CellSensor® ESRE-*bla* HeLa Cell Line

Cat. no. K1817

CellSensor® Cell-Based Assay Validation Packet

This cell-based assay has been thoroughly tested and validated by Invitrogen and is suitable for immediate use in a screening application. The following information illustrates the high level of assay testing completed and the validation of assay performance under optimized conditions.

Pathway Description

Endoplasmic Reticulum (ER) stress is associated with a variety of pathophysiological conditions, such as neurodegenerative diseases, diabetes, tumor growth under hypoxic conditions, and ischemic heart disease. Proteins in the ER misfold or unfold, and accumulate under stress conditions, which promote the expression of ER stress responsive genes. One of the mechanisms for mediating ER stress response is the activation of transcription factor ATF6. The quiescent form of ATF6 (p90ATF6), a type II-transmembrane protein, is embedded in the ER membrane and proteolyzed in an ER stress-dependent manner. The liberated N-terminal fragment (p50ATF6) translocates to the nucleus, binding to ER stress response element (ERSE) present in the proximal promoter regions of many ER stress-responsive proteins including ER chaperones.

Cell Line Description

To better understand the pathological processes and provide novel avenues to potential therapies, ESRE–*bla* HeLa cells are engineered to express beta-lactamase under the control of ER stress response element. This is a clonal population isolated by FACS and its dose response curves with tunicamycin and thapsigargin are performed. This cell line also response to other known ER stress inducers.

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 tunicamycin EC_{50} = 189 nM
   Average Z'-Factor (EC_{100}) = 0.73
   Average Response Ratio = 4.8

   Recommended cell no. = 5,000 cells/well
   Recommended [DMSO] = up to 0.5 %
   Stimulation Time = 5 hours
   Max. [Stimulation] = 1000 nM
   tunicamycin

2. **Ligand panel**

   See Fig. 2 and Fig. 3

3. **Inhibitor panel**

   See Fig. 4

4. **Stealth™ RNAi Testing**

   See Fig. 5

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

Assay Testing Summary

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

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

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

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

10. **Assay performance with cryo-preserved cells**

Primary Agonist Dose Response

**Figure 1 — Tunicamycin dose response under optimized conditions**
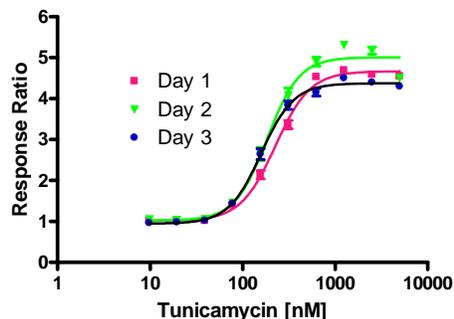
	Day 1	Day 2	Day 3
BOTTOM	1.016	1.023	0.9453
TOP	4.668	5.009	4.375
LOGEC50	2.355	2.265	2.201
HILLSLOPE	2.310	2.499	2.466
EC50	226.2	184.0	158.8

ESRE-*bla* HeLa cells were assayed on three separate days in 384-well assay format in Assay Medium at 5,000 cells/well. Following overnight incubation, serial dilutions of tunicamycin (EMD Biosciences, 654380) 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 2.5 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 tunicamycin treated wells from the 460/530 ratios obtained with the untreated control wells (n = 16 for each data point).

Alternative Agonists

Figure 2 — Thapsigargin dose response under the optimized condition
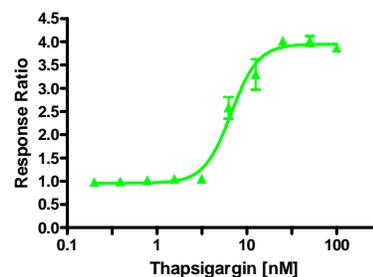
	Thapsigargin
BOTTOM	0.9592
TOP	3.947
LOGEC50	0.8237
HILLSLOPE	2.886
EC50	6.663

ESRE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 5,000 cells/well. Following overnight incubation, serial dilutions of Thapsigargin (Sigma, T9033) 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 2.5 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 thapsigargin treated wells from the 460/530 ratios obtained with the untreated control wells (n = 8 for each data point).

