TECHNICAL DATA SHEET

Alpha CETSA® Research Reagents

B-cell lymphoma 2 (Bcl-2) Alpha CETSA® Assay

Product number: **CETSA-BCL2-A500 / CETSA-BCL2-A5K1 / CETSA-BCL2-A5K2 / CETSA-BCL2-A5K3 / CETSA-BCL2-A5K4 / CETSA-BCL2-A5K5**

Caution: For Laboratory Use. A research product for research purposes only.

Contents

For an electronic version of this manual, please go to:
http://www.perkinelmer.com/category/alpha-CETSA-kits

Product Information

Application: The Alpha CETSA® kit presented here is designed for the quantitative determination of soluble human B-cell lymphoma 2 (Bcl-2) in Cellular Thermal Shift Assay (CETSA®) using a homogeneous AlphaLISA® assay (no wash steps). This kit is not cross reactive with mouse Bcl-2.

Sensitivity: Lower Detection Limit (LDL): 30 pg/mL

Lower Limit of Quantification (LLOQ): 72 pg/mL

EC_{50}: 20 ng/ml

Dynamic range: Kit designed to detect [Bcl-2] between: 30 – 1,000,000 pg/mL (Figure 1).

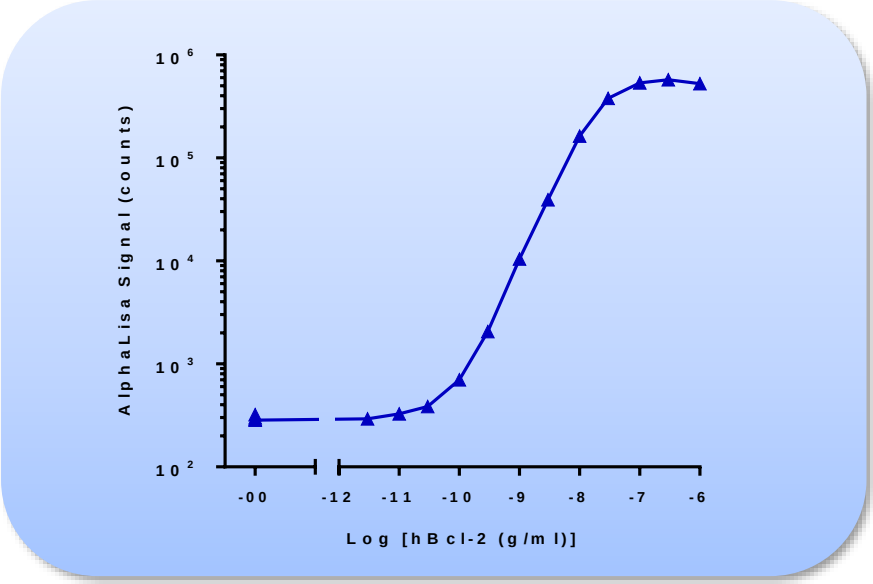


Figure 1. Typical sensitivity curve in AlphaLISA ImmunoAssay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4˚C. The human Bcl-2 analyte is stable for at least 6 months at 4°C. Store reconstituted analyte at -20°C for long term storage. Avoid freeze-thaw cycles.

Stability: This kit is stable for at least 6 months from the shipping date when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

B-cell lymphoma 2 (Bcl-2) is an oncogenic protein that inhibits apoptosis. It is a protein with a MW of 24-26 kDa, located within the mitochondrial membrane, endoplasmic reticulum and nuclear envelope. Bcl-2 is considered an important anti-apoptotic protein and is thus classified as a proto-oncogene. Bcl-2 overexpression has been implicated in a wide variety of malignancies including (but not limited to) breast, prostate, skin, colon and pancreatic cancers as well as neurodegenerative and autoimmune diseases. Related anti-apoptotic proteins include Bcl-xL, Mcl-1 and Bcl-w. Pro-apoptotic proteins in the Bcl-2 family include Bax, BAD, Bak, Blk and Bid. The AlphaLISA kit presented here has been designed for the detection of Bcl-2 in cell culture media and serum.

CETSA® Assay Principle

The Cellular Thermal Shift Assay (CETSA) assesses the thermal stability of proteins in living cells and cell lysate based on denaturation and aggregation upon heating. The relative amount of remaining soluble protein after heating can be measured, and a thermal melting curve of a known target protein can be generated. Compound binding often affects the thermal stability of proteins, and the shift in the melting curve is indicative of cellular target engagement. Target engagement by compound binding can result in thermal stabilization of the protein target, leading to a right-shifted thermal denaturation profile (as exemplified in Fig 2), but can also result in protein destabilization, then leading to a left-shifted thermal denaturation profile.

The CETSA® assay is run by incubating intact cells or disrupted cells with the test compound and the assay will reflect the ability of the compound to interact with the target protein in a cellular context. In the case of intact cells, the CETSA® assay data is affected by cellular metabolism and permeability. The CETSA® assay takes into account the complexity of the cellular context, and as such provides very valuable and physiologically relevant target engagement information.

Typically, a melting curve is first generated, where the sample, in the presence and in the absence of a reference compound, is heat challenged at 12 different temperatures. From the melting curve a single temperature is selected, where about 80-90% of the target signal is lost, and where there is an obvious shift of the reference compound. This single temperature is then used to perform single concentration or concentration-response curve compound screening.

The concentration-response experiment determines the potency as the concentration yielding 50% of the maximal stabilization or destabilization effect (EC_{50}) at a single selected temperature. This EC_{50} value is in the literature also sometimes referred as “isothermal dose−response fingerprint” ($ITDRF_{CETSA}$), to signify its known dependence on assay conditions. The CETSA® assay EC_{50} can be used for ranking of compounds and reflects the direct compound binding to the native protein target in a cellular context. This data can and used for SAR analysis and correlation with other assay data.

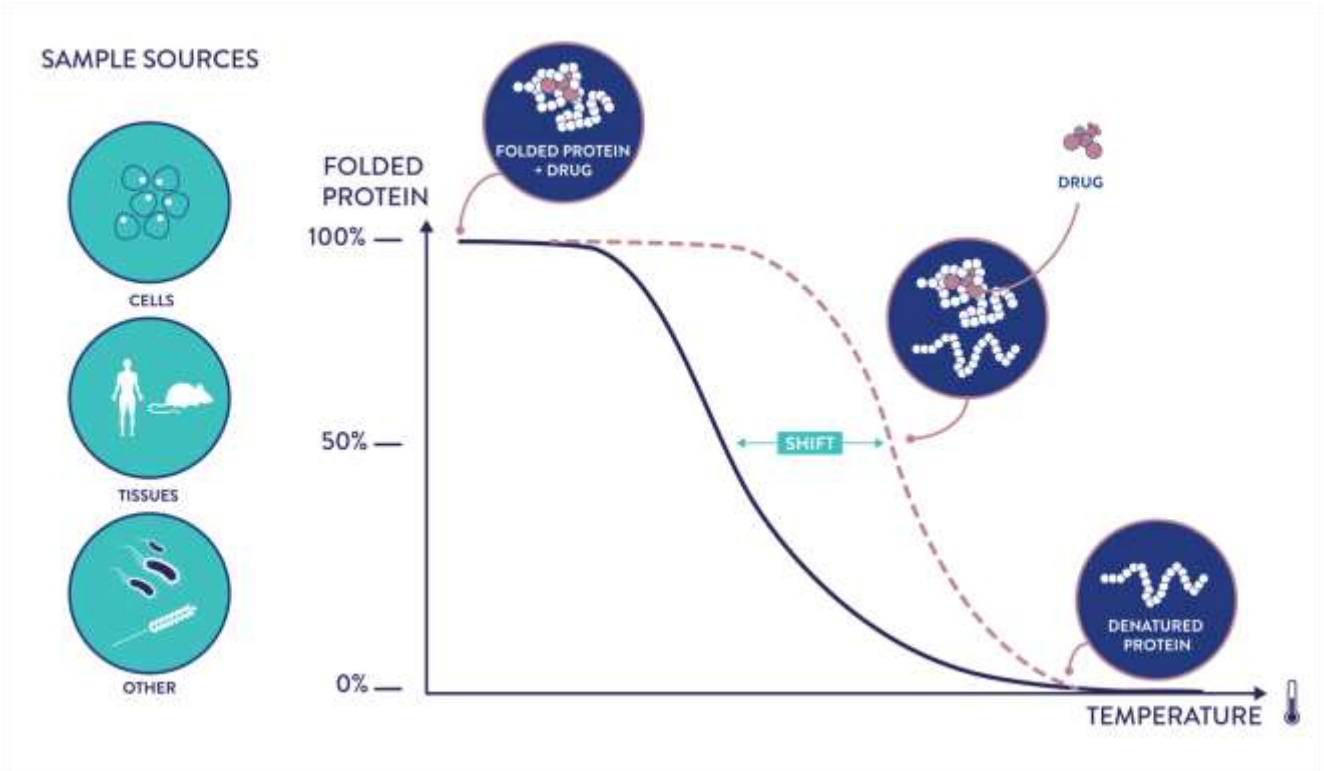
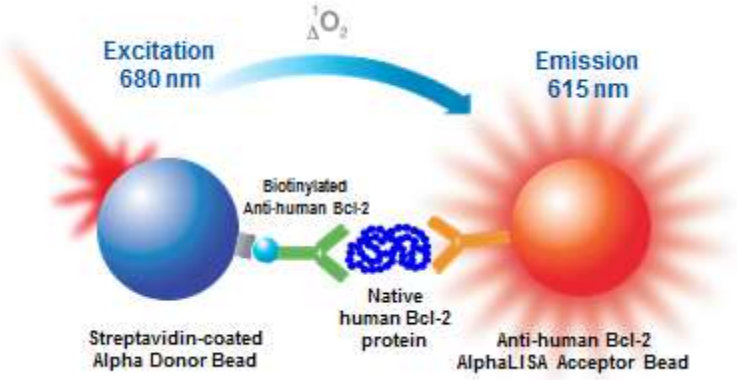


