Validation & Assay Performance Summary

CellSensor® HRE-*bla* HCT-116 Cell Line

Cat. no. K1643

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

Hypoxia is an almost universal hallmark of solid tumors. Adaptation to hypoxia is critical for tumors survival and growth and is mediated largely by transcriptional activation of genes that facilitate short-term (e.g. glucose transport) and long-term (e.g. angiogenesis) adaptive mechanisms. This coordinated homeostatic response is mediated in large part through the activation of the heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1). Under conditions of normal oxygenation, the regulated HIF-1α is hydroxylated and degraded by the proteasome system. As oxygen becomes rate limiting, hydroxylation diminishes and HIF-1α accumulates and heterodimerizes with the constitutively present β-subunit. The binding of this complex to the cognate hypoxia-response element (HRE) results in transcriptional activation of genes containing such elements within promoter or enhancer elements.

Cell Line Description

The CellSensor® HRE-*bla* HCT-116 cell line contains a beta-lactamase reporter gene under control of the Hypoxia Response Element (HRE) stably integrated into HCT-116 cells. HCT-116 is a colon cancer cell line. This cell line has been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and validated for Z' and EC_{50} concentrations of Deferoxamine (DFO) and Cobalt Chloride. Additional information using Stealth™ RNAi testing 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)**

DFO EC_{50}	= 97 µM
Z'-Factor (EC_{100})	= 0.68
Response Ratio	= 18
Recommended cell no.	= 15K cells/well
Recommended [DMSO]	= up to 0.5%
Recommended Stim. Time	= 24 hours
Max. [Stimulation]	= 200µM

2. **Alternate agonist dose response**

Cobalt Chloride EC_{50}	= 36 µM
Z'-Factor (EC_{100})	= 0.69

3. **Stealth™ RNAi Testing**
   See Stealth™ RNAi Testing section

4. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section

Assay Testing Summary

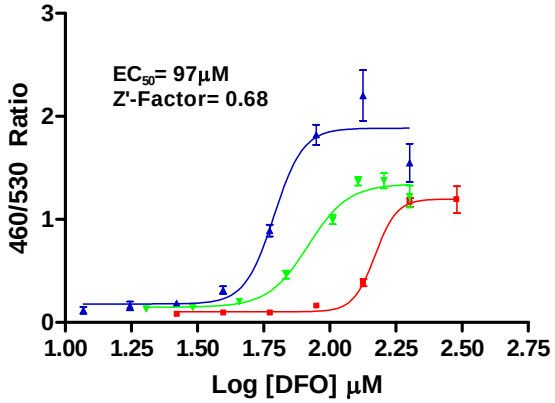
5. **Assay performance with variable cell number**

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

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

Primary Agonist Dose Response

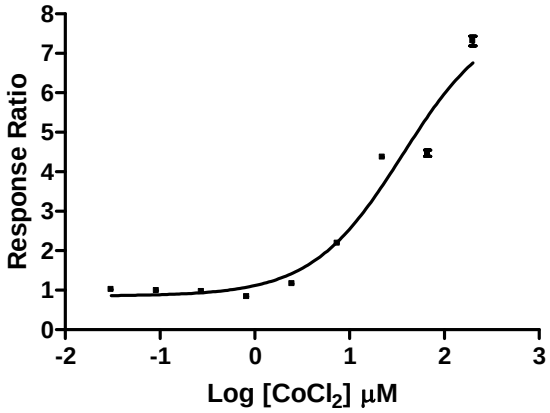
Figure 1 — HRE-*bla* HCT-116 dose response to DFO under optimized conditions



HRE-*bla* HCT-116 cells (15,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with Deferoxamine(Sigma #D-9533) over the indicated concentration range in the presence of 0.5% DMSO for 24 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 against the indicated concentrations of DFO (n=5 for each data point).

Alternate Agonist Dose Response

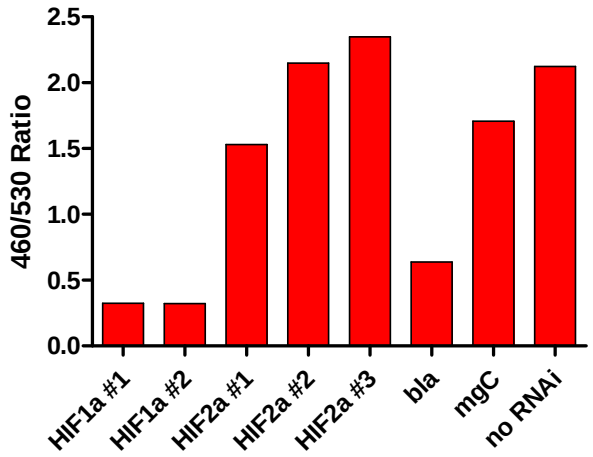
Figure 2 — HRE-*bla* HCT-116 dose response to Cobalt Chloride under optimized conditions



HRE-*bla* HCT-116 cells (15,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with Cobalt Chloride over the indicated concentration range 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. $CoCl_2$ treatment of cells at 600 µM was cytotoxic, as observed using a fluorescence microscope. Emission values for the non-cytotoxic values were obtained at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 Ratios plotted against the indicated concentrations of $CoCl_2$ (n=16 for each data point).

