Validation & Assay Performance Summary

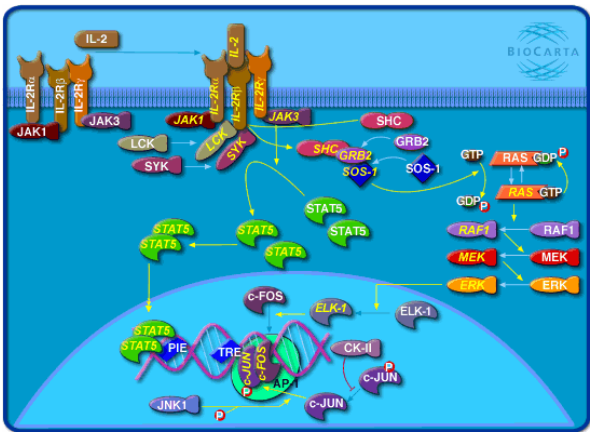
CellSensor® irf1-*bla* CTLL-2 Cell Line

Cat. no. K1653

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

Jak/Stat signaling pathways play essential roles in the cellular responses to distinct cytokines. One of Jak/Stat pathways, Jak1/Jak3/Stat5, is involved in the activation and proliferation of B-Cells and many T cells in response to Interleukin-2 (IL-2). The activated transcription factor Stat5 dimers recognize and bind to a specific palindromic DNA sequence, TTCNNNGAA. This sequence is found in the promoter region of β-casein, interferon regulatory factor-1 (IRF-1) and a number of other genes.



Cell Line Description

The CellSensor® irf1-*bla* CTLL-2 cell line contains a beta-lactamase reporter gene under control of the interferon regulatory factor-1 (irf1) response element stably integrated into CTLL-2 cells. CTLL-2 cells are a clone of cytotoxic T cells derived from a C57/BL/6 mouse, which is cell-growth dependent on mouse IL-2. This cell line validated for IC_{50} and Z'-Factor under optimized conditions using IL-2. This cell line has also been tested under variable experimental conditions, including cell number, stimulation time, and substrate loading time, and has also been screened for responsiveness to a panel of ligands.

Validation Summary

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

1. **Small molecule inhibitor dose response under optimized conditions(n=3)**

IL-2 IC_{50}	= 1.813 ng/mL
Z'-Factor (EC_{100})	= 0.84
Response Ratio	= 15.22
Optimum cell no.	= 50K cells/well
Optimum [DMSO]	= 0.5%
Optimum Stim. Time	= 5 hours
Max. [Inhibition]	= 5.55 ng/mL

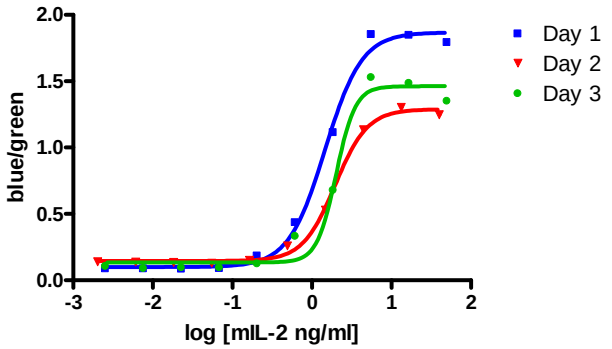
2. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

3. **Assay performance with variable cell number**
4. **Assay performance with variable stimulation time**
5. **Assay performance with variable substrate loading time**
6. **Assay performance with variable DMSO concentration**
7. **Ligand panel results**

Primary Agonist Dose Response

Figure 1 —irf1-*bla* CTLL-2 dose response to IL-1 under optimized conditions



irf1-*bla* CTLL-2 cells (50,000 cells/well) were assayed on three separate days represented by the three curves shown on the graph. Cells were plated the day of the assay in a 384-well format and stimulated with IL-2 over the indicated concentration range in the presence of 0.5% 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 and the 460/530 Emission Ratios plotted for the indicated concentrations of IL-2 (n=16 for each data point).

