# NanoLuc and HiBIT CETSA assays to assess cellular target engagement of compounds in cells.
# Version: 1.0
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## 1. Rationale/Aim

Cellular thermal shift assay (CETSA) is a cellular target engagement method used for monitoring the stabilization of the target protein by a small molecule binding in cells. Traditionally, protein stabilization was analyzed by SDS-PAGE and Western blotting of soluble fraction from cells treated or untreated with compound and heated at varying temperatures (Jafari et. al., 2014, DOI: 10.1038/nprot.2014.138). Recently, a simpler and higher throughput way to screen for compound target engagement in cells using NanoLuc technology was developed (NanoLuc thermal shift assay, NaLTSA, Dart et. al., 2018, DOI: 10.1021/acsmedchemlett.8b00081). In this method, the protein of interest (POI) is fused to 19 kDa NanoLuc luciferase (NL), and the remaining luminescence signal after thermal treatment is monitored. To avoid the possible effects of a large NL tag, split reporter system, HiBIT CETSA, was developed and successfully implemented for high throughput screening (Ramachandran et. al., 2023, DOI: 10.1007/978-1-0716-3397-7_11). Here, the NL is split into two fragments, and the activity is reconstituted upon fragment complementation. The POI is tagged with a small 11 amino acid HiBIT tag and expressed in cells; after heat exposure, the soluble protein–HiBIT fusion binds to added LgBIT protein to reconstitute the functional NL and produce a luminescence signal.

## 2. Experimental conditions

### 2.1 Key Requirement:

#### 2.1.1. Preparation of target DNA linked N-terminal or C-terminal to NL or HiBIT

Target DNA is cloned into commercially available plasmids (Promega)
pBiT3.1-N (#N2361)
pBiT3.1-C (#N2371)
pNLF1-N (#N1351)
pNLF1-C (#N1361)

#### 2.1.2. Instrumentation

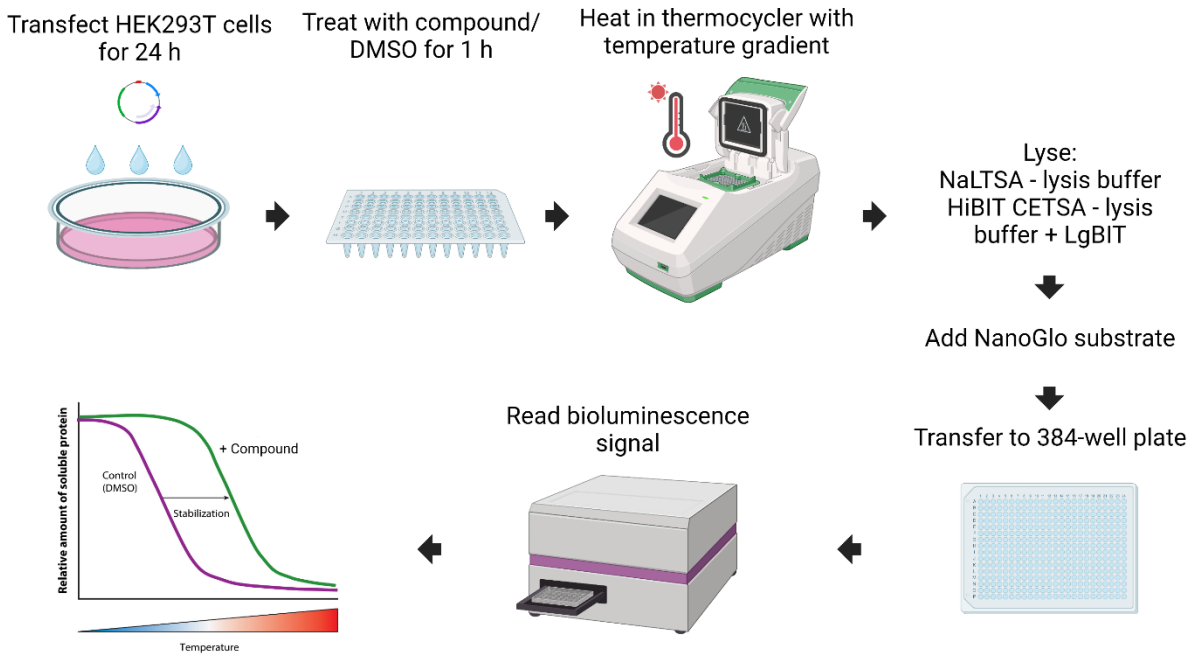
Microplate reader with luminescence detection
PCR thermocycler with temperature gradient

