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Caution: Research use only.

Alpha *SureFire*® CETSA®
ErbB2 Assay Kit

Products: CETSA-ERBB2-A500, CETSA-ERBB2-A5K1, CETSA-ERBB2-A5K2, CETSA-ERBB2-A5K3, CETSA-ERBB2-A5K4, CETSA-ERBB2-A5K5

Kit-Specificity Information

This assay kit contains antibodies which recognize invariant epitopes on erythroblastic leukemia viral oncogene homolog 2 (ErbB2). The protein detected by this kit corresponds to GenBank Accession NP_001005862. ErbB2 is also known as NEU, NGL, HER2, TKR1, CD340, HER-2, MLN 19 and HER-2/neu.

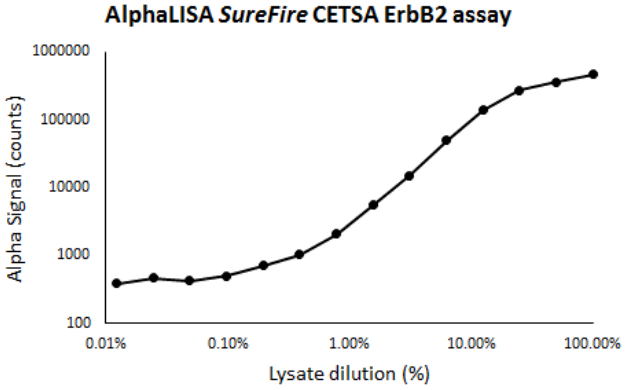
These antibodies recognize ErbB2 of human, rat and mouse origin. Other species should be tested on a case-by-case basis.

Control Lysate Information

Positive Control Lysate: Prepared from A431 cells, cultured in a T175 flask in 10% FBS containing medium, starved overnight, then treated with 1µg/mL EGF for 5min 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

Human epidermis skin epithelial carcinoma cell line A549 (ATCC® CCL-1555 ™) 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: 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 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 ErbB2 using the Alpha *Surefire®* CETSA® assay kit, with a cell density of 2.5 million/mL (25 000 cells/well). These parameters yield a signal-to-background ratio of 29 (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.