Cell Culture and Maintenance

Cells are grown in a 90:10 mix of complete growth media: T-STIM™ Culture Supplement with conA (BD Biosciences, 354115). The T-STIM™ Culture Supplement should be kept at 4°C and should not be warmed, due to loss in activity. It should be mixed with growth media just before using with cell cultures. Cell culture medium is changed every other day to replenish fresh IL-2. Cells are maintained at a cell density between 2 x 10^4 and 2 x 10^5 cells/ml.

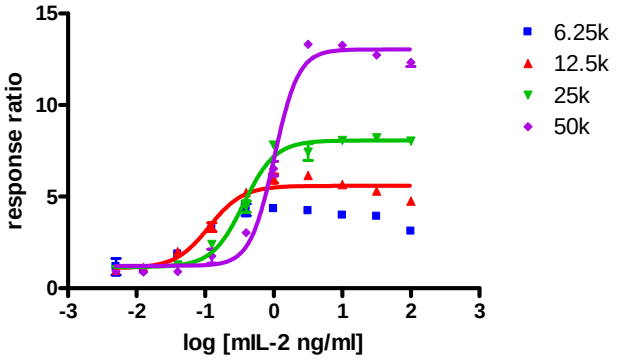
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For proper cell line performance, use dialyzed FBS (Invitrogen# 26400-036). For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	81%	—	—
Opti-MEM®	—	97%	
Dialyzed FBS **Do not substitute!**	9%	0.5%	—
NEAA	0.09 mM	0.1 mM	—
Sodium Pyruvate	0.9 mM	1 mM	—
T-STIM	10%	—	—
Penicillin (antibiotic)	90 U/mL	100 U/mL	—
Streptomycin (antibiotic)	90 µg/mL	100 µg/mL	—
Blasticidin (antibiotic)	5 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

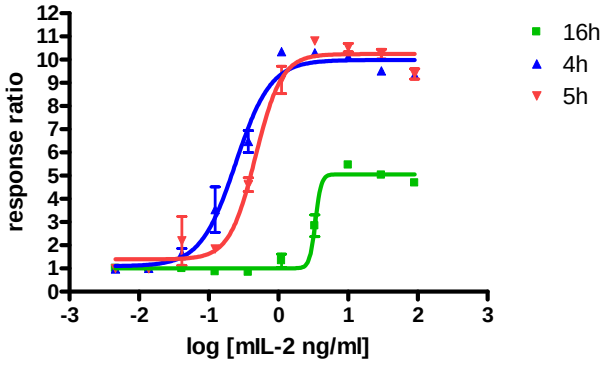
Figure 2 — irf1-*bla* CTLL-2 dose response to mIL-2 with 6.25K, 12.5K, 25K and 50K cells/well



irf1-*bla* CTLL-2 cells were plated the day of the assay at 6,250 12,500, 25,000 or 50,000 cells/well in a 384-well format. Cells were treated with mIL-2 in the presence of 0.5% 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 for the various cell numbers were obtained using a standard fluorescence plate reader and the Response Ratios plotted for each cell number against the indicated concentrations of mIL-2 (n=4 for each data point).

Assay Performance with Variable Stimulation Time

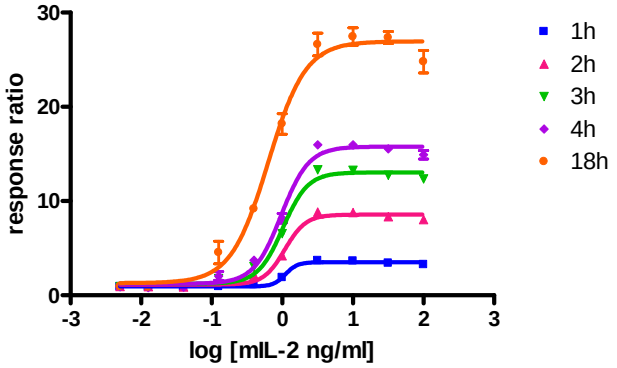
Figure 3 – irf1-*bla* HEL dose response to mIL-2 with 4, 5 and 16 hour stimulation times



