Validation & Assay Performance Summary

CellSensor® HSE-*bla* HeLa Cell Line

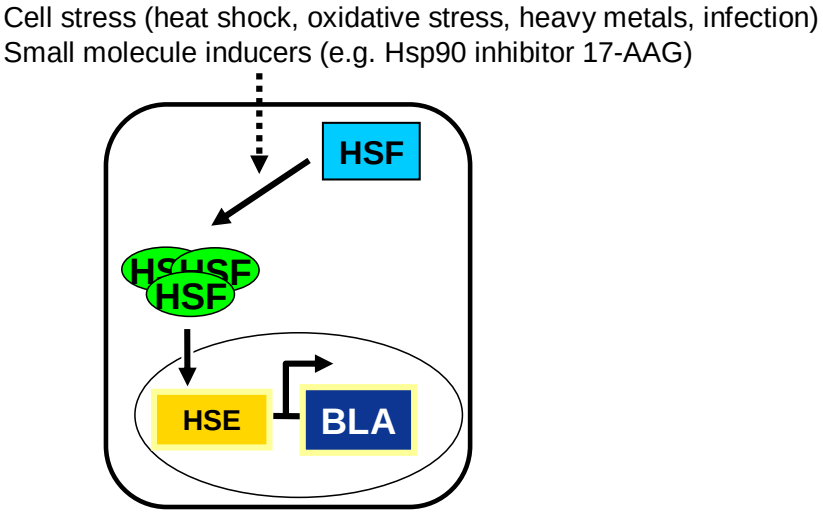
Cat. no. K1813

Pathway Description

Activation of the heat shock response/unfolded protein response (HSR/UPR) occurs in response to a diversity of chemical, environmental, and physiological stress conditions. Transcriptional regulation of the human HSR is mediated by a family of three heat shock transcription factors (HSFs), HSF-1, -2, and -4. Stress-induced activation of quiescent HSF monomers results in their trimerization and accumulation in the nucleus, wherein they bind to and upregulate transcription of target genes (e.g. molecular chaperones, certain proteases, and other stress response genes) harboring a heat shock element (HSE). Downstream expression of heat shock protein family members (e.g. Hsp90 and Hsp70) that function as molecular chaperones to guide conformational states of client proteins is essential to maintaining the health of cells and protecting them from various acute and chronic stress conditions. As a result, HSR activation may provide therapeutic benefit to certain types of tissue trauma (e.g. brain and heart ischemia) and neurodegenerative disorders (e.g. Huntington disease, Alzheimer disease, and Parkinson disease). Conversely, since aberrant expression of chaperones has been associated with tumorigenesis, compounds that down-regulate the HSR and chaperone levels could provide useful tools for combating cancer.

Cell Line Description

To generate an effective readout for interrogating the HSR pathway, we engineered HeLa cervical cancer cells with an HSE driving beta-lactamase reporter gene expression (HSE-bla). A stably integrated pool of heat shock responsive cells was isolated by FACS and further evaluated using an inhibitor of Hsp90, 17-AAG. Hsp90 inhibition is known to upregulate HSF-1 activity leading to potent induction of the HSR, which can be readout using HSE-bla HeLa cells.



Validation Summary

Testing and validation of this assay was evaluated in 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **Primary agonist dose response under optimized conditions (n=3)**

Average 17-AAG EC_{50}	= 50 nM
Average Z'-Factor (EC_{100})	= 0.70
Average Response Ratio	= 10.1
Recommended cell no.	= 8,000 cells/well
Recommended [DMSO]	= up to 0.5 %
Stimulation Time	= 5 hours
Max. [Stimulation]	= 600 nM 17-AAG

2. **Activator panel**

<u>5 hour stimulation</u>	
EC_{50} 17-AAG	= 34 nM
EC_{50} Bortezomib/Velcade	= 3 μM
EC_{50} Celastrol	≤ 1.3 μM
<u>Overnight stimulation</u>	
EC_{50} 17-AAG	= 64 nM
EC_{50} Bortezomib/Velcade	= 1.1 μM
EC_{50} Celastrol	= 1.9 μM