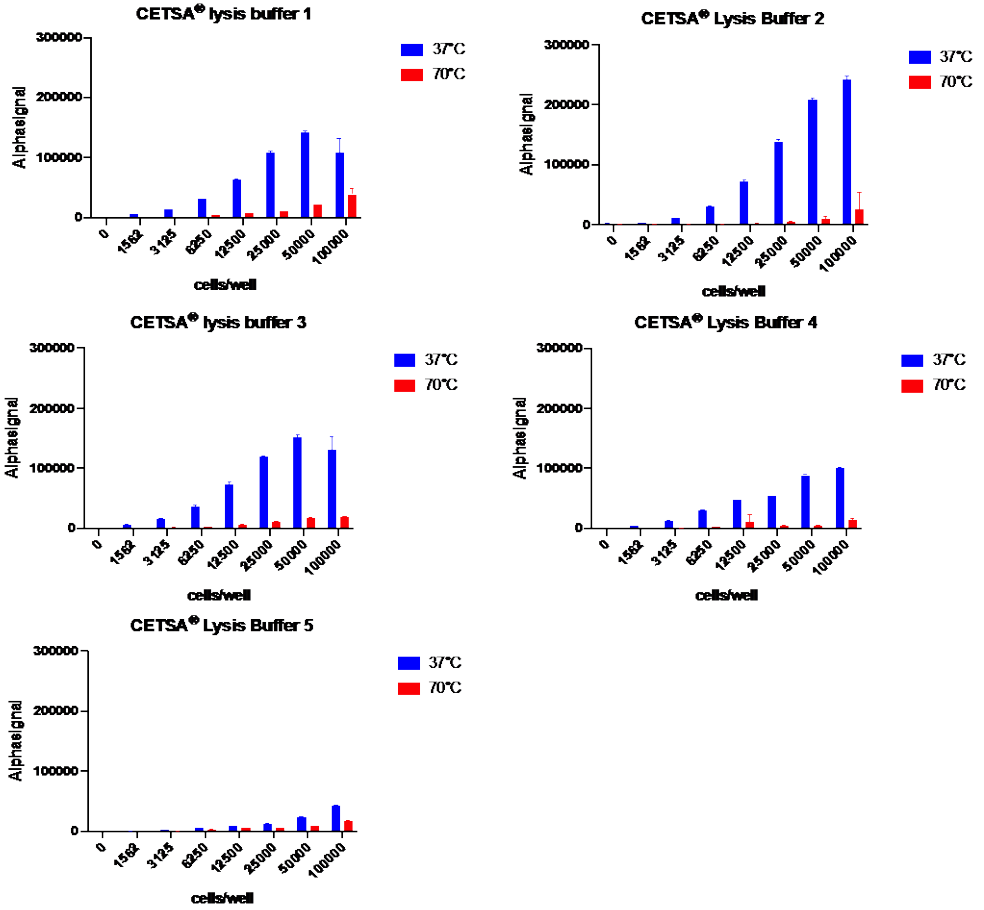


Figure 1. Cell density titration and Lysis Buffer optimization.
Panel A-E show dilution series of intact A549 cells ranging from 1 562 to 100 000 cells/well plus negative control (HBSS) treated with the respective CETSA® Cell Lysis Buffer. A. CETSA® Cell Lysis Buffer 1, B. CETSA® Cell Lysis Buffer 2, 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	11	36	11	16	3
6 250	8	40	12	12	1
12 500	5	29	11	13	1
25 000	10	29	11	19	2
50 000	6	21	9	7	3
100 000	3	9	7	4	2

CETSA® assay melting and shift curve analysis

Method

Melting and shift curves were generated of ErbB2 in intact A549 cells. The cells were harvested and diluted in HBSS to 10 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 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. The plate was then incubated on a plate shaking table for 30 minutes at RT. 10 µL of the cell lysates, (corresponding to 25 000 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 ErbB2 in intact A549 cells were generated with four reference compounds (Table 3) at a final concentration of 10 µM 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). Resulting T_m values, calculated from the GraphPad curve fitting, are listed in Table 2. These data indicate a stabilization to ErbB2 following treatment with Lapatinib and a destabilization following treatment with the other 3 compounds. It is to be noted that Feldinger and Kong (Breast Cancer: Targets and Therapy 2015:7 147–162) have reported that Lapatinib is a reversible inhibitor, while the other 3 compounds are irreversible inhibitors of ErbB2. The shift allows for selection of a temperature with a significant amplitude between compound treated and control samples. Such temperatures of 45°C (destabilizing compounds) and 47°C (stabilizing compounds), marked by a dotted line (Figure 2A-D), were selected for isothermal concentration-response analysis of the compounds.

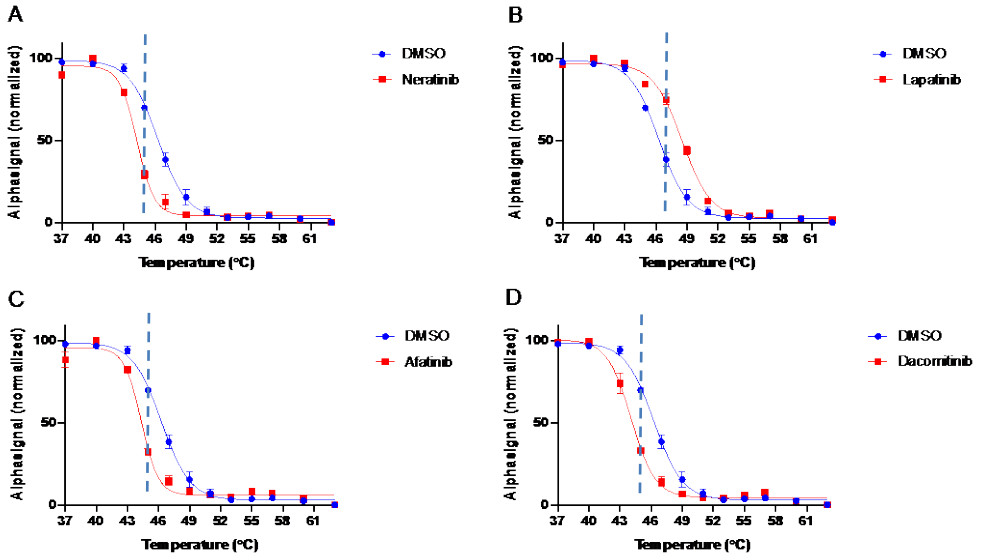


Figure 2. CETSA® assay melting and shift curve analysis of ErbB2 inhibitors in A549 intact cells. A549 cells incubated with 10 µM A.Neratinib. B. Lapatinib., C.Afatinib., D. Dacomitinib or DMSO control. The dotted line indicates the temperature that has been selected to generate isothermal concentration-response 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 ErbB2 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	46.3	46.0-46.5	2;4
A549	Neratinib	44.3	44.1-44.5	2;4
A549	Lapatinib	48.5	48.3-48.8	2;4

