

Caution: Research use only.

Alpha SureFire® CETSA® NFκB p65 Assay Kit

**Products: CETSA-NFKB-A500, CETSA-NFKB -A5K1,
CETSA-NFKB-A5K2, CETSA-NFKB-A5K3, CETSA-NFKB-A5K4,
CETSA-NFKB-A5K5**

Kit-Specificity Information

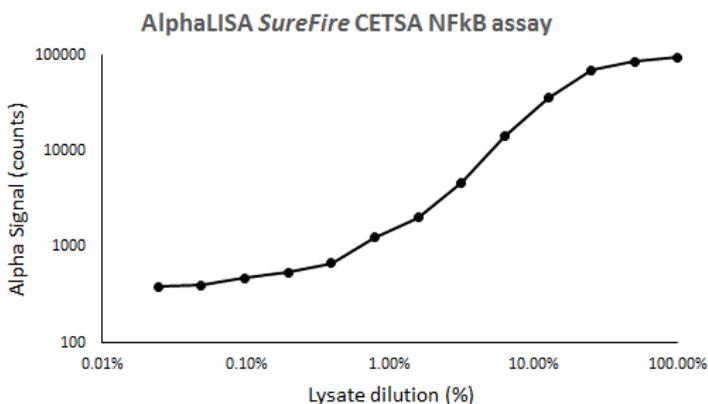
This assay kit contains antibodies which recognize invariant epitopes on v-rel reticuloendotheliosis viral oncogene homolog A (avian) (NF-κB p65). The protein detected by this kit corresponds to GenBank Accession NP_068810. NF-κB p65 is also known as p65, NFKB3, MGC131774 and RELA. These antibodies recognize NFκB p65 of human, rat and mouse origin. Other species should be tested on a case-by-case basis.

Control Lysate Information

Positive Control Lysate: Prepared from HeLa cells, cultured to confluence in T175 flasks in 10% FBS containing medium, then treated with 50ng/mL TNFα plus 20ng/mL Calyculin A 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 mammary gland cell line SK-BR-3 (ATCC®-HTB-30™) was cultured using the following conditions:

SK-BR-3 culture medium:

DMEM hg (Thermo Fisher, #41965-062) supplemented with 10% FBS (Thermo Fisher, #10500064), 100 units/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher, #15140-122).

SK-BR-3 culture conditions:

The cells were cultured in complete DMEM at 37°C, 5% CO₂, in a humidified atmosphere to confluency. For harvesting, the cells were first washed in Hanks Balanced Salt Solution (HBSS, ThermoFisher, #14025-050) and then incubated with TrypLE (ThermoFisher, #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 SK-BR-3 cells were harvested and resuspended to the desired density in HBSS. In a volume of 30 µL, the cells were serially diluted to: 80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 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 40 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 400 000 to 761 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 SK-BR-3 cell density titrations (761 to 400 000 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 NFκB using the Alpha *Surefire*® CETSA® assay kit, with a cell density of 0.625 million cells/mL (6250 cells/well). These parameters yield a signal-to-background ratio of 603 (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.

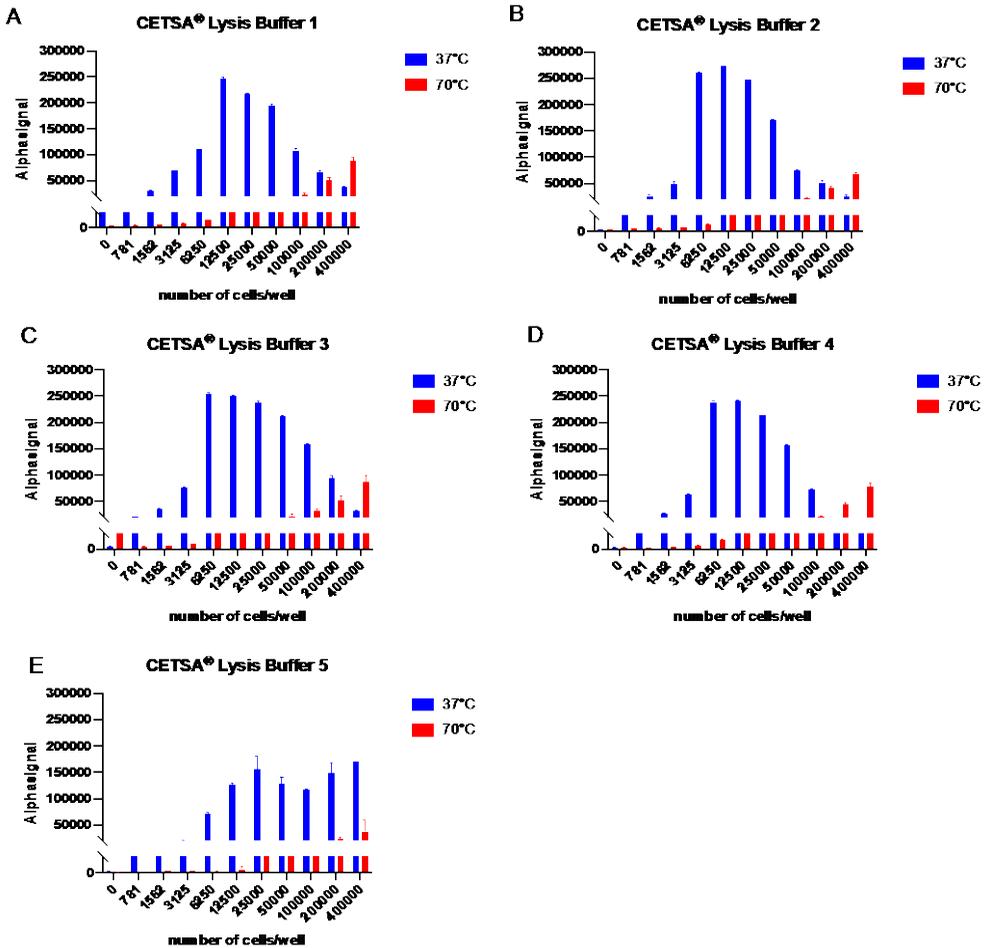


Figure 1. Cell density titration and Lysis Buffer optimization.