Figure 2 : Principle of the CETSA® melting curve, and thermal stabilization by compound binding.

Description of the Alpha CETSA® Assay

The Alpha CETSA® assay kits allow the rapid, sensitive and quantitative detection of the target protein remaining soluble after heat treatment of compound-treated cells. In an Alpha CETSA® assay, a biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaLISA® Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfers in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 3). This Alpha signal generated upon illumination of Donor Beads can be read with an Alpha-enabled plate reader, such as the EnVision® Multilabel Plate Reader, the EnSight™ or the Victor® Nivo™ Multimode Plate Readers. The amount of light emission is directly proportional to the amount of soluble protein present in the sample.

A.



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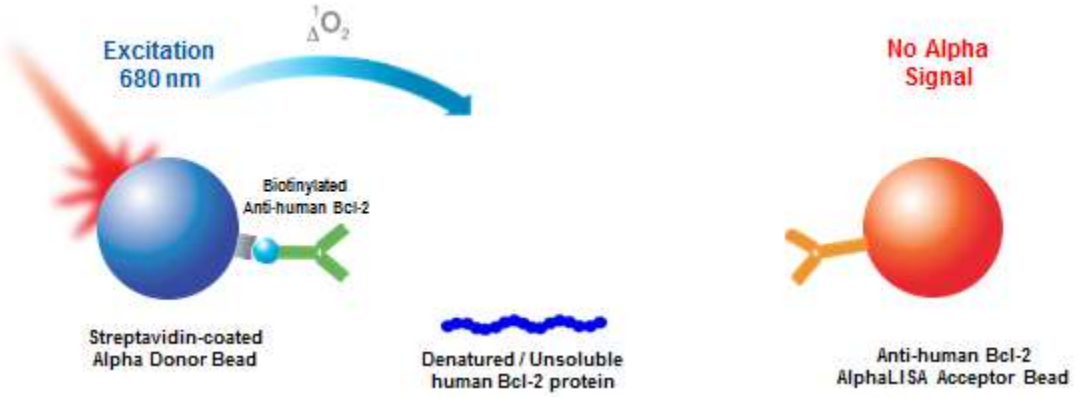


Figure 3. Principle of detection of the soluble protein by the Alpha CETSA® assay. Only the soluble protein will lead to the generation of an Alpha signal (A), while the thermally denaturated insoluble protein does not generate the proximity needed for an Alpha signal (B).

License

Please note that CETSA® is a registered trademark of Pelago Bioscience AB who hold the exclusive rights to the CETSA® patent family. If you do not have yet a valid license, please contact Pelago Biosciences at sales@pelagobio.com to discuss your needs.

The CETSA® method is patented in the following territories

United Kingdom:	Reg.no. 2490404
US:	Reg.no. 8969014, 9523693 and 9528996
Singapore:	Reg.no. 194137
China:	Reg.no. ZL201280025677.X
Korea:	10-1940342
Hong Kong:	HK1192612
India:	311112
Japan:	6032715
Australia:	2012246069
European states:	Austria, Belgium, Switzerland, Germany, Denmark, Spain, Finland, France, Great Britain, Ireland, Italy, the Netherlands and Sweden with Reg.no. 2699910

The granted and pending patents are based on patent application PCT/GB2012/050853.

Precautions

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures. The Donor Beads should NOT be used under red/orange light as can be found in photographic work darkrooms, as red light (680nm) excites the beads.

- All blood components and biological materials should be handled as potentially hazardous.

- The Biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	CETSA-BCL2-A500 (500 assay points)	CETSA-BCL2-A5K1 to 5 (5000 assay points)
CETSA® Cell Lysis Buffer 1 (5X) *	10 mL, 1 small bottle	100 mL, 1 large bottle (-K1 kit)
CETSA® Cell Lysis Buffer 2 (5X) *	10 mL, 1 small bottle	OR 100 mL, 1 large bottle (-K2 kit)
CETSA® Cell Lysis Buffer 3 (5X) *	10 mL, 1 small bottle	OR 100 mL, 1 large bottle (-K3 kit)
CETSA® Cell Lysis Buffer 4 (5X) *	10 mL, 1 small bottle	OR 100 mL, 1 large bottle (-K4 kit)
CETSA® Cell Lysis Buffer 5 (5X) *	10 mL, 1 small bottle	OR 100 mL, 1 large bottle (-K5 kit)
AlphaLISA® Anti-Bcl-2 Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	100 µL @ 5 mg/mL (1 brown tube, black cap)	1 mL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated Anti-Bcl-2 Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	100 µL @ 500 nM (1 tube, black cap)	1 mL @ 500 nM (1 tube, black cap)
Lyophilized Human Bcl-2 Analyte **, ***	1 µg, lyophilized (1 tube, clear cap)	1 µg, lyophilized (1 tube, clear cap)
AlphaLISA® ImmunoAssay Buffer (10X)****	10 mL, 1 small bottle	100 mL, 1 large bottle

* The choice of the **Cell Lysis Buffer** is an important part of the optimization of the CETSA® assays, and therefore 5 different Cell Lysis Buffers are provided in the 500 datapoints kit to experimentally determine which buffer is optimal for a particular cellular context. Once the optimal buffer has been found, 5 different versions of the 5000 datapoint kits are available, each containing a different Cell Lysis Buffer. The final number in the part number of these kits indicates which Cell Lysis Buffer is included in each of these kits. For example the CETSA-XXXX-X5K1 part number indicates that the kit contains CETSA Cell Lysis Buffer #1, and the CETSA-XXXX-X5K3 part number indicates that the kit contains CETSA Cell Lysis Buffer #3. Extra CETSA Cell Lysis buffers can be ordered separately (cat # CETSA-BUF1-100ML, CETSA-BUF2-100ML, CETSA-BUF3-100ML, CETSA-BUF4-100ML, CETSA-BUF5-100ML).

** Reconstitute hBcl-2 in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes, or can be aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid repeated freezing and thawing. One vial contains an amount of hBcl-2 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL384S).

