Validation & Assay Performance Summary

CellSensor® MMTV-*bla* HeLa Cell Line

Cat. no. K1641

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

The glucocorticoid receptor belongs to the nuclear receptor superfamily which represents one of the most abundant and important classes of transcriptional regulators. Glucocorticoid receptor (GR) signaling is critically involved in different aspects of metabolic homeostasis especially under conditions of physiological stress. The immunosuppressive activity of glucocorticoids is widely used to treat inflammatory and autoimmune diseases, but might be also of interest for the treatment of certain cancers.

GR shares a common domain structure with other members of the nuclear hormone receptor superfamily. The N-terminal A/B domain contains transactivation activity. It is followed by the highly conserved DNA-binding domain (C-domain) which is connected by a flexible hinge region (D domain), which includes the nuclear localization signal, to the C terminal ligand binding domain (LBD, E domain). The LBD combines a number of different functions including ligand binding, receptor dimerization, transactivation/repression and binding sites for numerous cofactors. In its inactive state GR is bound to a cytosolic protein complex including hsp90 and different inhibitory proteins, which prevent GR from translocation into the nucleus. Upon binding of its cognate ligand, GR undergoes a conformational change which leads to the dissociation of the cytosolic protein complex and the exposure of a nuclear localization signal, thus allowing the GR-ligand complex to translocate into the nucleus, where it mediates positive and negative gene regulatory effects by binding to GR specific DNA response element and association with a variety of cofactors. Transactivation requires the binding of GR to specific palindromic sequences in the regulatory regions of target genes called glucocorticoid response elements (GRE). The mouse mammary tumor virus (MMTV) long terminal repeat used for the development of this CellSensor lines contains several GRE's.

Cell Line Description

The CellSensor® MMTV-*bla* HeLa cell line contains a beta-lactamase reporter gene under the control of the MMTV. HeLa is a cervical cancer cell line which expresses glucocorticoid receptor. This CellSensor® line allows therefore the analysis of endogenous GR signaling. The construct was transduced into HeLa cells by lentivirus. This cell line is a clonal population isolated by flow cytometry after stimulation with Dexamethasone. This cell line is validated for EC_{50} and Z' under optimized conditions using Dexamethasone as ligand for GR stimulation. This cell lines has also been tested for assay performance under variable experimental conditions, including cell plating density, stimulation time, DMSO tolerance and substrate loading time. Additional information using alternate stimuli is 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)**

Z'-Factor (EC_{100})	= 0.66
Response Ratio (max stim.)	= 7.2
EC_{50} Dexamethasone	= 7.8 nM
Recommended cell no. cells/well	= 5000
Recommended [DMSO]	= 0.5-1%
Recommended Stim. Time	= 6 - 18 hrs
Recom. Substrate incubation	= 120 min
Max. [Stimulation]	= 1 μM

2. **Alternate Stimuli**

Hydrocortisone (EC_{50})	= 35.4 nM
Beclomethasone (EC_{50})	= 5.176 nM
Betamethasone (EC_{50})	= 3.654 nM
Triaminolone (EC_{50})	= 19.13 nM

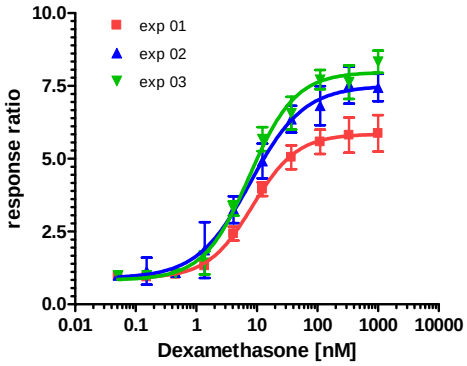
3. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

4. **Assay performance with variable cell number**
5. **Assay performance with variable agonist incubation time**
6. **Assay performance with variable substrate loading time**
7. **Assay performance with variable DMSO concentration**

Primary Agonist Dose Response

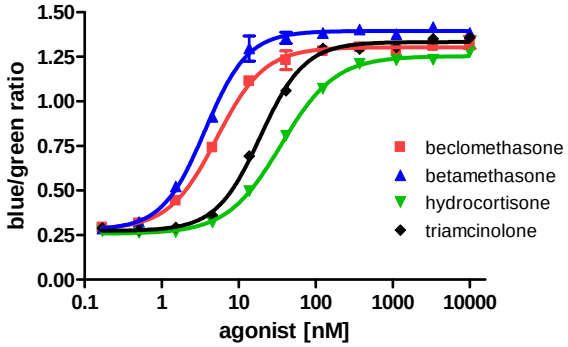
Figure 1 — MMTV-*bla* HeLa response to Dexamethasone treament under optimized conditions



MMTV-*bla* HeLa (5,000 cells/well) were assayed on three separate days represented by the three dose response curves shown on the graph. Cells were plated the day prior to the assay in a 384-well format and treated with the indicated concentration of Dexamethasone (Dex) in the presence of 0.5% DMSO for 18 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. 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 treatment (n=16 for each data point).

Alternate Agonist Dose Response

Figure 2 — MMTV-*bla* HeLa response to Hydrocortisone, Beclomethasone, Betamethasone and Triaminolone treament under optimized conditions



MMTV-*bla* HeLa cells were plated the day prior to the assay at 5000 cells/well in a 384-well format in assay medium. 24 hours later,cells were treated for 18 hrs with the indicated concentration of indicated agonist. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm for the various DMSO concentrations were obtained using a standard fluorescence plate reader and the emission ratios plotted for each DMSO concentration (n=6 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 85% confluency. Do not allow cells to reach confluence.