3. **Inhibitor Dose Response**

   IC_{50} Quercetin = 5.8 μM

4. **42°C Heat Shock Response**

5. **Stealth™ RNAi Testing**

6. **Assay-ready Cryopreserved Cells Testing**

7. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

8. **Assay performance with variable cell number**

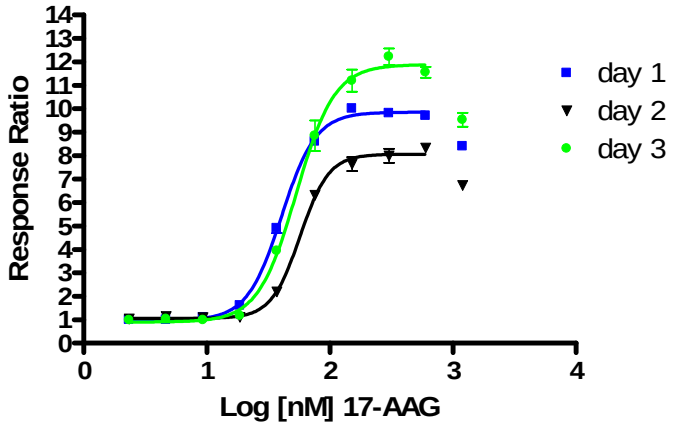
9. **Assay performance with variable DMSO concentration**

10. **Assay performance with variable substrate loading time**

11. **Assay performance with variable stimulation time**

Primary Agonist Dose Response

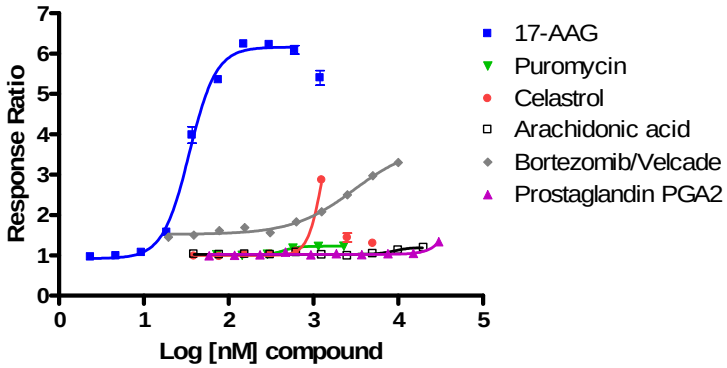
Figure 1 — 17-AAG dose response under optimized conditions



HSE-*bla* HeLa cells were assayed on three separate days in 384-well assay format in Assay Medium at 8,000 cells/well. Following overnight incubation, serial dilutions of the Hsp90 inhibitor 17-AAG were applied to the wells (0.1 % final DMSO) for 5 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1μM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the 17-AAG treated wells from the 460/530 ratios obtained with the untreated control wells (n = 16 for each data point).

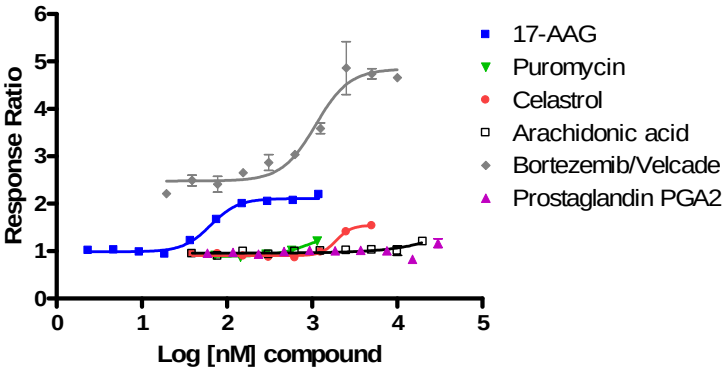
Activator Panel

Figure 2A — Activator Panel Dose Response (5 h stim)



HSE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 8,000 cells/well. Following overnight incubation, 10-point serial dilutions of compounds were applied to the wells (0.1 % final DMSO) for ~5 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the compound treated wells from the 460/530 ratios obtained with the untreated control wells (n = 4 for each data point). Note that high concentrations of Puromycin and Celastrol exhibited significant cytotoxicity and were excluded from the analysis.