And please note that the Bcl-2 analyte vial is provided as a control of the AlphaLISA® immunoassay (i.e. to control the ability of the reagents to detect the target), but that the Bcl-2 analyte provided should not be used to perform melting curves, or compound shift curve experiments, as it does not present the required characteristics for being heat-challenged successfully.

*** Not to be used in Thermoshift assay, only for controlling the immunoassay performance

**** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100 and 0.5% Proclin-300. Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the Alpha signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the Alpha signal (0.0001% final in the assay).

The CETSA® Cell Lysis Buffers 1 to 5 all contain a proprietary mixture of pH buffers, detergents, and salts. The different CETSA® Cell Lysis Buffers each use different types and concentrations of detergents, contain various concentrations of glycerol, or no glycerol, contain divalent cation chelators or not, contain different types of pH buffers and range from pH 7.0 to 7.5, and contain different types and concentrations of salts, in order to provide a variety of Cell Lysis Buffers so that an optimal one can be found for each target or cell type. The optimal Cell Lysis Buffer for a particular type of sample and target will need to be tested on a case-by-case basis.

The CETSA® Cell Lysis Buffers do not contain protease inhibitors as they are commonly not needed to perform CETSA® assays; however, when working with sample types particularly rich in proteases (such as pancreatic cells) the addition of protease inhibitors to the CETSA® Cell Lysis Buffers may be considered.

The table below highlights key differences between the Lysis Buffers.

Cell Lysis Buffer #	**Key Considerations**
CETSA® Cell Lysis Buffer 1	Most aggressive detergent formulation, contains divalent cation chelators, low salt concentration, moderate glycerol concentration
CETSA® Cell Lysis Buffer 2	Less aggressive detergent formulation; optimized for lysis of a broad range of cells without releasing nuclear DNA and minimally disrupting protein interactions; physiological salt concentrations
CETSA® Cell Lysis Buffer 3	Medium detergent concentration, strongest pH buffering capacity, no glycerol, low salt, 0.1% casein
CETSA® Cell Lysis Buffer 4	Medium-High detergent concentration, contains divalent cation chelators, close to physiological osmotic strength, no glycerol
CETSA® Cell Lysis Buffer 5	Medium-High detergent concentration, contains divalent cation chelator, close to physiological osmotic strength, highest glycerol concentration Please note that the CETSA® Cell Lysis Buffer 5 may turn yellow over time, which has no impact on assay performance.

Recommendations

General recommendations:

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec).
- Re-suspend all reagents by vortexing before use.
- Use Milli-Q® grade H_2O (18 MΩ•cm) to dilute 10X AlphaLISA ImmunoAssay Buffer.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- Alpha signal is detected using an EnVision® Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).
- AlphaLISA® signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment. The standard curve should be performed in AlphaLISA® ImmunoAssay buffer.

Materials Required but Not Provided

Item	Suggested source	Catalog #	Size
HardShell PCR Plate, 96 wells, Blue	PerkinElmer Inc.	6008870	50/box
HardShell PCR Plate, 384 wells, Blue	PerkinElmer Inc.	6008910	50/box
TOPSEAL-B FOR PCR PLATE	PerkinElmer Inc.	6050174	100/box
HBSS (1x) Hank's Balanced Salt Solution (with $CaCl_2$ and $MgCl_2$)	Gibco	14025-050	500 mL
Optiplate-384, White Opaque assay plate (1)	PerkinElmer Inc.	6007290	50/box
AlphaPlate-384, Light Gray Opaque assay plate (2)	PerkinElmer Inc.	6005350	50/box
TopSeal-A 384, clear adhesive sealing film	PerkinElmer Inc.	6050185	100/box
Veriti 96 Well Thermal Cycler: 96-well format PCR Machine, with 6 temperature zones (6 x 2 columns), useful for performing melting curves experiments	Applied Biosystems	-	-
ProFlex PCR System: Can accommodate 2 x 384-well plates at once; with a single temperature across the plate	Applied Biosystems	-	-
Microplate shaking table	-	-	-
Envision®, Ensight™ or Victor® Nivo™ Alpha-reader	PerkinElmer Inc.	-	-

(1) Plates used for the immunoassay; (2) Same as (1) but optimal if cross-talk needs to be reduced; For more assay plates options, please go to www.perkinelmer.com/microplates

Buffer Preparation and Subsequent Storage Conditions

2X Lysis Buffers	Dilute each 5X Lysis Buffer in MilliQ water to a final concentration of 2X. For example: for 5 mL of 2X Lysis Buffer, add: 2 mL of 5X Lysis Buffer to 3 mL MilliQ water. Excess 2X Lysis Buffer should be discarded.
1X AlphaLISA ImmunoAssay Buffer	Add 10 mL of 10X AlphaLISA ImmunoAssay Buffer to 90 mL MilliQ water.
5X Acceptor Mix	Add 50 µL of 5 mg/mL AlphaLISA Anti-Bcl-2 Acceptor beads to 4950 µl of 1X AlphaLISA ImmunoAssay Buffer
5X biotinylated Anti-Bcl-2 Antibody	Add 100 µL of 500 nM biotinylated Anti-Bcl-2 Antibody to 4900 µl of 1X AlphaLISA ImmunoAssay Buffer.
2X Donor Mix	Add 100 µL of 5 mg/mL SA-Donor beads to 12400 µL of 1X AlphaLISA ImmunoAssay Buffer
Lyophilized Human Bcl-2	Reconstitute with 100 µL water. Store at -20°C in single use aliquots and use within 3 months. Dilute as required in 1X CETSA Cell Lysis Buffer.

All buffer and beads dilutions should be prepared the same day as the experiment is run and used immediately.

Assay Procedure for Standard Curve Generation

The Alpha CETSA® assay is primarily intended to detect changes in soluble analyte following compound treatment. Therefore, conversion of Alpha signal into analyte concentration value is normally not done in a CETSA® assay.

However, performing a standard curve is still useful for controlling the AlphaLISA immunoassay performance.

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The protocol described below is an ***example*** for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations) and 452 samples. The protocols also include testing samples in 384 well plates. If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly, as shown in the table below. ****These calculations do not include excess reagents to account for losses during transfer of solutions or dead volumes.*
- The standard dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

		Volume					
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA beads**	**Biotin Antibody**	**SA-Donor beads**	**Plate recommendation**
CETSA 500	500	50 µL	5 µL	10 µL	10 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
CETSA 5K	5 000	50 µL	5 µL	10 µL	10 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)

Protocol for Bcl-2 Alpha CETSA® Assay Standard Curve

3 Step Protocol – Dilution of standards in 1X AlphaLISA ImmunoAssay Buffer. The protocol described below is for one standard curve (48 wells) and 452 sample wells. *If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

Steps for Preparing Reagents

1) Preparation of 1X AlphaLISA ImmunoAssay Buffer:

Add 10 mL of 10X AlphaLISA ImmunoAssay Buffer to 90 mL H_2O.

2) Preparation of hBcl-2 analyte standard dilutions:

a. Reconstitute lyophilized hBcl-2 (1 µg) in 100 µL H_2O.
b. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of hBcl-2 (µL)	Vol. of diluent (µL) *	[hBcl-2] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted hBcl-2	90	1.00E-06	1 000 000
B	60 µL of tube A	140	3.00E-07	300 000
C	60 µL of tube B	120	1.00E-07	100 000
D	60 µL of tube C	140	3.00E-08	30 000
E	60 µL of tube D	120	1.00E-08	10 000
F	60 µL of tube E	140	3.00E-09	3 000
G	60 µL of tube F	120	1.00E-09	1 000
H	60 µL of tube G	140	3.00E-10	300
I	60 µL of tube H	120	1.00E-10	100
J	60 µL of tube I	140	3.00E-11	30
K	60 µL of tube J	120	1.00E-11	10
L	60 µL of tube K	140	3.00E-12	3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Dilute standards in diluent (e.g. 1X AlphaLISA ImmunoAssay Buffer).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X AlphaLISA Anti-hBcl-2 Acceptor beads (50 µg/mL)

a. Add 50 µL of 5 mg/mL AlphaLISA Anti-hBcl-2 Acceptor beads to 4950 µL of 1X AlphaLISA ImmunoAssay Buffer.
b. Prepare just before use.

