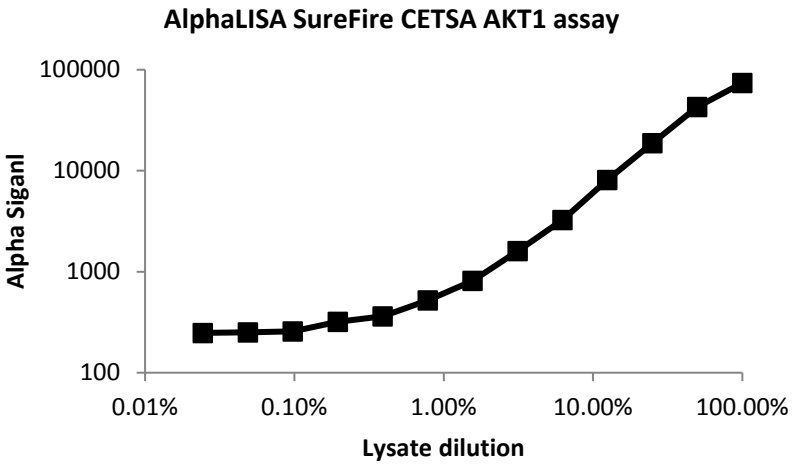
This assay kit contains antibodies which recognize two distinct epitopes on Akt 1. The protein detected by this kit corresponds to GenBank Accession NP_001014431. Also known as PKB, RAC, PRKBA, PKB-ALPHA, RAC-ALPHA. These antibodies recognize Akt 1 of human, mouse and rat origin. Other species should be tested on a case-by-case basis.

Control Lysate Information

Positive Control Lysate: Prepared from HEK293 cells, cultured to confluence in T175 flasks in 10% FBS containing medium, then treated with 20% FBS for 15min 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.



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

Lung epithelial 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).

A4549 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: 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- 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 A549 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 Akt1 using the Alpha *Surefire®* CETSA® kit, with a cell density of 1.25 million/mL (12 500 cells/well). These parameters yield a signal-to-background ratio of 93 (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.

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® Lysis Buffer #5
3 125	47	41	75	60	57
6 250	84	57	134	94	67
12 500	178	93	234	184	110
25 000	270	162	419	296	232
50 000	386	226	524	433	268
100 000	276	207	549	487	403

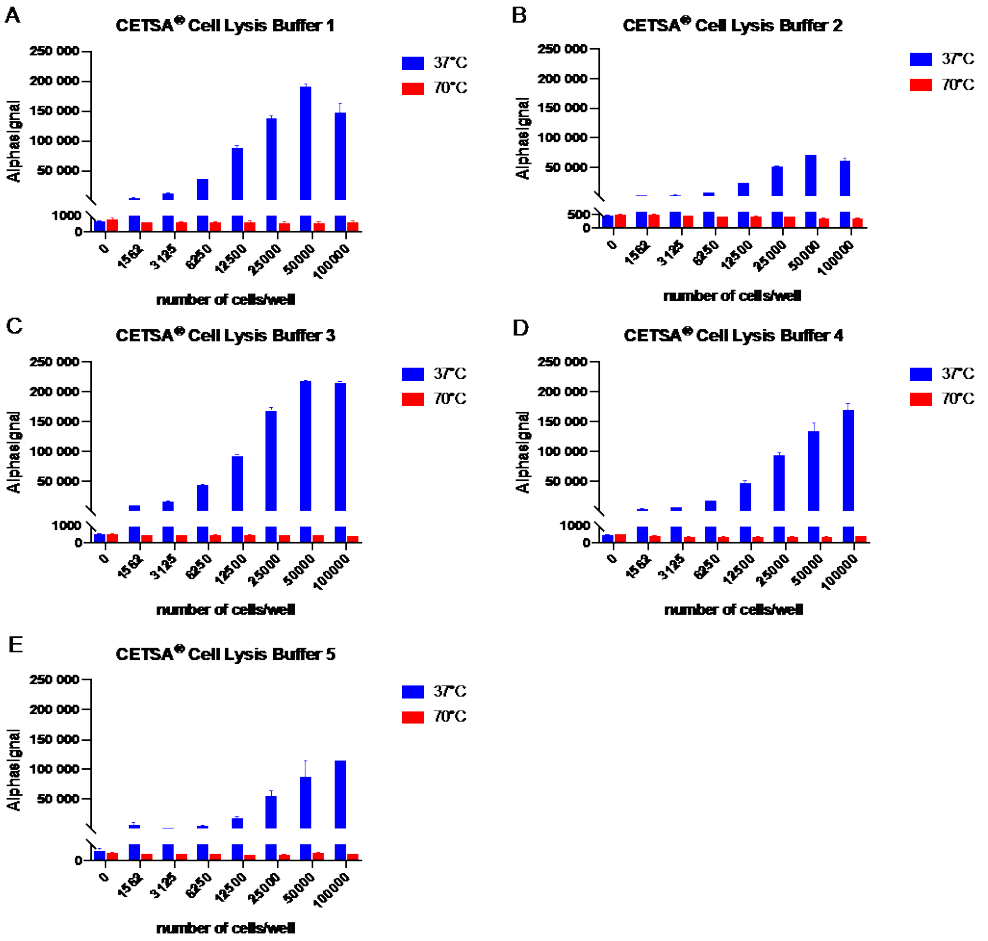


Figure 1. Cell density titration and Lysis Buffer optimization.
Panel A-E show dilution series of intact A549 cells ranging from 1 562 to 100 000 cells/well plus negative control (HBSS) treated with the respective CETSA® Cell Lysis Buffer. A. CETSA® Cell Lysis Buffer 1, B. CETSA® Cell Lysis Buffer 2, 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.