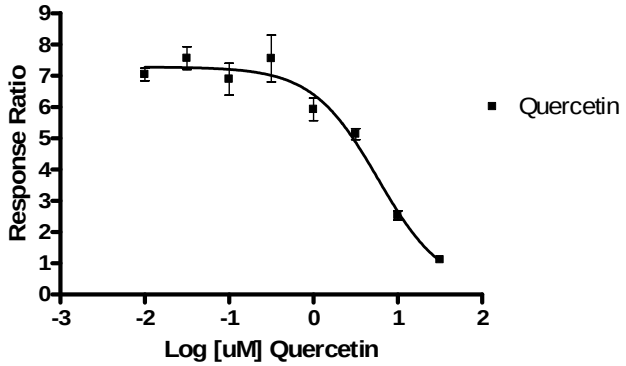
Figure 2B — Activator Panel Dose Response (16 h stim)



HSE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 8,000 cells/well. Immediately following cell plating, 10-point serial dilutions of compounds were applied to the wells (0.1 % final DMSO) for ~16 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the compound treated wells from the 460/530 ratios obtained with the untreated control wells (n = 2 for each data point). Note that high concentrations of Puromycin and Celastrol exhibited significant cytotoxicity and were excluded from the analysis.

Inhibitor Dose Response

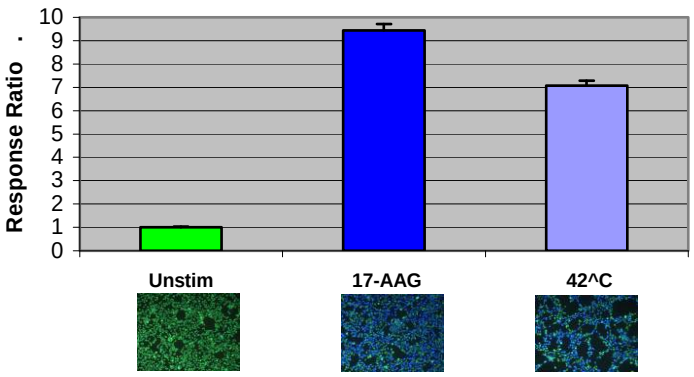
Figure 3 — Inhibitor Dose Response



HSE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 8,000 cells/well. Following overnight incubation, 10-point serial dilutions of inhibitor Quercetin were applied to the wells and incubated for ~1 h prior to stimulating them with 65 nM 17-AAG for ~5 h (0.2 % final DMSO) and then loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the inhibitor treated wells from the 460/530 ratios obtained with the untreated control wells (n = 4 for each data point). The highest concentrations of Quercetin exhibited significant cytotoxicity and were excluded from the analysis.

42°C Heat Shock Response

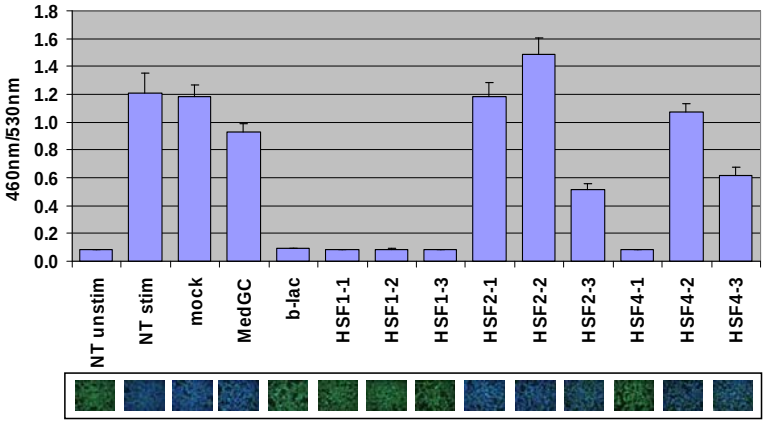
Figure 4 — 42°C Heat Shock Response vs. 17-AAG



HSE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 10,000 cells/well on two different plates. Following overnight incubation at 37°C, Assay Medium containing DMSO (to simulate compound addition with final 0.1% DMSO) was applied to first plate, which was then subjected to heat shock at 42°C for 1 hour followed by a 5 hour incubation at 37°C before loading with substrate. To the second plate, Assay Medium containing DMSO was applied to the unstimulated control wells while 450 nM final 17-AAG was applied to the stimulated wells (0.1 % final DMSO) followed by a 6 hour incubation at 37°C prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the heat shock treated wells or the compound treated wells from the 460/530 ratios obtained with the unstimulated control wells (n = 4 for each data point). Below are shown representative fluorescent images.