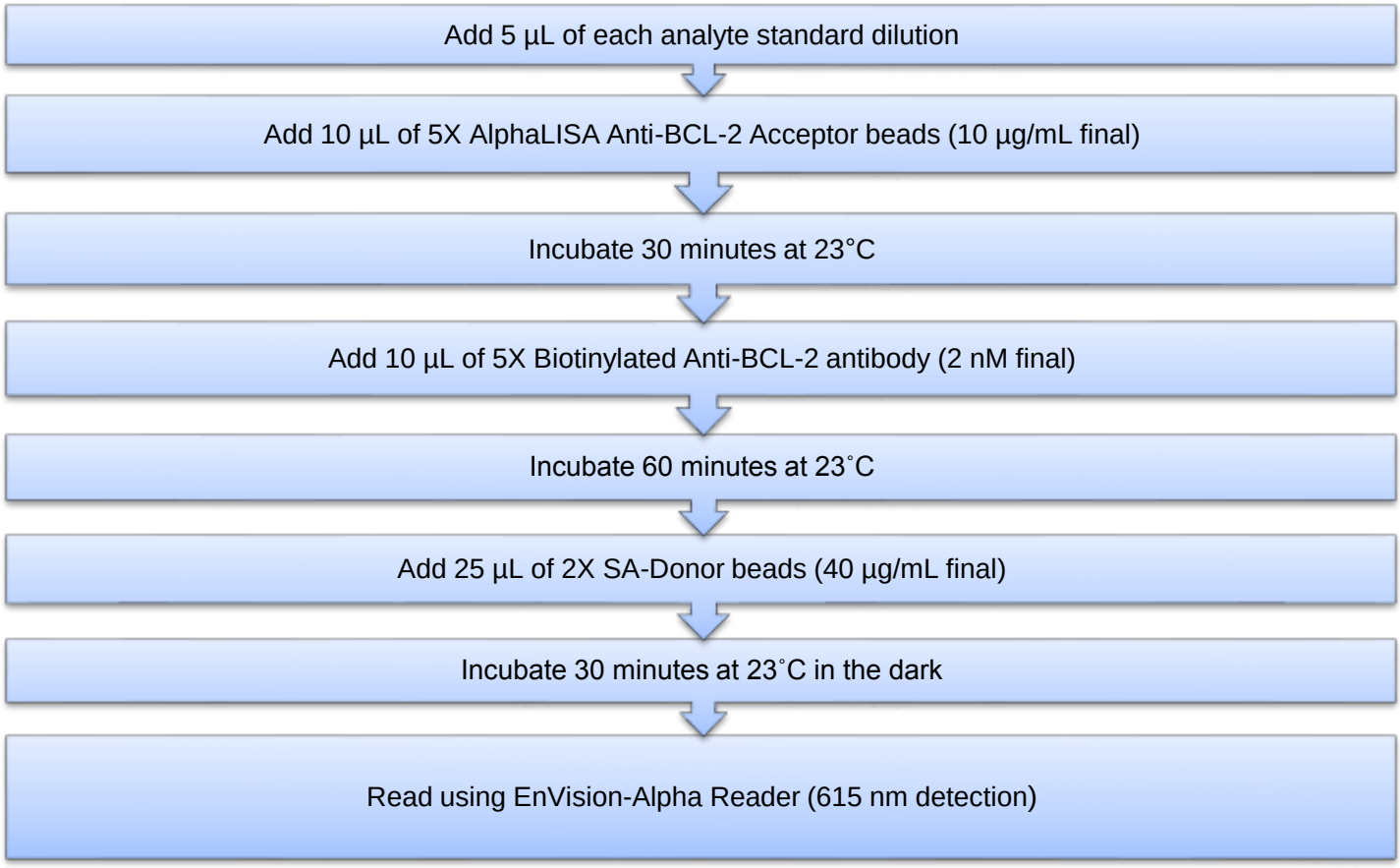
4) Preparation of 5X AlphaLISA Biotinylated Anti-hBcl-2 (10 nM)

a. Add 100 µL of 500 nM Biotinylated Anti-hBcl-2 antibody to 4900 µL of 1X AlphaLISA Immunoassay Buffer.
b. Prepare just before use.

5) Preparation of 2X Streptavidin (SA) Donor beads (40 µg/mL):

a. Prepare just before use and keep the beads under subdued laboratory lighting.
b. Add 100 µL of 5 mg/mL SA-Donor beads to 12400 µL of 1X AlphaLISA Immunoassay Buffer.

6) In a white Optiplate (384 wells):



Read Settings: Alpha signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

Standard Curve Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the Alpha counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).

Specificity

Cross-reactivity of the hBcl-2 Alpha CETSA® Kit was tested using the following protein at 10 ng/mL in IAB.

Protein	% Cross-reactivity
Mouse Bcl-2	0

Alpha CETSA® Assay Protocols

The most relevant CETSA® assay is performed on living cells, as data obtained in such protocols reflect the ability of the compound to reach and bind the target in a close-to-physiological situation.

However, there is a possibility to run CETSA® assays on cells disrupted by freeze-thawing prior to being placed in the presence of the test compounds. This may provide valuable information about the ability of the compound to engage the target, independently of its permeability and metabolization properties by a living cell.

A. Intact Cells CETSA® assay

Cell Preparation

1. Recover cells directly (suspension cells) or by trypsination (adherent cells) from T-Flasks, Petri Dishes or Cell Factories (or any other culture method in place for the cell type used). Then wash the cells in HBSS to remove trypsin and resuspend cells in HBSS. A common density to start with is 1 to 2 million cells/mL, but cell density is part of the initial optimization of CETSA® assays and this can vary from cell type to cell type and according to the target to be detected.

Cell Treatment

2. In a PCR plate, add 10 µL/well of 2X concentrated test compound diluted in HBSS. When performing concentration-response testing, it is recommended to do a first dilution in 100% DMSO and then further dilute each compound concentration in HBSS, in order to keep the final DMSO concentration the same in all samples.
   Note: DMSO concentration should be kept at maximum 0.1% final on cells in order to avoid toxic effects.
3. Add 10 µL/well of cells resuspended in HBSS.
4. Incubate at 37°C / 5% CO_2 (in a cell culture incubator) for 60 minutes.
   Note: 30 minutes is often sufficient for intracellular targets, but the optimal incubation time may vary according to the target and cell type used.
   Note: Some targets are unstable and incubation at lower temperatures than 37°C may be required.
5. Pre-heat the PCR thermocycler to the selected temperature.
   Note: For measuring a target melting curve, a thermocycler with variable temperature zones is very useful at this stage. - Please pay attention that “gradient PCR machines” do not provide a precise control of the temperature over different zones and therefore are not recommended for running melting curves as this could introduce a bias in the true melting temperature calculation. - A standard melting curve typically includes 12 different temperatures ranging between 37-63°C. When one temperature for CETSA® screening applications has been selected, a thermocycler with a single temperature zone can be used.
   Important Note: The heating of the cover should be inactivated, else this would result in higher than desired sample temperatures and inaccuracy of melting temperature calculation.
6. Transfer the plate into the PCR thermocycler and heat the samples for 3 minutes.
   Note: Heating time is an important parameter, and it is important to strictly control it. Using shorter or longer heating times may result in a different concentration-response profile. In particular, compounds with different retention times by the target (off-rates) are expected to be impacted differently by changes in plate heating time.
7. Remove the plate from the thermocycler and allow it to cool down to 4°C on ice or use the thermocycler for fastest possible cooling. Incubate for at least 3 minutes.

Lysate Preparation

8. Add 20 µL of freshly prepared 2X CETSA Cell Lysis Buffer. Agitate on a plate shaking table (350 to 700 rpm) for 30 minutes at room temperature.
   Note: An additional lysis step of flash freezing in liquid nitrogen and thawing at 20°C using the thermocycler can be implemented at this stage if desired as it may in some cases improve the day-to-day and/or well-to-well signal variability.