CETSA® assay melting and shift curve analysis

Method

Melting and shift curves were generated of Akt1 in intact A549 cells. The cells were harvested and diluted in HBSS to a concentration of 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 2.5 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 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 12 500 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 Akt1 in intact A549 cells were generated with two 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 Akt1 following treatment with the two compounds. The shift allows for selection of a temperature with a significant amplitude between compound treated and control samples. Such a temperature of 49°C, marked by a dotted line (Figure 2A-B), was selected for isothermal concentration-response analysis of the compounds.

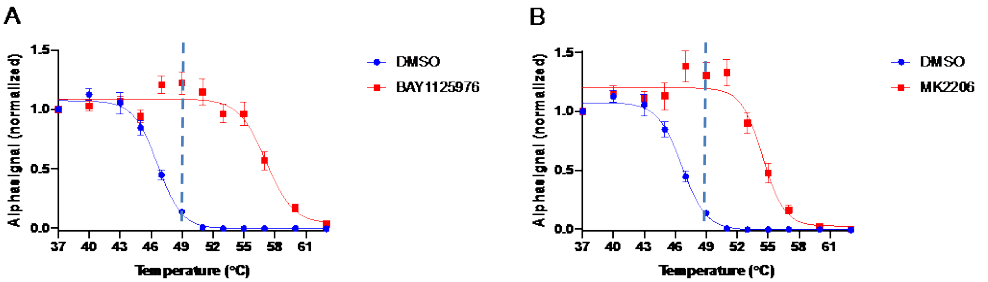


Figure 2. CETSA® assay melting and shift curve analysis of Akt1 inhibitors in A549 intact cells. A549 cells incubated with 10 µM A. BAY1125976 and B.MK2206 or DMSO control**.** The data is normalized to the corresponding value at 37°C. The dotted line indicates the temperature that has been selected 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® assay T_m values of Akt1 reference compounds in A549 intact cells.
CI=confidence interval, N=Biological repeats, n=technical repeats

Cell Type	Compound	T_m (°C)	95% CI	N; n
A549	DMSO control	46.6	46.3-46.9	5; 10
A549	BAY1125976	57.1	56.4-58.3	3; 6
A549	MK2206	54.5	53.8-55.2	3;6

CETSA® assay concentration-response analysis

Method

CETSA® assay concentration-response (C-R) curves were generated of Akt1 in intact A549 cells. The cells were harvested and diluted in HBSS to 5 million cells/mL, which is 2X the final cell concentration before the lysis step. 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 2.5 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 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 12 500 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$_{100µM}$) 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 Akt1 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 a rank order of the compounds tested.

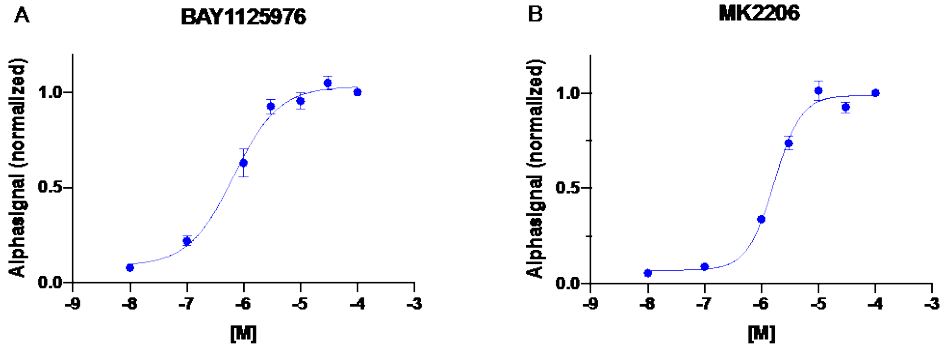


Figure 3. CETSA® assay isothermal concentration-response curves for Akt1 in A549 intact cells. The solid line represents the logistic curve fit to the data. The concentration-response curves represent Akt1 in intact A549 cells incubated with a serial dilution of A. BAY1125976 and B. MK2206. The plotted values are normalized to the obtained Alpha signals at 100 µM.

Table 3. CETSA® assay EC_{50} values for Akt1/2/3 reference compounds in A549 cells. CI= confidence interval, N= biological repeat, n= technical repeat.

Compound	CETSA® EC_{50}* (µM)	95% CI (µM)	N; n
BAY1125976	0.7	0.4-1.0	4;12
MK2206	1.7	1.4-1.9	4;12

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

Table 4. Reference compounds used in the Akt1/2/3 CETSA® assays.

Compound	Provider	Cat #	Stock Solution
BAY1125976	MedChemExpress	HY-100018	10 mM in 100% DMSO
MK2206	Selleckchem	S1078	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