Figure 3 — Known ER stress inducing agent panel
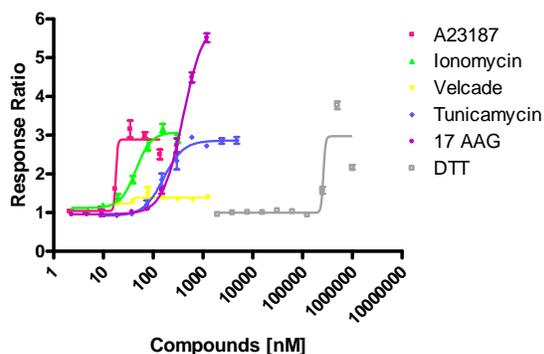
	A23187	Ionomycin	Velcade	Tunicamycin	17 AAG	DTT
BOTTOM	1.040	1.120	1.236	0.9154	0.9586	0.9973
TOP	2.886	3.072	1.390	2.861	5.969	2.971
LOGEC50	1.251	1.651	1.602	2.209	2.599	5.417
HILLSLOPE	23.14	2.802	27.90	2.178	2.094	20.38
EC50	17.81	44.81	40.02	162.0	397.6	260929

ESRE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 5,000 cells/well. Following overnight incubation, serial dilutions of indicated agents 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 2.5 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 treated wells from the 460/530 ratios obtained with the untreated control wells (n = 8 for each data point).

Inhibitor Panel

### Figure 4 – ESRE- *bla* HeLa dose response to salubrinal under optimized conditions
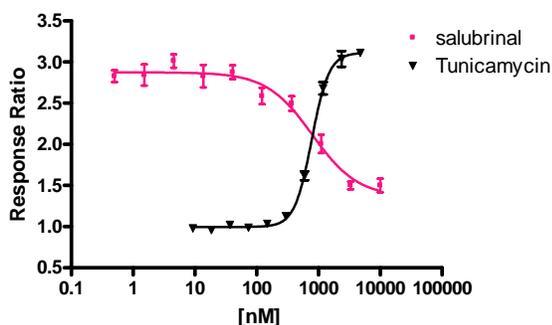
	salubrinal	Tunicamycin
BOTTOM	1.359	0.9962
TOP	2.875	3.113
LOGEC50	2.901	2.892
HILLSLOPE	-1.171	3.122
EC50	795.3	779.4

ESRE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 5,000 cells/well. Following overnight incubation, serial dilutions of salubrinal were applied to the wells (0.1 % final DMSO) for 30 minutes prior to the treatment with tunicamycin for 5 hours and loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2.5 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 treated wells from the 460/530 ratios obtained with the untreated control wells (n = 8 for each data point).

Stealth™ RNAi Testing

Figure 5 — ESRE-*bla* HeLa response to various RNAi oligos
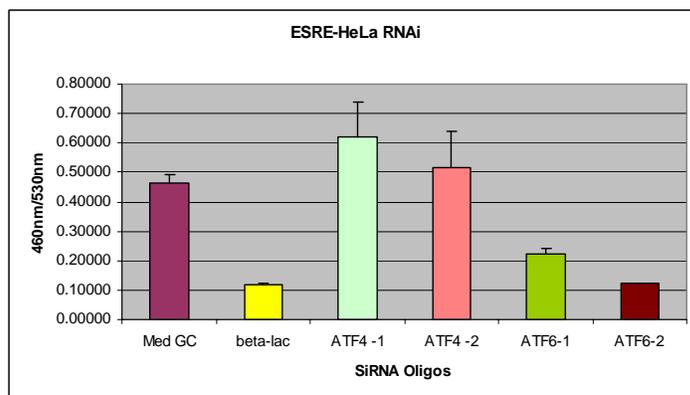
ESRE-*bla* HeLa cells (6,000 cells/well) were plated with growth medium in a 96-well format and incubated at 37°C overnight. Cells were then treated with RNAiMax mixtures containing the listed Stealth™ RNAi oligos (ATF4, Invitrogen # HSS141298; ATF6, Invitrogen # HSS117913) for 32 hrs. Following an Assay Media exchange and a 37°C incubation for 16 hours, cells were then stimulated with Tunicamycin (2µg/mL) for 5 hours, and then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 Ratio was plotted for each RNAi Oligos.