Panel A-E show dilution series of intact SK-BR-3 cells ranging from 761 to 400 000 cells/well plus negative control (HBSS) treated with the respective CETSAs[®] Cell Lysis Buffer. A. CETSAs[®] Cell Lysis Buffer 1, B. CETSAs[®] Cell Lysis Buffer 2, C. CETSAs[®] Cell Lysis Buffer 3, D. CETSAs[®] Cell Lysis Buffer 4, E. CETSAs[®] 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
3125	262	220	234	334	164
6250	115	603	105	419	911
12500	212	250	39	202	559
25000	57	72	21	55	112
50000	21	17	10	17	28
100000	5	3	5	4	10

CETSA® assay melting and shift curve analysis

Method

Melting and shift curves were generated of NFkB in intact SK-BR-3 cells. The cells were harvested and diluted in HBSS to 2.5 million cells/mL. The reference compound (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 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. 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 NFKB in intact SK-BR-3 cells were generated with four reference compounds (Table 4) at a final concentration of 100 μ M in comparison to DMSO control. Each technical repeat of the obtained 12 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 a non-linear regression curve fit with four parameters variable slope (GraphPad Prism 8.1.1, GraphPad Software) (Figure 2). The resulting T_m value, calculated from the GraphPad curve fitting, is listed in Table 2. The data indicate a stabilization of NFKB following treatment with the compound. The shift allows for selection of a temperature with a significant amplitude between compound treated and control samples. Such a temperature of 51°C, marked by a dotted line (Figure 2), was selected for isothermal concentration-response analysis of the compound.

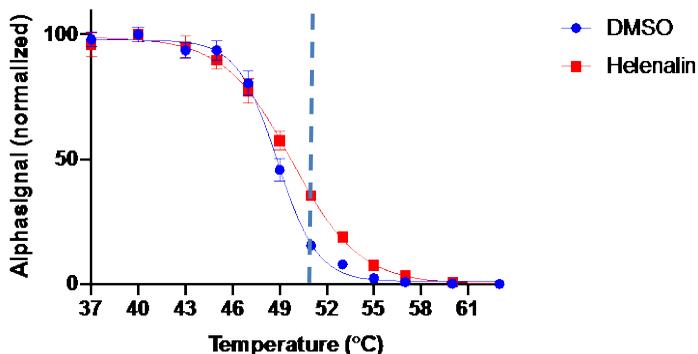


Figure 2. CETSA[®] assay melting and shift curve analysis of NFKB inhibitor in SK-BR-3 intact cells. SK-BR-3 cells incubated with 100 μ M Helenalin or DMSO control. The dotted line indicates the temperature that has been selected to generate isothermal concentration-response curves. Data are shown as mean values with error bars indicating \pm SEM. No error bars are shown visible if SEM is smaller than the symbol.

Table 2. CETSA[®] T_m values of NFKB reference compound in SK-BR-3 intact cells. CI=confidence interval, N=Biological repeats, n=technical repeats

Cell Type	Compound	T_m (°C)	95% CI	N; n
SK-BR-3	DMSO control	48.9	48.6-49.1	3; 6
SK-BR-3	Helenalin	49.8	49.4-50.2	3; 6

CETSA® assay concentration-response analysis

Method

CETSA® assay concentration-response (C-R) curves were generated of NFκB in intact SK-BR-3 cells. The cells were harvested and diluted in HBSS to 2.5 million cells/mL. The reference compound (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. 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. 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 ($y = x/\text{Alphasignal}_{100\mu\text{M}}$) 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 NFKB C-R curve is shown in Figure 3 and the CETSA[®] assay EC_{50} -value is presented in Table 3. The CETSA[®] assay EC_{50} value was obtained from the curve fitting.

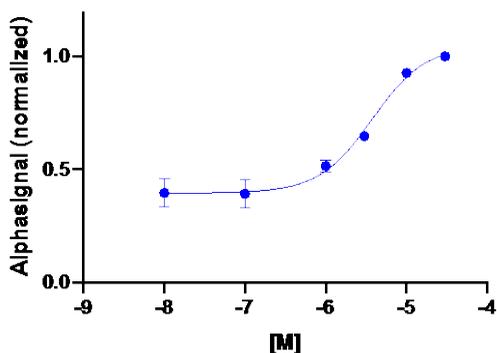


Figure 3. CETSA[®] assay isothermal concentration-response curve for NFKB in SK-BR-3 intact cells. The solid line represents the logistic curve fit to the data. The C-R curve represent NFKB in intact SK-BR-3 cells incubated with a serial dilution of Helenalin. The plotted values are normalized to the obtained Alpha signals at 100 μM .

Table 3. CETSA[®] assay EC_{50} values for NFKB reference compound in SK-BR-3 cells. CI= confidence interval, N= biological repeat, n= technical repeat, n.a.= not applicable.

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

Compound	CETSA [®] EC_{50} * (μM)	95% CI (μM)	N; n
Helenalin	3.8	2.3-11.1	2:6

Table 4. Reference compounds used in the NFKB CETSA[®] assays

Compound	Provider	Cat #	Stock Solution
Helenalin	Cayman Chemicals	17050	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>