

Validation & Assay Performance Summary



CellSensor® ARE-*bla* HepG2 Cell Line

Cat. no. K1633

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

Reactive Oxygen Species (ROS) can damage biological macromolecules and are detrimental to cellular health. Electrophilic compounds, xenobiotics and antioxidants are sources of Reactive Oxygen Species, creating oxidative stress that can harm cells. Enzymes involved in the Phase II detoxification of xenobiotics to reduce cellular stress include glutathione transferases, quinone reductase, epoxide hydrolase, heme oxygenase, UDP-glucuronosyl transferases, and gamma-glutamylcysteine synthetase. Expression of these genes protects cells from oxidative damage and can prevent mutagenesis and cancer. Transcription of these genes is coordinately regulated through antioxidant response elements (AREs). Nrf2 (NF-E2-related factor 2) and Nrf1 are transcription factors that bind to AREs and activate these genes. Inactive Nrf2 is retained in the cytosol by association a complex with the cytoskeletal protein Keap1. Cytosolic Nrf2 is phosphorylated and translocates into the nucleus in response to protein kinase C activation and Map kinase pathways. In the nucleus, Nrf2 activate genes through AREs by interacting with transcription factors in the bZIP family, including CREB, ATF4 and fos or jun. Nrf2 activation of genes is opposed by small maf proteins, including MafG and MafK, maintaining a counterbalance to Nrf2 and balancing the oxidation level of the intracellular environment.

Cell Line Description

The CellSensor® ARE-*bla* HepG2 cell line contains a beta-lactamase reporter gene under control of the Antioxidant Response Element (ARE) stably integrated into HepG2 cells. This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time and validated for Z' and EC₅₀ concentrations of t-BHQ. Additional testing information using alternate stimuli are also provided.

Validation Summary

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

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

tBHQ EC ₅₀	= 11μM
Z'-Factor (EC ₁₀₀)	= 0.82
Response Ratio	= 6.8
Optimum cell no.	= 12.5Kcells
Optimum [DMSO]	= 0.5-1%
Optimum Stim. Time	= 16 hours
Max. [Stimulation]	= ~150μM

2. Alternate Stimuli

See Compound Panel Section

3. Stealth™ RNAi Testing

Pending

4. Small molecule inhibitor Testing

Pending

5. Cell culture and maintenance

See Cell Culture and Maintenance Section and Table 1

Assay Performance Under Variable Conditions

6. Assay performance with variable cell number

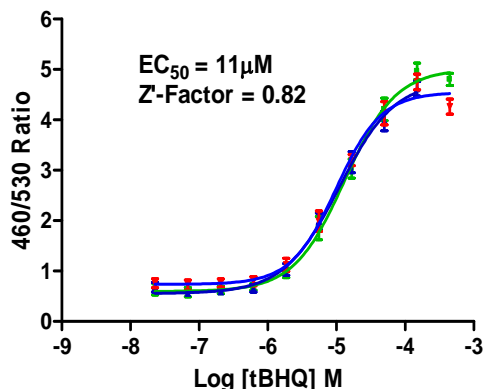
7. Assay performance with variable stimulation time

8. Assay performance with variable substrate loading time

9. Assay performance with variable DMSO concentration

Primary Agonist Dose Response

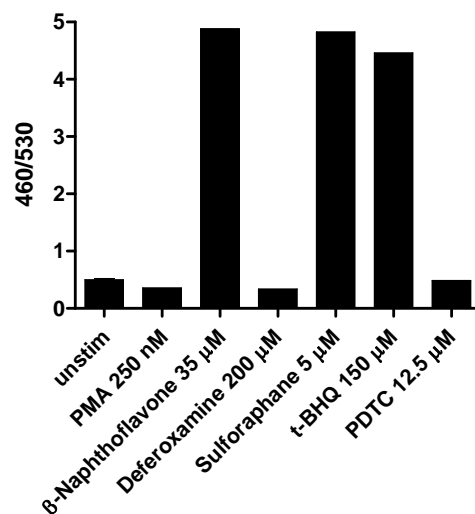
Figure 1 — ARE-*bla* HepG2 dose response to t-BHQ under optimized conditions



ARE-*bla* HepG2 cells (12,500 cells/well) were assayed on three separate days. Cells were plated the day of the assay in a 384-well format and stimulated with t-BHQ (Sigma #11294-1) over the indicated concentration range in the presence of 0.5% DMSO for 15 hours. Cells were 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 Emission Ratios plotted for the indicated concentrations of t-BHQ (n=16 for each data point).

Compound Panel

Figure 2 — ARE-*bla* HepG2 response to various stimuli



ARE-*bla* HepG2 cells (12,500 cells/well) were plated the day of the assay in a 384-well format and treated with PMA, B-Naphthoflavone, DFO, Sulforaphane, t-BHQ (Sigma #11294-1) or PDTC at the indicated concentrations in 0.5% DMSO for 15 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 Ratios plotted for each stimuli (n=3 for each data point).

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blasticidin and culture them in Growth Medium with Blasticidin. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO₂ incubator. Maintain cells between 10% and 90% confluency. Do not allow cells to reach confluence.