### 2.1.3 Reagents

- Opti-MEM Reduced Serum Medium (Gibco) no phenol red
- Any 96-well PCR plates
- 96-well breathable film cover
- Nano-Glo® HiBiT Lytic solution (# N3030 Promega) for HiBIT CETSA, alternatively you can make the lytic solution containing: 200 nM LgBIT protein, 2 %NP-40, protease inhibitors in Opti-MEM (no phenol red)
- 96-well plate adhesive cover compatible with your specific PCR plates and thermocycler
- 384-well white microplates
- NanoBRET™ Nano-Glo® substrate (# N1661, Promega). Note that Nano Glo® HiBiT Lytic solution contains the substrate.

## 3. Protocol

### 3.1 Workflow CETSA SGC-Toronto




### 3.2 Protocol

- Culture HEK293T cells in a 10 cm tissue culture plate in DMEM, supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, up to 90% density. Remove the media from the plate and wash it with PBS. Trypsinize and resuspend cells in culture media and mix gently to generate a single-cell suspension. Resuspend cells to a final density of $4 \times 10^5$ cells/mL in the cell culture medium and add 2 mL per/well of a 6-well plate.

- Prepare the transfection mixture according to the manufacturer's instructions (you can use any transfection reagent). Resuspend 0.2 µg target DNA and 1.8 µg of empty vector (e.g. pcDNA3.1) in 200 µL Opti-MEM™ I Reduced Serum Medium, vortex briefly, add 4 µL of X-tremeGENE™ HP DNA Transfection Reagent (Roche) straight into solution, vortex for 10 s and incubate for 15 min at RT. Add 200 µL of transfection mix per 6-well, mix gently by shaking the plate, and keep at 37 °C, 5% $CO_2$ overnight.
- The following day trypsinize cells and resuspend in DMEM (10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin). Pellet cells at 300 × g for 2 min, remove media, and resuspend in Opti-MEM™ I Reduced Serum Medium (no phenol red) to a final density of 2 × 105 cells/mL. Divide cells into separate tubes and add DMSO or compound at the required concentration. Transfer cell suspension (50 µL/well) into a 96-well PCR plate, cover with breathable film and incubate for 1 h at 37 °C and 5% $CO_2$. Include one well per intended temperature, compound, and concentration to be tested in the CETSA including control vehicle DMSO/solvent and control temperature 37 °C.
- Replace the breathable film on the PCR plate with a compatible PCR film. Heat samples as follows: 1 min at (22 °C) and 3 min at desired temperature gradient, followed by a cooling step to chill samples to RT (22 °C). Once samples reached RT, put an ice block on top of the plate for 30 s, while still in the thermocycler, to cool down the top of the plate.
- Add lytic buffer (50 µL/well) and incubate for 10 min at RT:
  NaLTSA: 2% NP-40, protease inhibitors, Opti-MEM™ I Reduced Serum Medium, (no phenol red)
  HiBIT CETSA: 2% NP-40, 200 nM LgBIT, protease inhibitors, Opti-MEM™ I Reduced Serum Medium, (no phenol red)
  Note: You can use Nano-Glo® HiBiT Lytic solution, according to the manufacturer's instructions.
- Add 25 µL/well of 100-fold NanoBRET™ Nano-Glo® substrate solution in Opti-MEM™ I Reduced Serum Medium, (no phenol red). Mix gently and transfer 20 µL/well to 384-well white plates in technical quadruplicates using a multichannel pipette and read the bioluminescence signal.
- To generate apparent $T_{agg}$ curves, the data are first converted to percent stabilized by relating the luminescence at temperature X to the luminescence at 37 °C.
- Data are then fitted to obtain apparent $T_{agg}$ values using the nonlinear curve fit.
- $T_{agg}$ shifts ($\Delta T_{agg}$) can be obtained by calculating the difference between $T_{agg}$ DMSO-treated curve and compound-treated curves.