Validation & Assay Performance Summary

CellSensor® irf1-*bla* Ba/F3 Cell Line

Cat. no. K1654

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, Jak2/Stat5, mediates cell proliferation in response to Interleukin-3 (IL-3), prolactin, erythropoietin (EPO), and granulocyte-macrophage colony stimulating factor (GM-CSF). JAK2 gene knock-out causes embryonic lethality due to defective erythropoiesis, suggesting the Jak2/Stat5 pathway plays important role in red blood cell formation. The recent discovery of an activating mutation in JAK2 (V617F) present in a high percentage of myeloproliferative disease (MPD) patients suggests Jak2/Stat5 pathway to be a potential therapeutic target for certain forms of MPD (Levine, *et al*). 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* Ba/F3 cell line contains a beta-lactamase reporter gene under control of the interferon regulatory factor 1 (irf1) response element stably integrated into Ba/F3 cells. Ba/F3 cells are a mouse pro B cell line that is growth dependent on IL-3 and have an intact IL-3–JAK2–STAT5 pathway. This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time and validated for Z′ and EC_{50} concentrations of mIL-3. Additional testing information using Jak Inhibitor I is also provided.

Validation Summary

Testing and validation of this assay was evaluated using LiveBLAzer™-FRET B/G Substrate.

1. **Primary agonist dose response under optimized conditions**

IL-3 EC_{50}	= 0.2 ng/mL
Z'-Factor (EC_{100})	= 0.82
Response Ratio	= 22
Optimum cell no.	= 30K cells/well
Optimum [DMSO]	= 0.5%
Optimum Stim. Time	= 5 hours
Max. [Stimulation]	= 2 ng/mL

2. **Small Molecule Inhibitor Dose Response**

   IC_{50} Jak Inhibitor I = 0.3µM

3. **Cell culture and maintenance**

   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

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

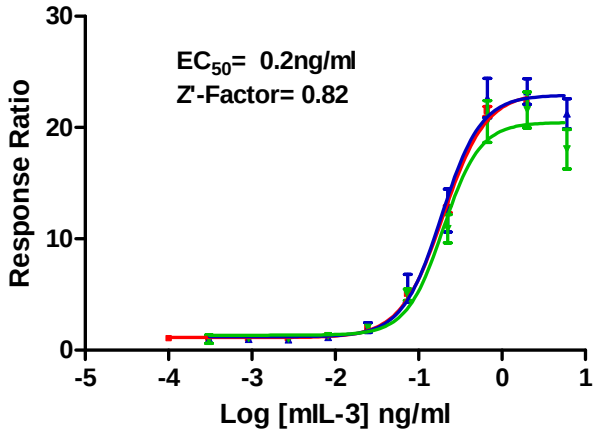
5. **Assay performance with variable stimulation time**

6. **Assay performance with variable substrate loading time**

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

Primary Agonist Dose Response

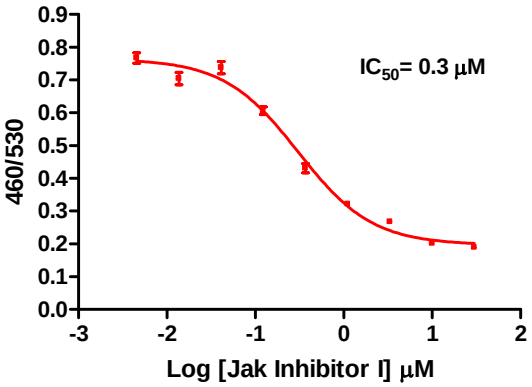
Figure 1 –irf1-*bla* Ba/F3 dose response to mIL-3 under optimized conditions



irf1-*bla* Ba/F3 cells (30,000 cells/well) were assayed on three separate days, represented by the three curves shown on the graph. Cells were plated in a 384-well plate and stimulated with mIL-3 (R&D Systems # 403-ML) 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 Response Ratios plotted for the indicated concentrations of mIL-3 (n=16 for each data point).

Small Molecule Inhibitor Dose Response

Figure 2–irf1-*bla* Ba/F3 dose response to Jak Inhibitor I under optimized conditions



irf1-*bla* Ba/F3 cells (30,000 cells/well) were plated in a 384-well plate in the absence of IL-3 for 16 hrs, followed by pre-treatment with the indicated concentrations of Jak Inhibitor I (EMD/ Calbiochem # 420099) for 30 min. Cells were then stimulated with mIL-3 (R&D Systems # 403-ML) at 0.5 ng/ml in the presence of 0.5% DMSO for 5 hours, and 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 Ratios plotted for the indicated concentrations of Jak Inhibitor I (n=16 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 10^5 and 10^6 cells/ml.