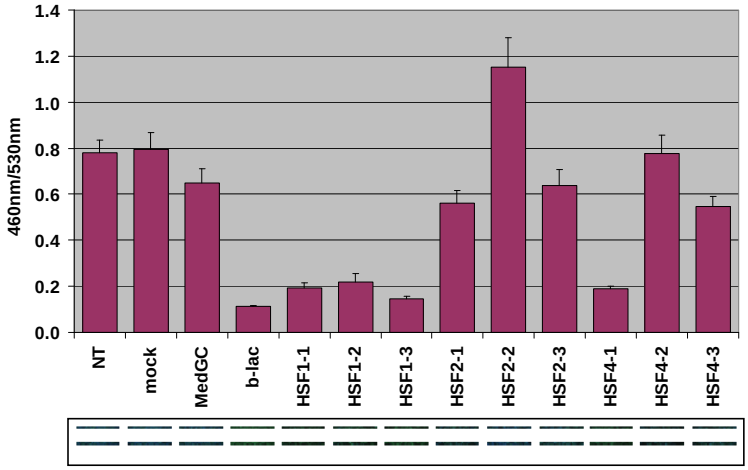
Stealth™ RNAi Testing

Figure 5A — RNAi panel, 17-AAG stimulated



HSE-*bla* HeLa cells were plated in 384-well format in Growth Medium at 2,000 cells/well and reverse transfected using using Lipofectamine™ RNAiMAX Transfection Reagent and 20 nM of Stealth™ RNAi duplexes against HSF1, HSF2, and HSF4. Controls were as follows: nontransfected and unstimulated cells (NT unstim), nontransfected and stimulated cells (NT stim), mock transfected (no RNAi duplex), Stealth™ RNAi Negative Control Med GC, and Beta-lactamase positive control RNAi duplex. At ~40 hours post-transfection, a 400 nM final concentration of 17-AAG was applied to the wells and the plate was incubated for an additional 6 hours prior to loading with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted (n = 4 for each data point). Below are shown representative fluorescent images.

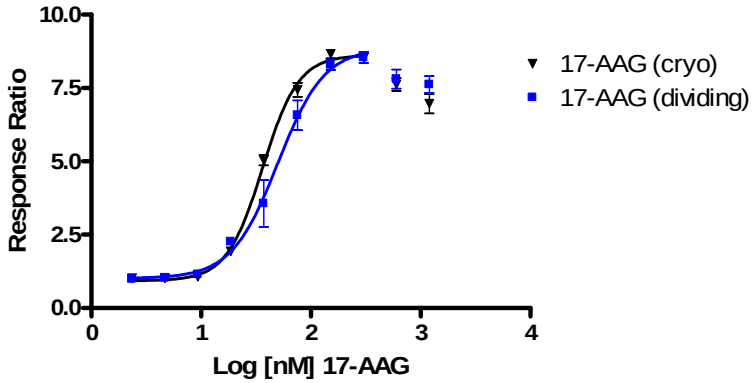
Figure 5B — RNAi panel, heat shock stimulated



HSE-*bla* HeLa cells were plated in 384-well format in Growth Medium at 2,000 cells/well and reverse transfected using using Lipofectamine™ RNAiMAX Transfection Reagent and 20 nM of Stealth™ RNAi duplexes against HSF1, HSF2, and HSF4. Controls were as follows: nontransfected cells (NT), mock transfected (no RNAi duplex), Stealth™ RNAi Negative Control Med GC, and Beta-lactamase positive control RNAi duplex. At ~40 hours post-transfection, the plate was subjected to heat shock at 42°C for 1 hour followed by a 6 hour incubation at 37°C prior to loading with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted (n = 4 for each data point). Below are shown representative fluorescent images.