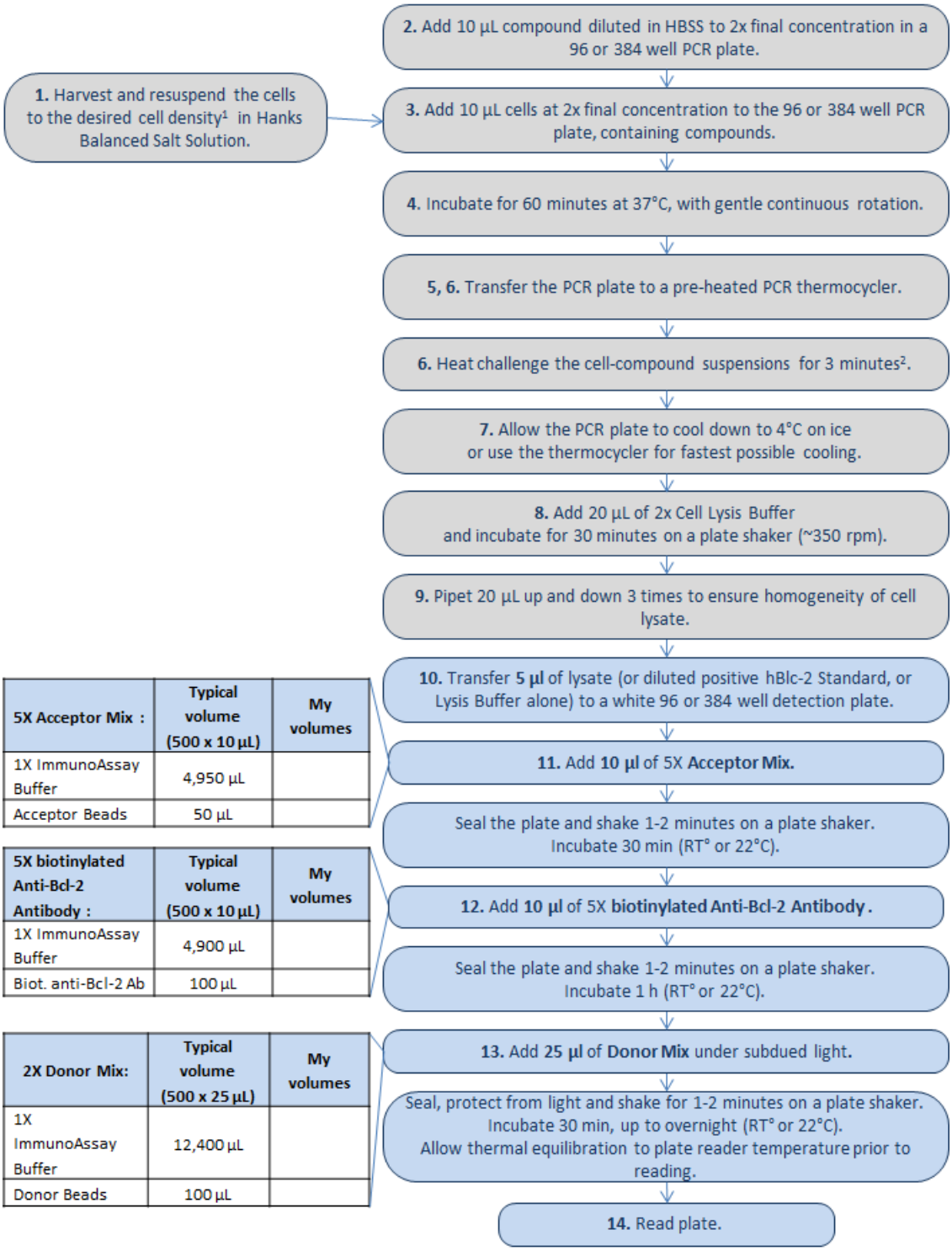
Note: make sure you comply with standard lab safety procedures when working with liquid nitrogen to avoid any burning by liquid nitrogen. In particular wear protective glasses and gloves.

9. Pipet 20 µL up and down three times to ensure homogeneity of the cell lysate solution.
10. Transfer 5 µL of the lysate to a 384-well Optiplate™ or AlphaPlate™ microplate for the immunoassay. Add 5 µL of diluted Bcl-2 Standard to separate wells. We recommend testing a serial dilution of Bcl-2 Standard diluted in 1X CETSA Cell Lysis Buffer.

Alpha CETSA® Assay

11. Add 10 µL of 5X Acceptor Mix to the wells. Seal plate with Topseal-A adhesive film and incubate for 30 minutes at room temperature.
12. Add 10 µL of 5X biotinylated Anti-Bcl-2 Antibody Mix to the wells. Seal plate with Topseal-A adhesive film and incubate for 1 hour at room temperature.
13. Add 25 µL of 2X Donor Mix to the wells under subdued light. Seal plate with Topseal-A adhesive film, and protect the plate from light. Incubate for 30 minutes at room temperature in the dark.
    Note: Longer incubation may give greater sensitivity. Plates can be incubated overnight if required.
14. Read plate on an Alpha Technology-compatible plate reader, using standard Alpha settings.

Alpha CETSA® Bcl-2 flowchart - Intact Cells assay



5X Acceptor Mix :	Typical volume (500 x 10 µL)	My volumes
1X ImmunoAssay Buffer	4,950 µL	
Acceptor Beads	50 µL	

5X biotinylated Anti-Bcl-2 Antibody :	Typical volume (500 x 10 µL)	My volumes
1X ImmunoAssay Buffer	4,900 µL	
Biot. anti-Bcl-2 Ab	100 µL	

2X Donor Mix:	Typical volume (500 x 25 µL)	My volumes
1X ImmunoAssay Buffer	12,400 µL	
Donor Beads	100 µL	

[1] A starting point is usually 1 to 2 million cells/mL, but optimal cell density will depend on the target and cell type
[2] A standard melt curve curve includes 12 different temperatures ranging between 37-63°C. Use a uniform temperature for single concentration or concentration-response compound screening.

B. Disrupted Cells CETSA® assay

Cell Preparation

1. Recover cells directly (suspension cells) or by trypsinization (adherent cells) from T-Flasks, Petri Dishes or Cell Factories (or any other culture method in place for the cell type used). Then wash the cells in HBSS to remove trypsin and resuspend cells in HBSS. A common density to start with is 1 to 2 million cells/mL, but cell density is part of the initial optimization of CETSA® assays and this can vary from cell type to cell type and according to the target to be detected.

Cell disruption

2. Dispense the cell suspension in 1 mL to 10 mL tubes.
3. Freeze the cells by plunging the tubes into liquid nitrogen, until the tube content is completely frozen.
   Note: make sure you comply with standard lab safety procedures when working with liquid nitrogen to avoid any burning by liquid nitrogen. In particular wear protective glasses and gloves and close the tubes tightly to avoid any risk of tube explosion consecutive to liquid nitrogen entering in the tubes.
4. Thaw cell suspensions by plunging the tubes in a water bath at 20°C, until the tube content is completely thawed.
5. Repeat the freeze thawing three times.
6. Optional: Centrifuge the tubes for 15 min at 20 000xg to remove cell debris and transfer the supernatant into a new tube, and use the disrupted cells suspension immediately
   Note 1: In some cases using non-centrifuged disrupted cell suspension can lead to elevated background. In such case, the suspension can be cleared by centrifugation, as this usually decreases the background signal.
   Note 2: Depending on the target and cell type used, there may be a possibility to store the disrupted cell solution at -80°C for later use. However, some targets may degrade over time when stored in such conditions, and this needs to be validated on a case-by-case basis.