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 10% 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	—	—
OPTI-MEM	—	—	500 mL	—
Dialyzed FBS (dFBS) **Do not substitute!**	50 mL	50 mL	2.5 mL	—
HEPES (1 M)	12.5 mL	12.5 mL	—	—
NEAA (100x)	5 mL	5 mL	5 mL	—
Pen/Strep (100x)	5 mL	5 mL	5 mL	—
Na Pyruvate (100x)	—	—	5 mL	—
Blasticidin	—	5 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

**Figure 5 — Tunicamycin dose response with varying cell plating density**
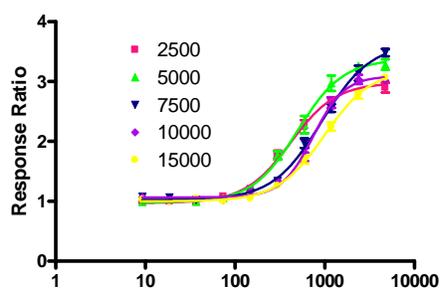
	2500	5000	7500	10000	15000
BOTTOM	0.9715	0.9832	1.022	1.065	0.9997
TOP	2.984	3.373	3.643	3.093	3.212
LOGEC50	2.618	2.698	2.967	2.869	2.999
HILLSLOPE	1.705	1.722	1.587	2.474	1.650
EC50	414.9	498.4	925.9	739.5	997.2

ESRE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at indicated number of cells/well. Following overnight incubation, serial dilutions of tunicamycin 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 2.5 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 treated wells from the 460/530 ratios obtained with the untreated control wells (n = 8 for each data point).

Assay Performance with variable DMSO concentration

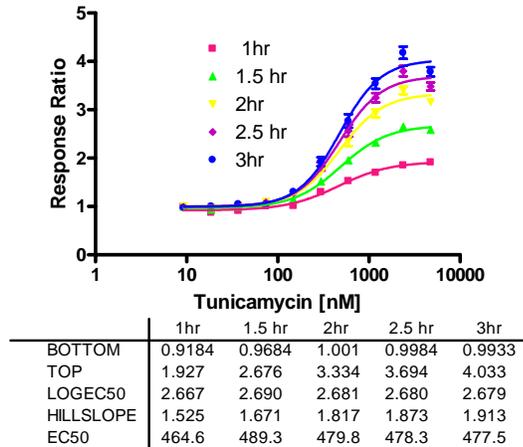**Figure 6 – Tunicamycin dose response with 0.1, 0.25, 0.5 and 1% DMSO.**
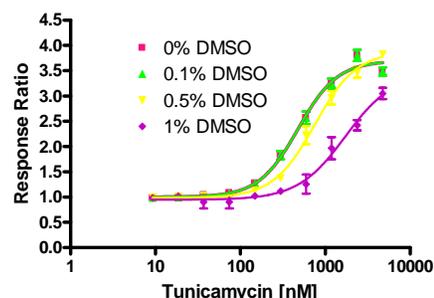
	0% DMSO	0.1% DMSO	0.5% DMSO	1% DMSO
BOTTOM	0.9984	0.9984	0.9812	0.9448
TOP	3.694	3.694	3.879	3.451
LOGEC50	2.680	2.680	2.877	3.237
HILLSLOPE	1.873	1.873	1.762	1.548
EC50	478.3	478.3	752.6	1726

ESRE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 5000 cells/well. Following overnight incubation, serial dilutions of tunicamycin were applied to the wells in the presence of indicated amount of final DMSO for 5 h prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2.5 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 treated wells from the 460/530 ratios obtained with the untreated control wells (n = 8 for each data point).