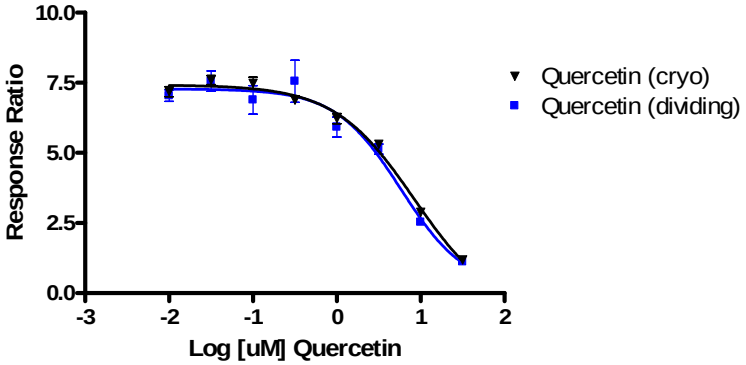
Assay-ready Cryopreserved Cells Testing

Figure 6A — 17-AAG activator dose response curve



Cryopreserved HSE-*bla* HeLa cells were thawed and plated in 384-well assay format in Assay Medium at 8,000 cells/well in comparison to an actively dividing culture. Following overnight incubation, serial dilutions of 17-AAG were applied to the wells (0.1 % final DMSO) for ~5 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the 17-AAG treated wells from the 460/530 ratios obtained with the untreated control wells (n = 4 for each data point).

Figure 6B — Quercetin inhibitor dose response curve



Cryopreserved HSE-*bla* HeLa cells were thawed and plated in 384-well assay format in Assay Medium at 8,000 cells/well in comparison to an actively dividing culture. Following overnight incubation, 10-point serial dilutions of Quercetin were applied to the wells and incubated for ~1 h prior to stimulating them with 65 nM 17-AAG for ~5 h (0.2 % final DMSO) and then loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the inhibitor treated wells from the 460/530 ratios obtained with the untreated control wells (n = 4 for each data point). The highest concentrations of Quercetin exhibited significant cytotoxicity and were excluded from the analysis.

Cell Culture and Maintenance

Thaw cells in Growth Medium without selection (Blasticidin) and culture them in Growth Medium with selection. Pass or feed cells 2-3 times a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 20% and 90% confluence.

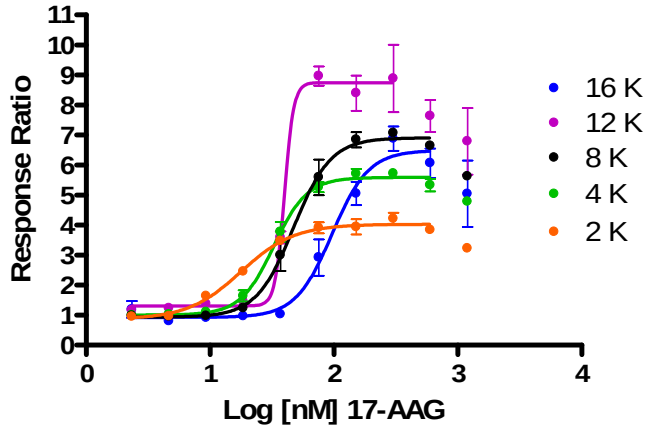
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium (–)	Growth Medium (+)	Assay Medium	Freeze Medium
DMEM with GlutaMAX™	500 mL	500 mL	500 mL	—
Dialyzed FBS (dFBS) **Do not substitute!**	50 mL	50 mL	0.5 mL	—
HEPES (1 M)	12.5 mL	12.5 mL	12.5 mL	
NEAA (100x)	5 mL	5 mL	5 mL	—
Pen/Strep (100x)	5 mL	5 mL	5 mL	—
Blasticidin	—	5 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

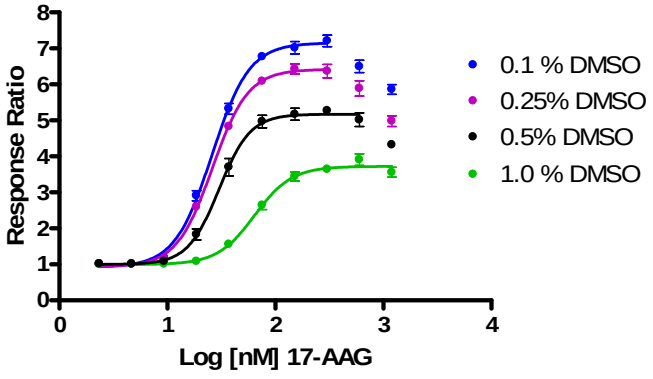
Figure 7 — 17-AAG dose response with varying cell plating density



HSE-*bla* HeLa cells were plated onto a 384-well assay plate in Assay Medium at varying cell densities. Following overnight incubation, serial dilutions of the Hsp90 inhibitor 17-AAG were applied to the wells (0.1 % final DMSO) for 5 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the 17-AAG treated wells from the 460/530 ratios obtained with the untreated control wells (n = 6 for each data point).