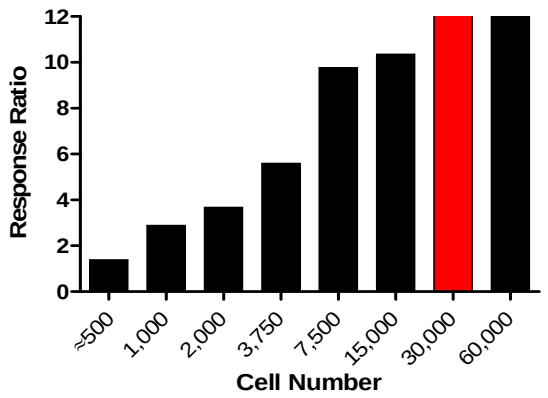
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. Freeze cells at 5 x 10^6 cells/ml in Freezing Medium. For optimal cell line performance, use Newborn Calf Serum (Invitrogen # 16010-159). For detailed growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	90%	—	—
Opti–MEM®	—	96.5%	—
Newborn Calf Serum Do not substitute!	10%	0.5%	90%
NEAA	_	0.1 mM	—
Sodium pyruvate	_	1 mM	—
HEPES (pH 7.3)	_	10 mM	—
Penicillin (antibiotic)	100 U/ml	100 U/ml	—
Streptomycin (antibiotic)	100 µg/ml	100 µg/ml	—
Blasticidin (antibiotic)	5 µg/ml	—	—
mIL-3	1 ng/ml	—	—
DMSO	—	—	10%

Assay Performance with Variable Cell Number

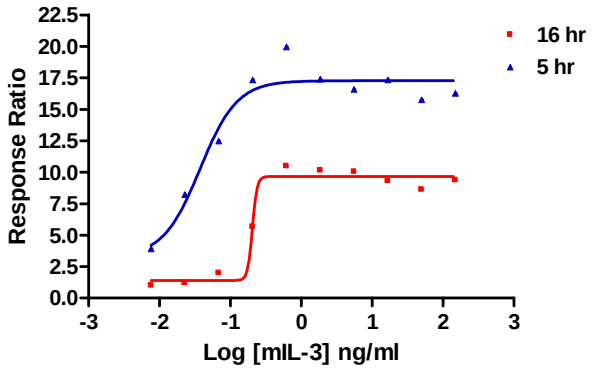
Figure 3 — irf1-*bla* Ba/F3 response to mIL-3 using ~500, 1000, 2000, 3750, 7500, 15000, 30000 or 60000K cells/well



irf1-*bla* Ba/F3 cells were plated at ~500, 1000, 2000, 3750, 7500, 15000, 30000 or 60,000 cells/well in a 384-well format. Cells were then stimulated with mIL-3 (R&D Systems # 403-ML) at 100 ng/ml 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 fixed concentration of mIL-3.

Assay Performance with Variable Stimulation Time

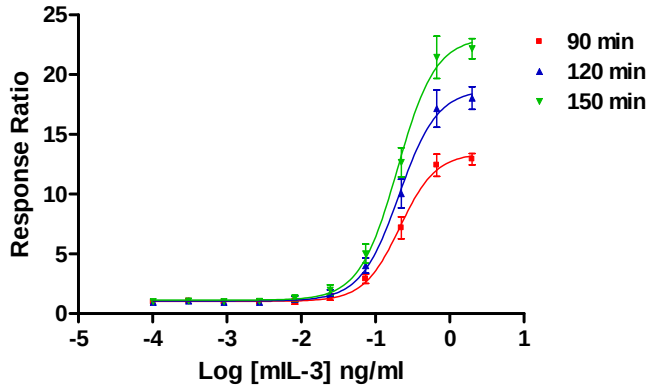
Figure 4 – irf1-*bla* Ba/F3 dose response to mIL-3 using 5 and 16 hour stimulation times



irf1-*bla* Ba/F3 cells (30,000 cells/well) were plated the in a 384-well assay plate. mIL-3 (Invitrogen # 403-ML) was then added to the plate at over the indicated concentration range. Plates were stimulated for 5 or 16 hrs with mIL-3 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.

Assay Performance with Variable Substrate Loading Time

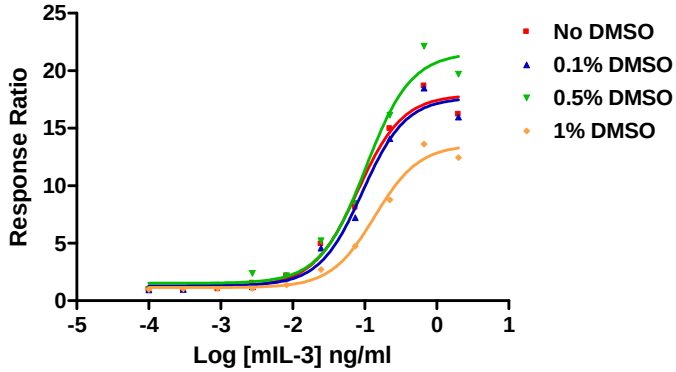
Figure 5 — irf1-*bla* Ba/F3 response to mIL-3 with 1.5, 2 and 2.5 hour substrate loading times



irf1-*bla* Ba/F3 cells were plated at 30,000 cells/well in a 384-well format. Cells were stimulated with 100 ng/ml IL-3 (R&D Systems# 403-ML) in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1.5, 2 or 2.5 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 the indicated substrate loading times.

Assay Performance with Variable DMSO Concentration

Figure 6 – irf1-*bla* Ba/F3 response to mIL-3 using 0, 0.25, 0.5 and 1% DMSO



irf1-*bla* Ba/F3 cells (30,000 cells/well) were plated in a 384-well plate and treated with the indicated concentrations of mIL-3 (Invitrogen # 403-ML) with final DMSO concentrations ranging from 0% to 1%. Plates were stimulated for 5 hrs and 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 DMSO concentration.