Disrupted cells Treatment

7. In a PCR plate, add 10 µL/well of 2X concentrated test compound diluted in HBSS. When performing concentration-response testing, it is recommended to do a first dilution in 100% DMSO and then further dilute each compound concentration in HBSS, in order to keep the final DMSO concentration the same.
8. Add 10 µL/well of disrupted cell suspension prepared above.
9. Incubate at 37°C / 5% CO_2 (in a cell culture incubator) for 30 minutes.
10. Pre-heat the PCR thermocycler to the selected temperature.
11. Transfer the plate into the PCR thermocycler and heat the samples for 3 minutes.
    Note: Heating time is an important parameter, and it is important to strictly control it. Using shorter or longer heating times may result in a different concentration-response profile. In particular, compounds with different retention times by the target (off-rates) are expected to be impacted differently by changes in plate heating time.
12. Remove the plate from the PCR thermocycler and allow it to cool down to 4°C on ice or use the thermocycler for fastest possible cooling. Incubate for at least 3 minutes.
13. To keep the same buffer conditions as when working with intact cells, add 20 µL of freshly prepared 2X CETSA Cell Lysis Buffer. Agitate on a plate shaking table (350 to 700 rpm) for 10 minutes at room temperature.
14. Pipet 20 µL up and down three times to ensure homogeneity of the cell lysate solution.
15. Transfer 5 µL of the lysate to a 384-well Optiplate™ or AlphaPlate™ microplate for the immunoassay. Add 5 µL of diluted hBcl-2 Standard to separate wells. We recommend testing a serial dilution of hBcl-2 Standard diluted in 1X CETSA Cell Lysis Buffer.

Alpha CETSA® Assay

16. Add 10 µL of 5X Acceptor Mix to the wells. Seal plate with Topseal-A adhesive film and incubate for 30 min at room temperature.
17. Add 10 µL of 5X biotinylated Anti-Bcl-2 Antibody Mix to the wells. Seal plate with Topseal-A adhesive film and incubate for 1 hour at room temperature.

18. Add 25 µL of 2X Donor Mix to the wells under subdued light. Seal plate with Topseal-A adhesive film, and protect the plate from light. Incubate for 30 minutes at room temperature in the dark.
    Note: Longer incubation may give greater sensitivity. Plates can be incubated overnight if required.
19. Read plate on an Alpha Technology-compatible plate reader, using standard Alpha settings.

Alpha CETSA® Bcl-2 flowchart - Disrupted Cells assay

1, 2. Harvest and resuspend the cells to the desired cell concentration[1] in Hanks Balanced Salt Solution.

3-5. Freeze-thaw the cells in liquid nitrogen 3x. Thaw in a water bath.

6. Optional: Centrifuge for 15 min at 20,000 x g to clear the cell lysate

7. Add 10 µL compound diluted in HBSS to 2x final concentration in a 96 or 384 well PCR plate.

8. Add 10 µL disrupted cells suspension to the 96 or 384 well plate, containing compounds.

9. Incubate for 30 minutes at 37°C, with gentle continuous rotation.

10, 11. Transfer the PCR plate to a pre-heated PCR thermocycler.

11. Heat challenge the cell-compound suspension for 3 minutes[2].

12. Allow the PCR plate to cool down to 4°C on ice or use the thermocycler for fastest possible cooling.

13. Add 20 µL 2x Cell Lysis Buffer and incubate for 10 minutes on a plate shaker (~350 rpm)[3].

14. Pipet 20 µL up and down 3 times to ensure homogeneity of cell lysate.

15. Transfer **5 µl** of lysate (or diluted positive hBcl-2 Standard, or Lysis Buffer alone) to a white 96 or 384 well detection plate.

5X Acceptor Mix :	Typical volume (500 x 10 µL)	My volumes
1X ImmunoAssay Buffer	4,950 µL	
Acceptor Beads	50 µL	

16. Add **10 µl** of 5X **Acceptor Mix.**

Seal the plate and shake 1-2 minutes on a plate shaker. Incubate 30 min (RT° or 22°C).

5X biotinylated Anti-Bcl-2 Antibody :	Typical volume (500 x 10 µL)	My volumes
1X ImmunoAssay Buffer	4,900 µL	
Biot. anti-Bcl-2 Ab	100 µL	

17. Add **10 µl** of 5X **biotinylated Anti-Bcl-2 Antibody.**

Seal the plate and shake 1-2 minutes on a plate shaker. Incubate 1 h (RT° or 22°C).

2X Donor Mix:	Typical volume (500 x 25 µL)	My volumes
1X ImmunoAssay Buffer	12,400 µL	
Donor Beads	100 µL	

18. Add **25 µl** of **Donor Mix** under subdued light.

Seal, protect from light and shake for 1-2 minutes on a plate shaker. Incubate 30 min, up to overnight (RT° or 22°C). Allow thermal equilibration to plate reader temperature prior to reading.

19. Read plate.

[1] A starting point is usually 1 to 2 million cells/mL, but optimal cell density will depend on the target and cell type.
[2] A standard melt curve includes12 different temperatures ranging between 37-63°C. Use a uniform temperature for single concentration or concentration-response compound compound screening.
[3] For a comparative buffer conditions to an intact Cells Assay, the Lysis Buffer is added at this step.

Alpha CETSA® Bcl-2 Assay Data

A. Cell line culture conditions

The Peripheral blood B-lymphocyte cell line (ATCC® TIB-196) was cultured using the following conditions:

U266B1 culture medium:

RPMI 1640 (ThermoFisher, # 61870-036) supplemented with 15% FBS (ThermoFisher, #10500064) and 100 units/mL penicillin and 100 µg/mL streptomycin (ThermoFisher, #15140-122).

U266B1 culture conditions:

The cells were cultured in complete RPMI 1640 medium at 37°C, 5% CO_2, in a humified atmosphere to confluency. For harvesting, the cells were collected and washed twice in Hanks Balanced Salt Solution (HBSS, ThermoFisher, #14025-050). Thereafter, the cells were resuspended to the desired density in HBSS.

B. Cell density titration and Lysis Buffer optimisation

Method

The U266B1 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, 0.62 and 0.31 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). 5 µL of the cell lysates (corresponding to 50 000 to 780 cells/well) was transferred to duplicate wells of the detection plate (Alpha plate, #6005350). The Alpha CETSA® 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 4 A-E show the Alpha signals generated in U266B1 cell density titrations (800 to 50 000 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 4). 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. CETSA Cell Lysis Buffer 1 was selected for detection of Bcl-2 using the Alpha CETSA® assay, with a cell density of 5 million/mL (25 000 cells/well). These parameters yield a signal-to-background ratio of 24 (Table 1).