Assay Performance with variable DMSO concentration

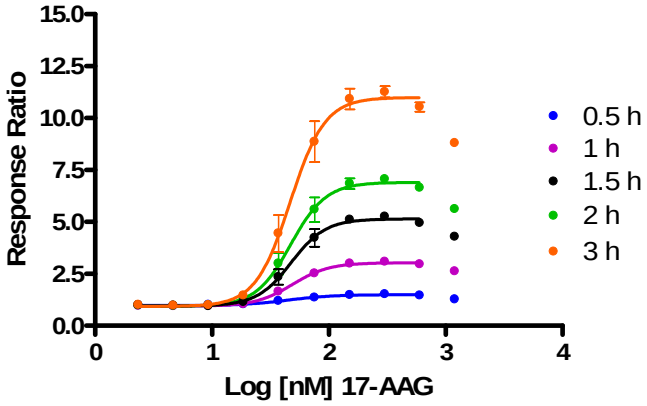
Figure 8 – 17-AAG dose response with 0.1, 0.25, 0.5 and 1% DMSO.



HSE-*bla* HeLa cells were plated onto a 384-well assay plate in Assay Medium at 8,000 cells/well. Following overnight incubation, serial dilutions of the Hsp90 inhibitor 17-AAG were applied to the wells in the presence of varying final DMSO concentrations for 5 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the 17-AAG treated wells from the 460/530 ratios obtained with the untreated control wells (n = 6 for each data point).

Assay performance with Variable Substrate Loading Time

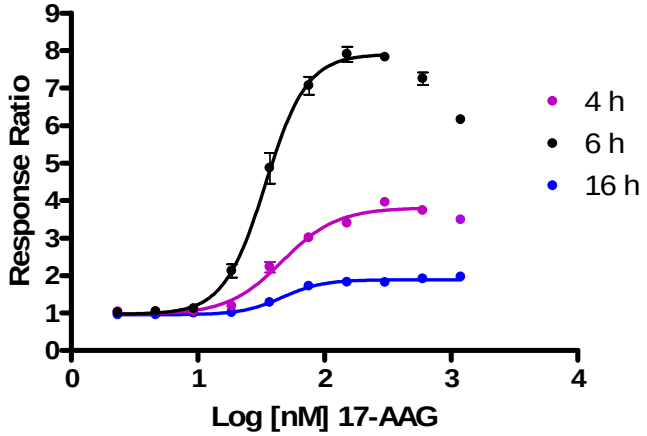
Figure 9 – 17-AAG dose response with increasing loading times



HSE-*bla* HeLa cells were plated onto a 384-well assay plate in Assay Medium at 8,000 cells/well. Following overnight incubation, serial dilutions of the Hsp90 inhibitor 17-AAG were applied to the wells (0.1 % final DMSO) for 5 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for and reading the plate at varying time points. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the 17-AAG treated wells from the 460/530 ratios obtained with the untreated control wells (n = 6 for each data point).

Assay performance with Variable Stimulation Time

Figure 10 – 17-AAG dose response with varying stimulation times



HSE-*bla* HeLa cells were plated onto a 384-well assay plate in Assay Medium at 8,000 cells/well. Serial dilutions of the Hsp90 inhibitor 17-AAG were applied to the wells immediately (for 16 h) or for 4 h or 6 h following O/N incubation in Assay Medium. Wells were loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were calculated by dividing the 460/530 ratios of the 17-AAG treated wells from the 460/530 ratios obtained with the untreated control wells (n = 6 for each data point).

References

1. Westerheide SD and Morimoto RI. (2005) **Heat shock response modulators as therapeutic tools for diseases of protein conformation**. *Journal of Biological Chemistry* 280:33097-33100.

2. Davenport EL, Morgan GJ, and Davies FE. (2008) **Untangling the unfolded protein response**. *Cell Cycle* 7:865-869.