Stealth™ RNAi Testing

Figure 3 — HRE-*bla* HCT-116 response to Stealth™ RNAi oligos for HIF1α, HIF2α, beta-lactamase (*bla*), MGC (control)



HRE-*bla* HCT-116 cells were plated the day of the assay at 7,000 cells per well in a 96-well format. Lipofectamine™ 2000 mixtures containing RNAi oligos HIF1α-1, HIF1α-2 (Invitrogen #s 50812 and 50813), beta-lactamase (*bla*), and Medium GC control were added to the plate and incubated for 48 hours. The media was changed, followed by addition of 100 µM $CoCL_2$ with 0.5% DMSO and cells were incubated at 37ºC & 5% CO_2 for 16 hrs. Cells were 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 460/530 values plotted for each RNAi (n=4 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% CO¬2 incubator. Maintain cells between 10% and 90% confluency. Do not allow cells to reach confluence.

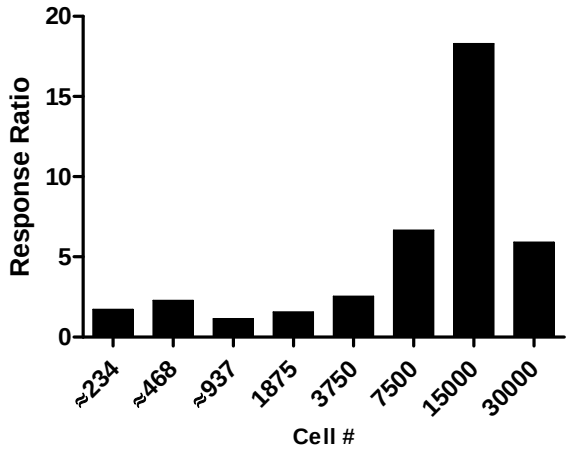
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For optimal cell line performance, use dialyzed FBS (Invitrogen #26400-010). Freeze cells at 2 x 10^6 cells/ml in Freezing Medium.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
McCoy's 5A Medium	90%	—	—
Opti–MEM®	—	96.5%	—
Dialyzed FBS Do Not Substitute!	10%	0.5%	—
NEAA	—	0.1 mM	—
Sodium pyruvate	—	1 mM	—
HEPES (pH 7.3)	—	5 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

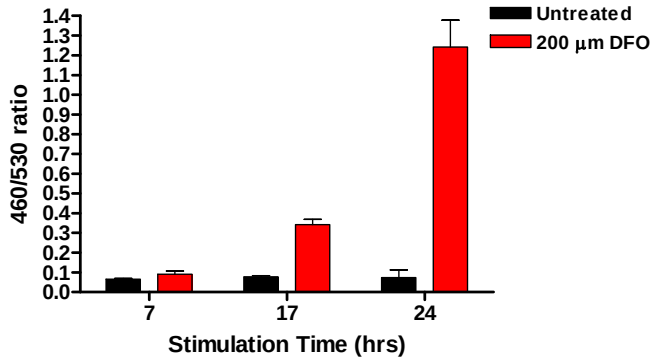
Figure 4 — HRE-*bla* HCT-116 response to DFO with variable cell #s



HRE-*bla* HCT-116 cells were plated the day of the assay at the indicated cell numbers in a 384-well format. Cells were stimulated with 200 µM DFO in the presence of 0.5% DMSO for 24 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 plotted against the indicated concentrations of DFO (n=8 for each data point).

Assay Performance with Variable Stimulation Time

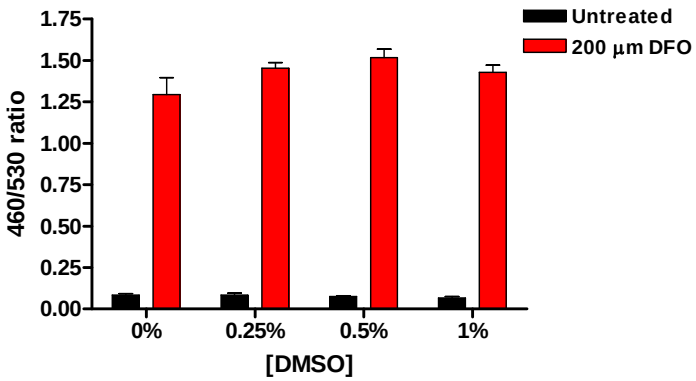
Figure 5 – HRE-*bla* HCT-116 response to DFO with 7, 17 and 24 hour stimulation times



HRE-*bla* HCT-116 cells (15,000 cells/well) were plated the day of the assay in a 384-well assay plate. 200 µM DFO was added to the plate and cells stimulated for 7, 14, or 24 hrs with 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 florescence plate reader and the Emission Ratios plotted for each stimulation time(n=8 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 6 – HRE-*bla* HCT-116 response to DFO with 0, 0.25, 0.5 and 1% DMSO



HRE-*bla* HCT-116 cells (15,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. 200 µM DFO was then added to the plate and DMSO was added to cells at final concentrations ranging from 0% to 1%. Plates were stimulated for 24 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 florescence plate reader and the 460/530 Ratios for each DMSO concentration were plotted (n=8 for each data point).