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

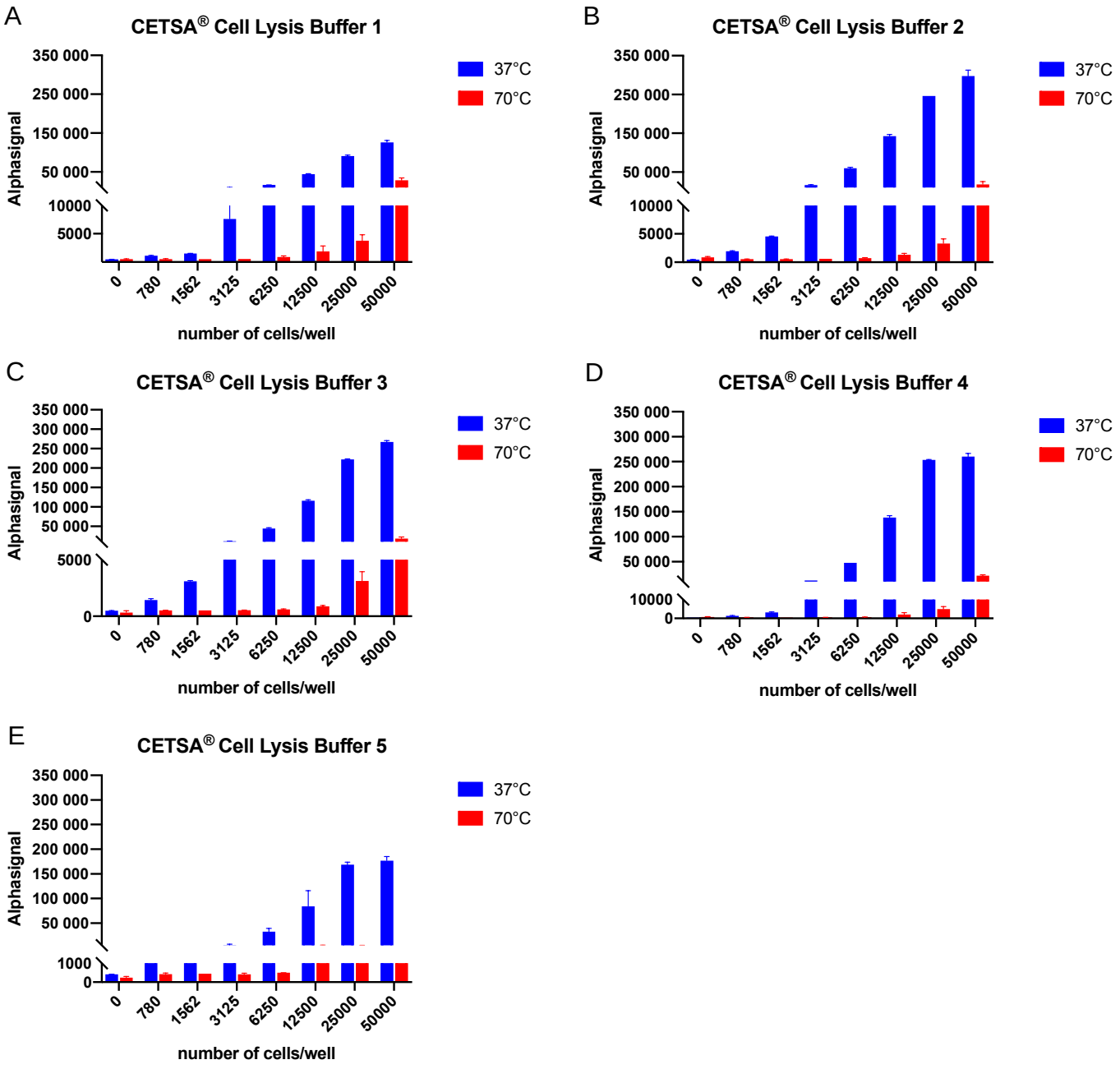


Figure 4. Cell density titration and Lysis Buffer optimization. Panel A-E show dilution series of intact U266B1 cells ranging from 800 to 50 000 cells/well plus negative control (HBSS) treated with the respective CETSA® Cell Lysis Buffer after the heat treatment. **A.** CETSA® Cell Lysis Buffer 1, **B.** 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 cells (positive control samples) and red bars samples heated to 70°C (negative control).

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

Number of cells/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
780	2	4	3	3	3
1 562	3	9	6	6	4
3 125	14	28	22	23	12
6 250	18	84	71	77	66
12 500	23	109	129	154	29
25 000	24	74	71	81	54
50 000	4	16	14	14	138

C. CETSA® assay Melting and shift curve analysis

Method

Melting and shift curves were generated of Bcl-2 in intact U266B1 cells. The cells were harvested and diluted in HBSS to a concentration of 20 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 10 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 1. The plate was then incubated on a plate shaking table for 30 minutes at RT. 5 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 CETSA® protocol was followed for Alpha signal detection.

Results

Melting and shift curves of Bcl-2 in intact U266B1 cells were generated with three 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” represents a blank control with no addition of cells). The data was analyzed using a non-linear regression curve fit with four parameters variable slope (GraphPad Prism 8.1.1, GraphPad Software) (Figure 5). Resulting Tm values, calculated from the GraphPad curve fitting, are listed in Table 2. These data indicate a destabilization to Bcl-2 following treatment with all five compounds. The shift allows for selection of a temperature with a significant amplitude between compound treated and control samples. Such a temperature of 58°C, marked by a dotted line (Figure 5A-C), was selected for isothermal concentration-response analysis of the compounds.

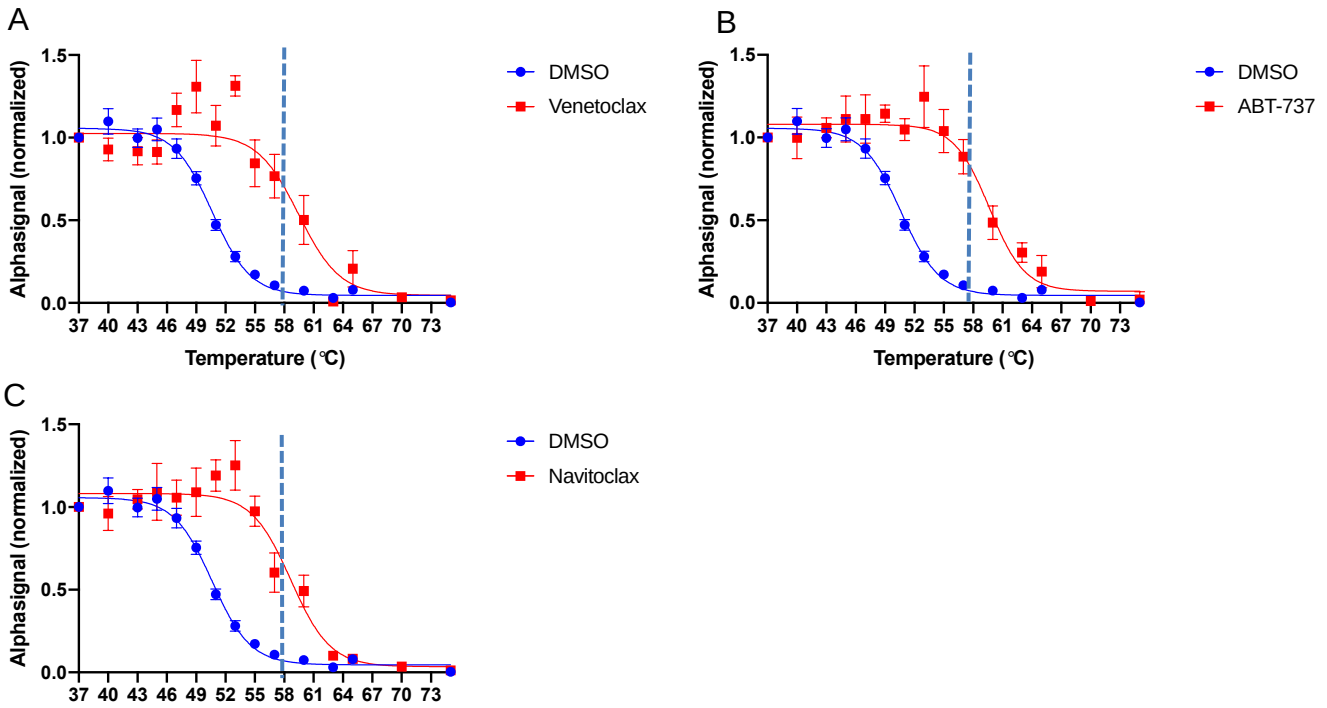


Figure 5. CETSA® Melting and shift curve analysis of Bcl-2 inhibitors in U266B1 intact cells. U266B1 cells incubated with 10 µM **A** Venetoclax, **B** ABT-737 and **C** Navitoclax or DMSO control. The data is normalized to the corresponding value at 37°C. The dotted line indicates the temperature that has been selected to generate 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 Bcl-2 reference compounds in U266B1 intact cells. CI=confidence interval, N=Biological repeats, n=technical repeats.

Cell type	Compound	T_m (°C)	95% CI (°C)	N;n
U266B1	DMSO control	50.6	50.1-51.2	3;6
U266B1	Venetoclax	59.5	57.8-61.6	3;6
U266B1	ABT-737	60.0	58.2-61.7	3;6
U266B1	Navitoclax	58.7	57.0-60.3	3;6

D. CETSA® assay concentration-response analysis

Method

CETSA® assay concentration-response (C-R) curves were generated of Bcl-2 in intact U266B1 cells. The cells were harvested and diluted in HBSS to a concentration of 20 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 10 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 minutes at 58°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 1. The plate was then incubated on a plate shaking table for 30 minutes at room temperature. 5 µL of the cell lysates (corresponding to 25 000 cells/well) was transferred to duplicate wells of the detection plate (Alpha plate, # 6005350). The Alpha CETSA® 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 ($y = x/Alphasignal_{100\mu M}$) 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 Bcl-2 C-R curves are shown in Figure 6 and the CETSA® EC_{50} -values are presented in Table 3. The CETSA® EC_{50} values were obtained from the curve fitting and allowed the determination of a rank order of potency of the compounds tested.