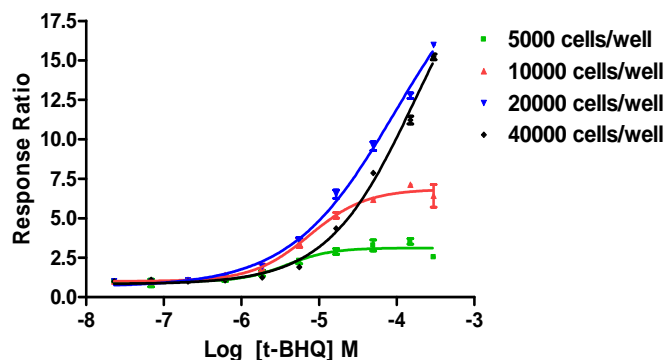
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For optimal cell line performance, use dialyzed FBS (Invitrogen #26400-010). For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	90%	—
Dialyzed FBS Do Not Substitute!	10%	10%	—
NEAA	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	25 mM	—
Penicillin (antibiotic)	100 U/ml	100 U/ml	—
Streptomycin (antibiotic)	100 µg/ml	100 µg/ml	—
Blasticidin (antibiotic)	5 µg/ml	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

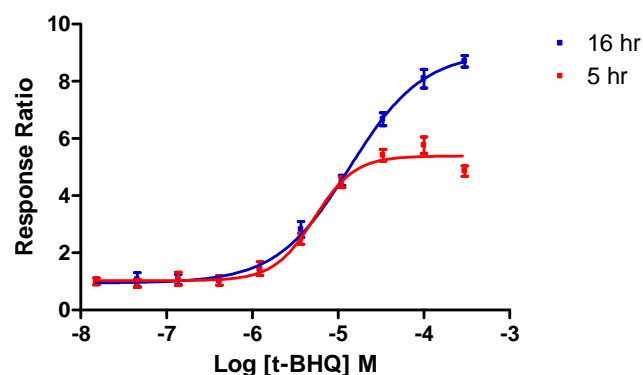
Figure 5 — ARE-*bla* HepG2 dose response to t-BHQ with 5, 10, 20 and 40K cells/well



ARE-*bla* HepG2 cells were plated the day of the assay at 5,000, 10,000, 20,000 or 40,000 cells/well in a 384-well format. Cells were stimulated with t-BHQ (Sigma #11294-1) in the presence of 0.5% DMSO for 15 hours. Cells were then loaded with LiveBLazer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm for the various cell numbers were obtained using a standard fluorescence plate reader and the Response Ratios for each cell number plotted against the indicated concentrations of t-BHQ (n=4 for each data point).

Assay Performance with Variable Stimulation Time

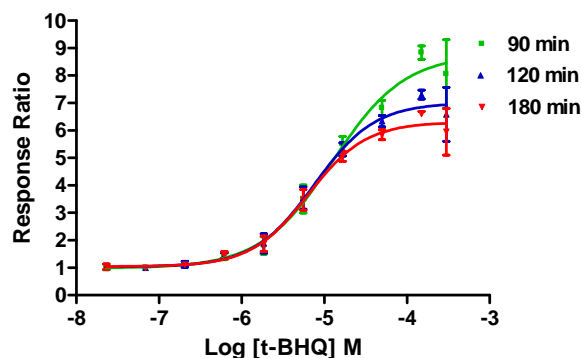
Figure 6 — ARE-*bla* HepG2 dose response to t-BHQ with 5 and 16 hour stimulation times



ARE-*bla* HepG2 cells (10,000 cells/well) were plated the day of the assay in a 384-well assay plate. t-BHQ (Sigma #11294-1) was then added to the plate over the indicated concentration range. Plates were stimulated for 5 or 16 hrs with t-BHQ in 0.5% DMSO and then loaded for 2 hours with LiveBLazer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios for each stimulation time plotted against the indicated concentrations of t-BHQ (n=5 for each data point).

Assay Performance with Variable Substrate Loading Time

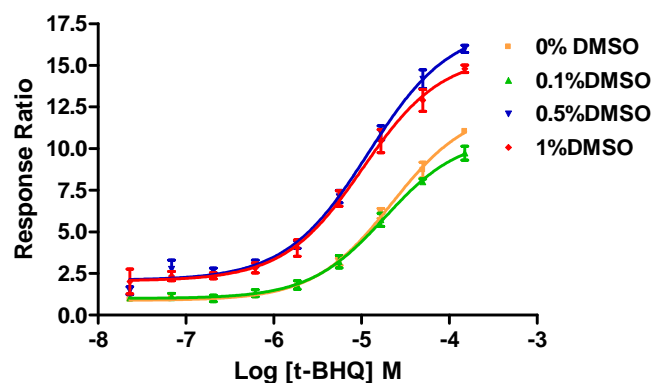
Figure 7 — ARE-*bla* HepG2 dose response to t-BHQ with 1.5, 2 and 3 hour substrate loading times



ARE-*bla* HepG2 cells were plated the day of the assay at 10,000 cells/well in a 384-well format. Cells were stimulated with t-BHQ (Sigma #11294-1) over the indicated concentration range in the presence of 0.5% DMSO for 15 hours. Cells were then loaded with LiveBLazer™-FRET B/G Substrate for either 1.5 or 2 or 3 hours. Fluorescence emission values at 460 nm and 530 nm for the various substrate loading times were obtained using a standard fluorescence plate reader and the Response Ratios for each substrate loading time plotted against the indicated concentrations of t-BHQ (n=4 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 8 — ARE-*bla* HepG2 dose response to t-BHQ with 0, 0.25, 0.5 and 1% DMSO



ARE-*bla* HEPG2 cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. t-BHQ (Sigma #11294-1) was then added to the plate over the indicated concentration range. DMSO was then added to the assay at concentrations from 0% to 1%. Plates were stimulated for 15 hrs and loaded for 2 hours with LiveBLazer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios for each DMSO concentration plotted against the indicated concentrations of t-BHQ (n=4 for each data point).