A549	Afatinib	44.4	44.0-44.8	2;4
A549	Dacomitinib	44.1	43.9-44.3	2;4

CETSA® assay concentration-response analysis

Method

CETSA® assay concentration-response (C-R) curves were generated of ErbB2 in intact A549 cells. The cells were harvested and diluted in HBSS to 10 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 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 45 or 47°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 25 000 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 (stabilizing compounds) or 10nM (destabilizing) 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 ErbB2 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.

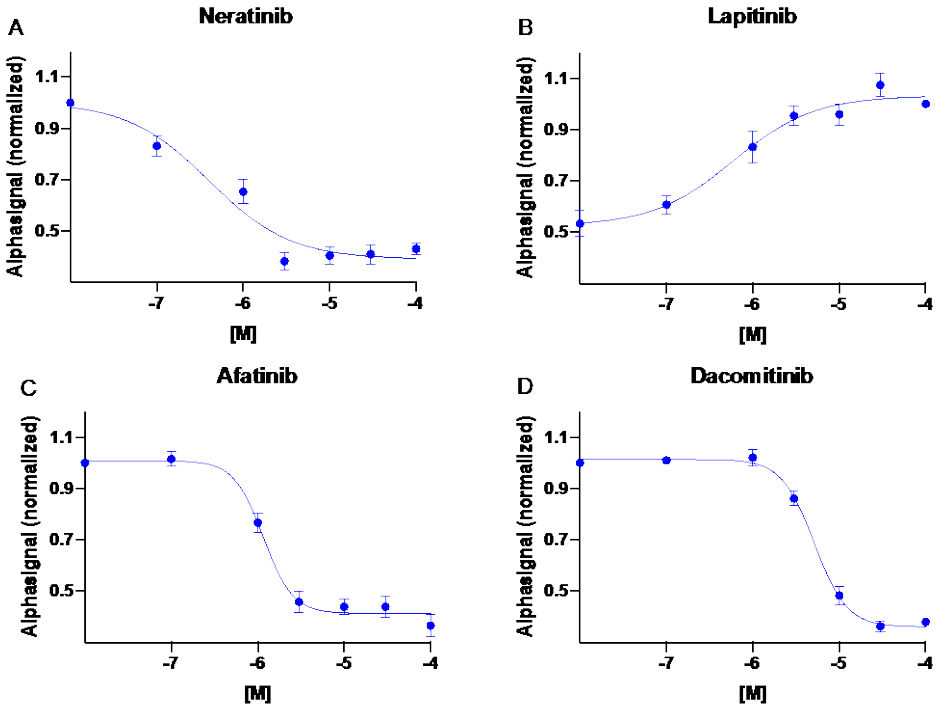


Figure 3. CETSA® assay isothermal concentration-response curves for ErbB2 in A549 intact cells. The solid line represents the logistic curve fit to the data. The concentration-response curves represent ErbB2 in intact A549 cells incubated with a serial dilution of A. Neratinib., B. Lapatinib., C.Afatinib., D. Dacomitinib. The plotted values are normalized to the obtained Alpha signals at 100 µM (stabilizing compounds) or 10nM (destabilizing compound).

Table 3. CETSA® assay EC_{50} values for ErbB2 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
Neratinib	0.4	0.12-1.3	3;9
Lapatinib	0.6	0.10-1.4	3;9
Afatinib	1.2	0.94-1.5	3;9
Dacomitinib	5.2	4.2-6.2	3;9

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

Table 4. Reference compounds used in the ErbB2 CETSA® assays.

Compound	Provider	Cat #	Stock Solution
Neratinib	Selleckchem	S2150	10 mM in 100% DMSO
Lapatinib	Selleckchem	S2111	10 mM in 100% DMSO
Afatinib	Selleckchem	S1011	10 mM in 100% DMSO
Dacomitinib	Selleckchem	S2727	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