Validation & Assay Performance Summary

CellSensor® HRE-*bla* ME-180 Cell Line

Cat. no. K1644

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* ME-180 cell line contains a beta-lactamase reporter gene under control of the Hypoxia Response Element (HRE) stably integrated into ME-180 cells. This cell line has been tested for assay performance under variable conditions, including DMSO concentration, stimulation time, and validated for Z' and EC_{50} concentrations of Deferoxamine (DFO) and Cobalt Chloride. Additional information using Hypoxia chamber conditions, Stealth™ RNAi, and small molecule inhibitor 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}	= 119 nM
Z'-Factor (EC_{100})	= 0.80
Response Ratio	= 14
Recommended cell no.	= 8K cells/well
Recommended [DMSO]	= up to 0.5%
Recommended Stim. Time	= 16 hours
Max. [Stimulation]	= 130-200µM

2. **Alternate agonist dose response**

Cobalt Chloride EC_{50}	= 32 µM

3. **Hypoxia Chamber Testing**
   See Hypoxia Chamber Testing section

4. **Small Molecule Inhibitor Testing**
   See Small Molecule Inhibitor Testing section

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

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

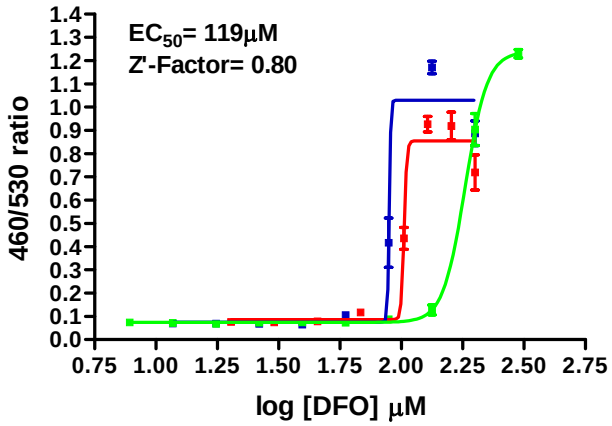
Assay Testing Summary

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

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

Primary Agonist Dose Response

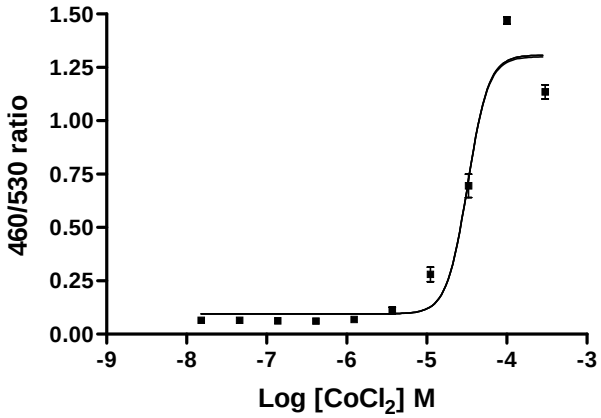
Figure 1 — HRE-*bla* ME-180 dose response to DFO under optimized conditions



HRE-*bla* ME-180 cells (8,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with Deferoxamine over the indicated concentration range in the presence of 0.5% DMSO for 16 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 are shown plotted for the indicated concentrations of DFO (n=16 for each data point).

Alternate Agonist Dose Response

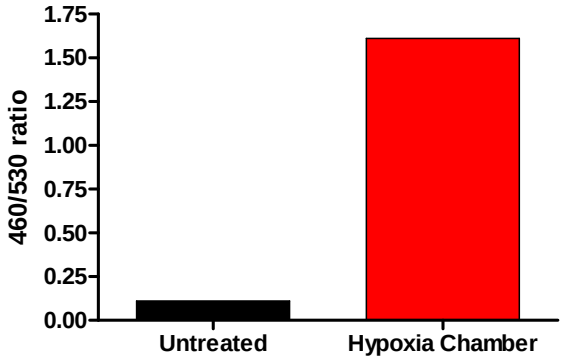
Figure 2 — HRE-*bla* ME-180 dose response to Cobalt Chloride under optimized conditions



HRE-*bla* HCT-116cells (8,000 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. Emission values 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 CoCl2 (n=8 for each data point).

Hypoxia Chamber Testing

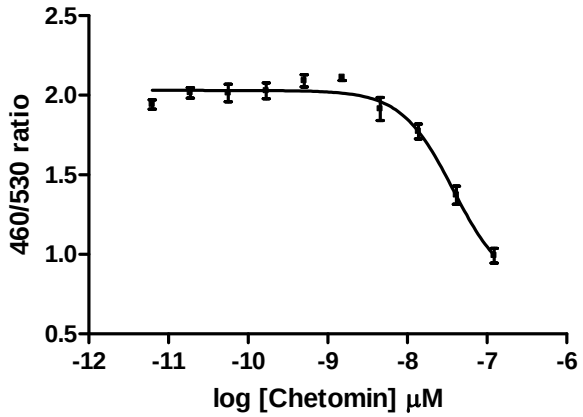
Figure 3 — HRE-*bla* ME-180 response to Hypoxia Chamber conditions



HRE-*bla* ME-180 cells were plated in two 96-well assay plates at 30,000 cells/well. One plate was incubated in 37°C /5%CO_2 incubator and the other plate was incubated in a hypoxia chamber with less than 1% O_2 for 16 hours. Cells were then loaded with LiveBLAzer™ FRET B/G substrate for 2 hours and the 460/530 emission ratios obtained and plotted for each condition.