irf1-*bla* CTLL-2 cells (50,000 cells/well) were plated the day of the assay in a 384-well assay plate. mIL-2 was then added to the plate over the indicated concentration range. Plates were treated for 4, 5 or 16 hrs with mIL-2 in 0.5% DMSO and then loaded for 2.5 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 plotted for each stimulation time against the indicated concentrations of mIL-2 (n=8 for each data point).

Assay Performance with Variable Substrate Loading Time

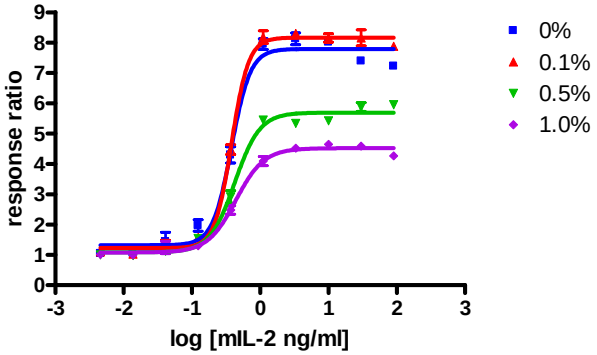
Figure 4 — irf1-*bla* CTLL-2 dose response to mIL-2 with 1, 2, 3, 4, and 18 hour substrate loading times



irf1-*bla* CTLL-2 cells were plated the day of the assay at 50,000 cells/well in a 384-well format. Cells were treated with mIL-2 over the indicated concentration range in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1, 2, 3, 4 or 18 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 plotted for each substrate loading time against the indicated concentrations of mIL-2 (n=4 for each data point).

Assay Performance with Variable DMSO Concentration

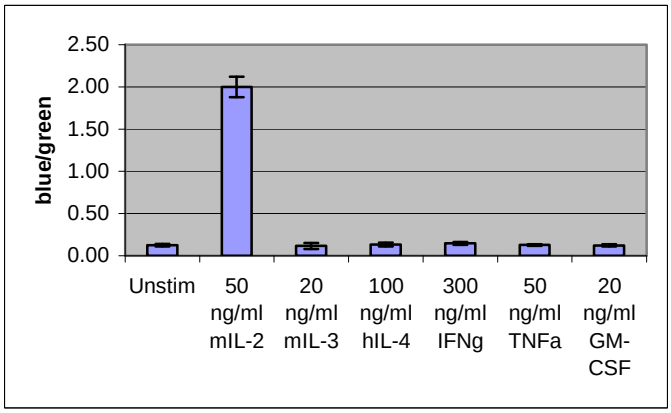
Figure 5 — irf1-*bla* CTLL-2 dose response to mIL-2 with 0%, 0.1%, 0.5% and 1.0% DMSO



irf1-*bla* CTLL-2 cells (50,000 cells/well) were plated the day of the assay in a 384-well assay plate. mIL-2 was then added to the plate over the indicated concentration range. Plates were treated for hrs with mIL-2 in 0.0%, 0.1%, 0.5% or 1.0% DMSO and then loaded for 2.5 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 plotted for each stimulation time against the indicated concentrations of mIL-2 (n=6 for each data point).

Ligand Panel

Figure 6 — irf1-*bla* CTLL-2 response to various stimulants



irf1-*bla* CTLL-2 cells (100,000 cells/well) were plated the day of the assay in a 96-well assay plate (100 µl/well). The cells were stimulated with the indicated ligands for 5 hours in the presence of 0.5% DMSO. They were then loaded for 2.5 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 ratio for each stimulant was plotted. As shown in the graph, irf1-*bla* CTLL-2 cells responded significantly only to mIL-2. (n=4 for each data point).