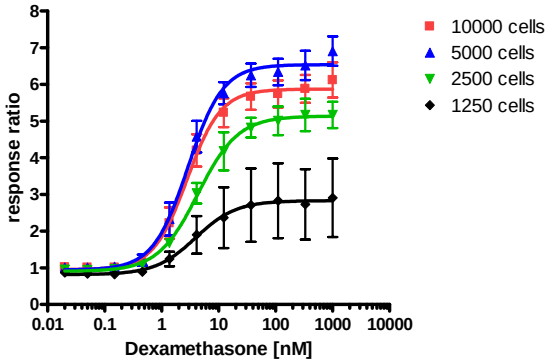
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	-	—
OPTIMEM		99%	
FCS **Do Not Substitute!**	10%	-	—
FCS charcoal/dextran treated		1%	
NEAA	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	25 mM	—
Sodium Pyruvate	1 mM	1 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

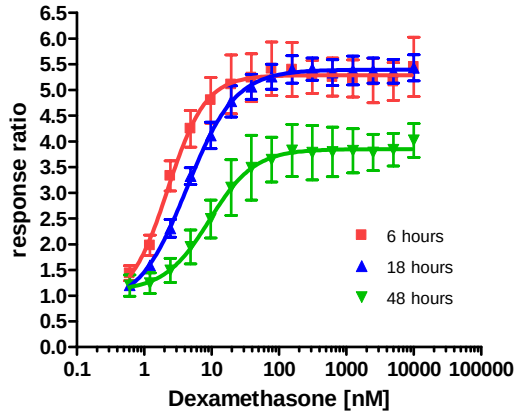
Figure 3 —Dexamethasone dose response with 10K, 5K, 2.5K or 1.25K cells/well



MMTV-*bla* HeLa cells were plated the day prior to the assay at the indicated number of cells/well in a 384-well format in assay medium. 24 hours later cells were treated with the indicated concentration of Dexamethasone for 18 hrs. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 90 minutes. Fluorescence emission values at 460 nm and 530 nm for the various cell numbers were obtained using a standard fluorescence plate reader and the emission ratios plotted for each cell number. (n=6 for each data point).

Assay Performance with Variable Agonist Incubation Times

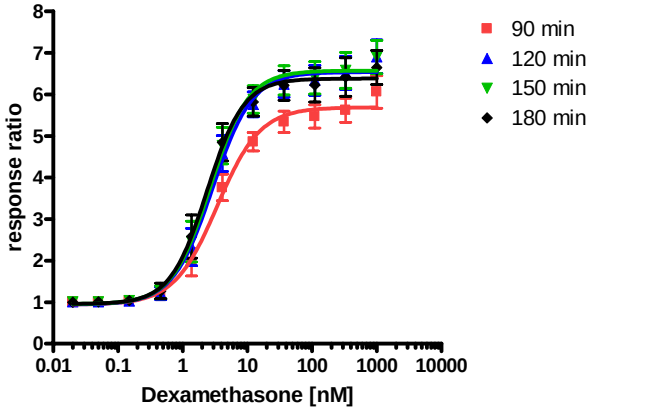
Figure 4 – Incubation of MMTV-*bla* HeLa with Dexamethasone for 5, 18 or 48 hours



MMTV-*bla* HeLa cells were plated the day prior to the assay at 5000 cells/well in a 384-well format in assay medium. 24 hours later,cells were treated for 6, 18 or 48 hrs with the indicated concentration of indicated agonist. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm for the various DMSO concentrations were obtained using a standard fluorescence plate reader and the emission ratios plotted for each DMSO concentration (n=12 for each data point).
Note: We recommend the 18 hr agonist stimulation for MMTV-*bla* HeLa based on our observation that shorter stimulation times occasionally result in less reliable results (increased EC50, decreased Z').

Assay Performance with Variable Substrate Loading Time

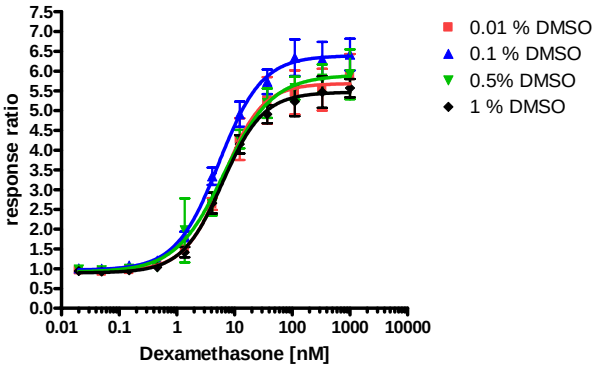
Figure 5 — Dexamethasone dose response with 90, 120, 250 or 180 minute substrate loading time



MMTV-*bla* HeLa cells were plated the day prior to the assay at 5000 cells/well in a 384-well format in assay medium. 24 hours later, cells were treated with the indicated concentration of Dexamethasone for 18 hrs. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for indicated time. Fluorescence emission values at 460 nm and 530 nm for the various loading times were obtained using a standard fluorescence plate reader and the emission ratios plotted for each loading time against the indicated concentrations of Dexamethasone (n=6 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 6 – Dexamethasone dose response with 0.01%, 0.1%, 0.5% and 1% DMSO



MMTV-*bla* HeLa cells were plated the day prior to the assay at 5000 cells/well in a 384-well format in assay medium. 24 hours later,cells were treated for 18 hrs with the indicated concentration of Dexamethasone the presence of the indicated concentration of DMSO. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm for the various DMSO concentrations were obtained using a standard fluorescence plate reader and the emission ratios plotted for each DMSO concentration (n=6 for each data point).
Note: We observed that the cells showed changes in morphology at DMSO concentrations above 0.5% indicating a potential stress response. The assay performance (Dex EC_{50} and Z') was not affected aside from a slightly diminished response ratio.