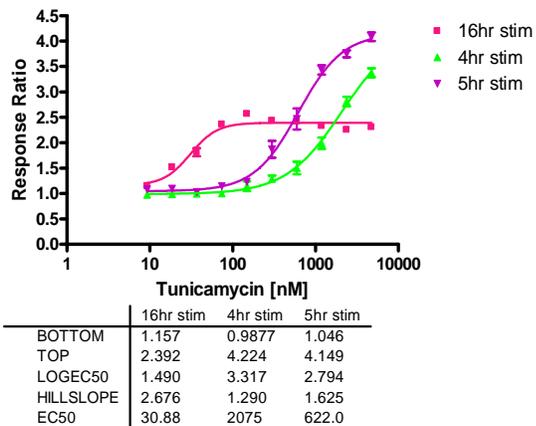## Assay performance with Variable Substrate Loading Time

Figure 7 – Tunicamycin dose response with increasing substrate loading times

	1hr	1.5 hr	2hr	2.5 hr	3hr
BOTTOM	0.9184	0.9684	1.001	0.9984	0.9933
TOP	1.927	2.676	3.334	3.694	4.033
LOGEC50	2.667	2.690	2.681	2.680	2.679
HILLSLOPE	1.525	1.671	1.817	1.873	1.913
EC50	464.6	489.3	479.8	478.3	477.5

ESRE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 5000 cells/well. Following overnight incubation, serial dilutions of tunicamycin 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 indicated amount of time. 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 treated wells from the 460/530 ratios obtained with the untreated control wells (n = 8 for each data point).

## Assay performance with Variable Stimulation Time

Figure 8 – Tunicamycin dose response with varying stimulation times

	16hr stim	4hr stim	5hr stim
BOTTOM	1.157	0.9877	1.046
TOP	2.392	4.224	4.149
LOGEC50	1.490	3.317	2.794
HILLSLOPE	2.676	1.290	1.625
EC50	30.88	2075	622.0

ESRE-*bla* HeLa cells were assayed in 384-well assay format in Assay Medium at 5000 cells/well. Following overnight incubation, serial dilutions of tunicamycin were applied to the wells (0.1 % final DMSO) for 4, 5 and 16 hrs prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1μM final concentration of CCF4-AM) for 2.5 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 treated wells from the 460/530 ratios obtained with the untreated control wells (n = 8 for each data point).

Assay performance with cryo-preserved cells

Figure 10 – Cryo-preserved ESRE-*bla* HeLa dose response to Tunicamycin

	experiment 1
BOTTOM	1.032
TOP	3.164
LOGEC50	2.406
HILLSLOPE	3.398
EC50	254.5
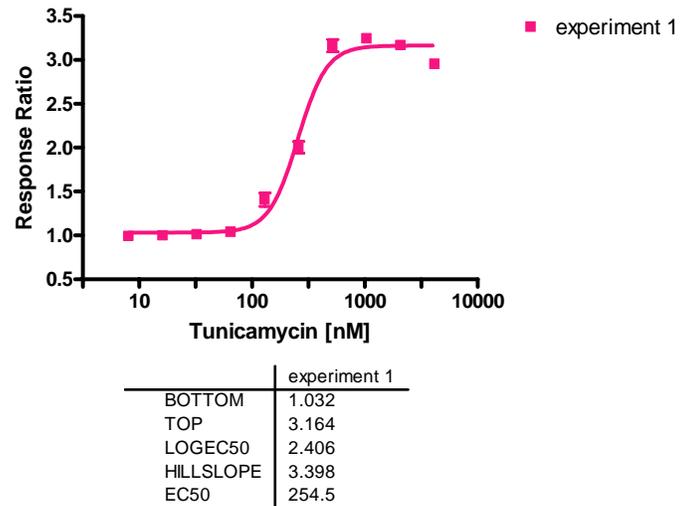
Cryo-preserved ESRE-*bla* HeLa cells (passage# 15) were thawed, resuspended with assay medium and plated (5,000 cells/well) the day before the assay in a 384-well format. Next morning, cells were stimulated with Tunicamycin (EMD Biosciences # 654380) over the indicated concentration range in the presence of 0.1% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2.5 hours. Fluorescence 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 treated wells from the 460/530 ratios obtained with the untreated control wells (n = 8 for each data point).