Small Molecule Inhibitor Testing

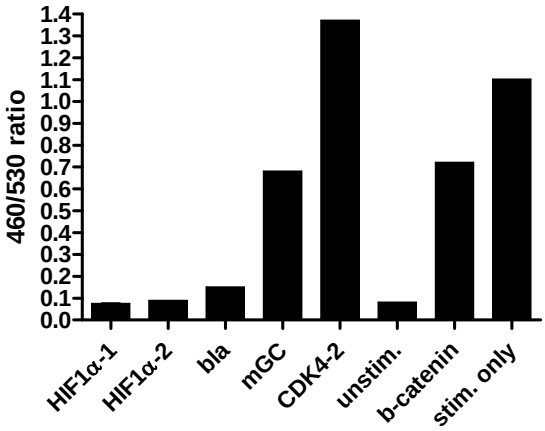
Figure 6 — HRE-bla ME-180 dose response to Chetomin



HRE-*bla* ME-180 cells were plated in a 96-well format and treated with Chetomin over the indicated concentrations followed by treatment with 100 µM $CoCl_2$ for 16 hours. LiveBLAzer™ FRET B/G Substrate was then added to the plate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and plotted against the indicated concentrations of inhibitor (n=4 for each data point).

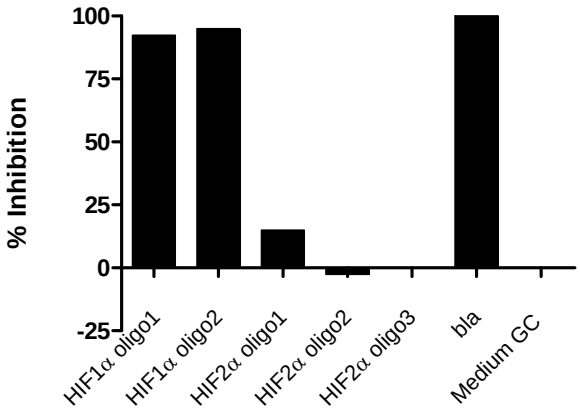
Stealth™ RNAi Testing

Figure 4 — HRE-*bla* ME-180 response to various Stealth™ RNAi oligos.



HRE-*bla* ME-180 cells were plated the day of the assay at 10,000 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*), Medium GC control, CDK4-2, or Beta-catenin were added to the plate and incubated for 60 hours. 100 µM CoCL2 was then added to the plate 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 ratios converted to % inhibition relative (n=4 for each data point).

Figure 5 — HRE-*bla* ME-180 response to Stealth™ RNAi oligos for HIF1α, HIF2α, and beta-lactamase (*bla*).



HRE-*bla* ME-180 cells were plated the day of the assay at 10,000 cells per well in a 96-well format. Lipofectamine™ 2000 mixtures containing RNAi oligos HIF1α-1 & 2, HIF2α 1-3, beta-lactamase (*bla*), or Medium GC control were added to the plate and incubated for 60 hours. 100 µM $CoCL_2$ was then added to the plate 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 Emission Ratios used to calculate % inhibition for each RNAi.

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blasticidin and culture them in Growth Medium with Blasticidin. Passage 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.

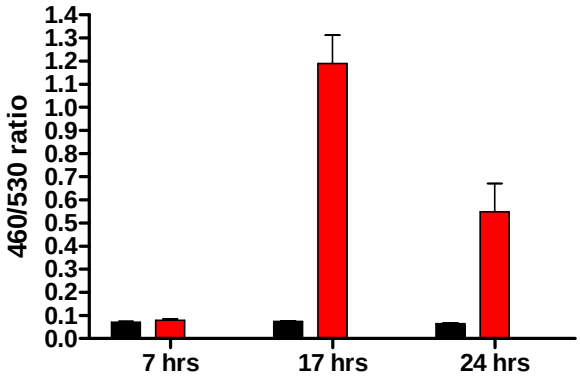
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. Freeze cells at $2x10^6$ cells/ml in Freezing Medium. For optimal cell line performance, use dialyzed FBS (Invitrogen #26400-010).

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM	90%	—	—
Opti–MEM®	—	99.5%	—
Dialyzed FBS **Do Not Substitute!**	10%	0.5%	—
NEAA	0.1 mM	0.1 mM	—
Sodium pyruvate	1 mM	1 mM	—
HEPES (pH 7.3)	25 mM	10 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 Stimulation Time

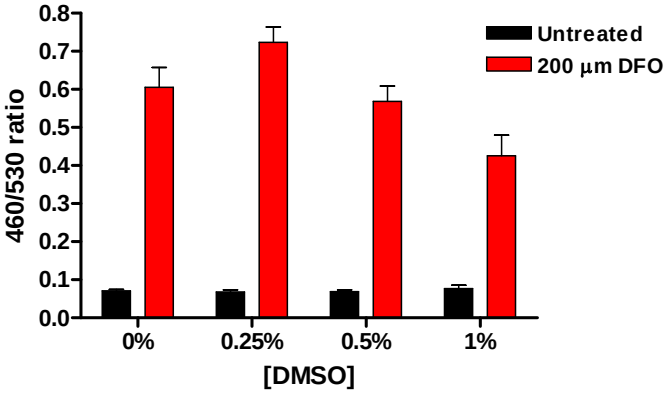
Figure 6 – HRE-*bla* ME-180 response to DFO with 7, 17 and 24 hour stimulation times



HRE-*bla* ME-180 cells (5,000 cells/well) were plated the day of the assay in a 384-well assay plate. 200 µM DFO was then added to the plate and cells were stimulated for 7, 17, or 24 hrs 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 florescence plate reader and the 460/530 Ratios for each stimulation time were plotted(n=8 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 8 – HRE-*bla* ME-180 response to DFO with 0, 0.25, 0.5 and 1% DMSO



HRE-*bla* ME-180 cells (5,000 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 the assay at concentrations 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 for untreated and 200 µM DFO treated samples (n=8 for each data point).