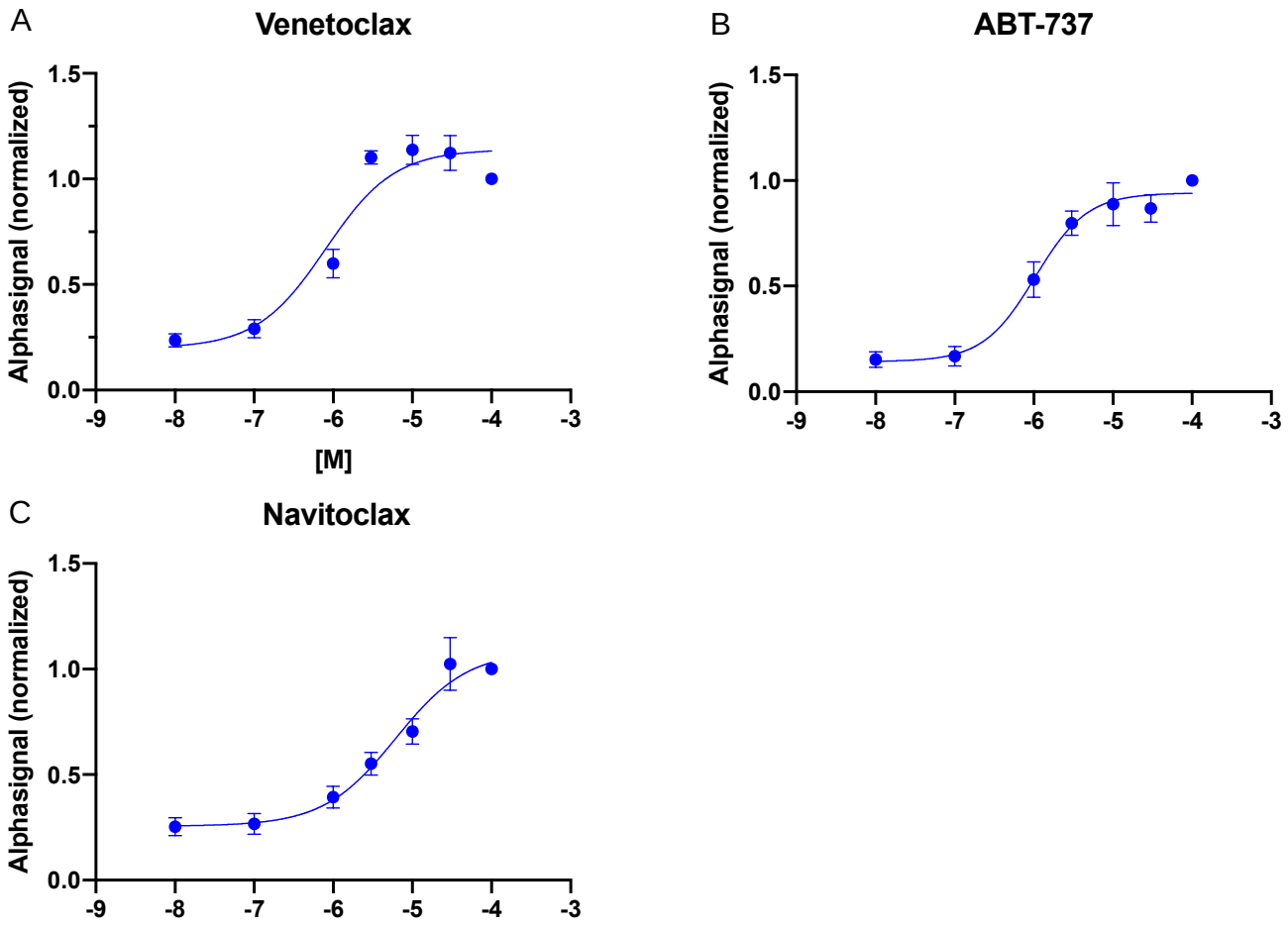


Figure 6. CETSA® concentration-response curves for Bcl-2 in U266B1 intact cells. The solid line represents the logistic curve fit to the data. The concentration-response curves represent Bcl-2 in intact U266B1 cells incubated with a serial dilution of **A.** Venetoclax, **B.** ABT-737, **C.** Navitoclax. The plotted values are normalized to the Alpha signals obtained at 100 µM.

Table 3. CETSA® assay EC_{50} values for Bcl-2 reference compounds in U266B1 cells. CI= confidence interval, N= biological repeat, n=technical repeat n.a = not applicable.

Cell type	Compound	CETSA® EC_{50}* (µM)	95% CI (µM)	N; n
U266B1	Venetoclax	0.8	n.a- 1.3	3; 9
U266B1	ABT-737	1.0	0.6-1.8	3; 9
U266B1	Navitoclax	6.1	3.0-38	3; 9

Table 4. Reference compounds used in the Bcl-2 CETSA® assays.

Compound	Provider	Cat #	Stock Solution
Venetoclax	Selleckchem	S8048	10 mM in 100% DMSO
ABT-737	Selleckchem	S1002	10 mM in 100% DMSO
Navitoclax	Selleckchem	S1001	10 mM in 100% DMSO

Supplementary Buffers and Beads

If using the standard protocol, sufficient amounts of buffers and beads are provided in the kit. However if the standard protocol is modified, more buffers or Streptavidin beads may be needed. In this case, additional buffers and Streptavidin beads can be ordered using the following catalog numbers:

Item	Suggested source	Catalog #	Size
CETSA® Cell Lysis Buffer 1	PerkinElmer Inc.	CETSA-BUF1-100mL	100mL
CETSA® Cell Lysis Buffer 2	PerkinElmer Inc.	CETSA- BUF2-100mL	100mL
CETSA® Cell Lysis Buffer 3	PerkinElmer Inc.	CETSA- BUF3-100mL	100mL
CETSA® Cell Lysis Buffer 4	PerkinElmer Inc.	CETSA- BUF4-100mL	100mL
CETSA® Cell Lysis Buffer 5	PerkinElmer Inc.	CETSA- BUF5-100mL	100mL
Alpha Streptavidin Donor Beads -5mg/mL	PerkinElmer Inc.	6760002S	1 mg
	PerkinElmer Inc.	6760002	5 mg
	PerkinElmer Inc.	6760002B	50 mg

Please note that AlphaLISA Acceptor Beads are kit-specific and cannot be ordered separately.

Useful Links

For FAQ and troubleshooting, please go to:
www.perkinelmer.com/CETSAFAQ

For a complete list of Alpha CETSA® kits, please go to: **http://www.perkinelmer.com/category/alpha-CETSA-kits** or **https://www.pelagobio.com/**

For technical support please go to:
www.perkinelmer.com/ASK

Recommended CETSA® Reading

Martinez-Molina. D. (2013). Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay. Science **341**:84-87.
https://science.sciencemag.org/content/341/6141/84

Seashore-Ludlow B, Axelsson H, Almqvist H, Dahlgren B, Jonsson M, Lundbäck T. (2018) Quantitative Interpretation of Intracellular Drug Binding and Kinetics Using the Cellular Thermal Shift Assay. Biochemistry **57**:6715-6725.
https://dx.doi.org/10.1021/acs.biochem.8b01057

Shaw J, Dale I, Hemsley P, Leach L, Dekki N, Orme JP, Talbot V, Narvaez AJ, Bista M, Martinez-Molina D, Dabrowski M, Main MJ, Gianni D. (2019) Positioning High-Throughput CETSA in Early Drug Discovery through Screening against B-Raf and PARP1. SLAS Discovery **24**:121-132.
https://journals.sagepub.com/doi/10.1177/2472555218813332

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