Validation & Assay Performance Summary

CellSensor® irf1-*bla* HEL Cell Line

Cat. no. K1647

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 high percentage of myeloproliferative disease (MPD) patients suggests that the Jak2/Stat5 pathway is a potential therapeutic target for certain forms of MPD. The activated transcription factor Stat5 dimers recognize and bind to a specific palindromic DNA sequence 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* HEL cell line contains a beta-lactamase reporter gene under control of the interferon regulatory factor-1 (irf1) response element stably integrated into HEL cells. HEL cells are a human erythroleukemia cell line that is growth factor independent and contains an endogenous homozygous JAK2V617F mutation which leads to constitutive STAT5 activity. This cell line validated for IC_{50} and Z'-Factor under optimized conditions using Jak Inhibitor 1. This cell line has also been tested under variable experimental conditions, including cell number, stimulation time, and substrate loading time. Comparison data using a commonly used cell proliferation assay 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. **Small molecule inhibitor dose response under optimized conditions(n=3)**

Jak Inhibitor I IC_{50}	= 0.3µM
Z'-Factor (EC_{100})	= 0.68
Response Ratio	= 4.0
Optimum cell no.	= 30K cells/well
Optimum [DMSO]	= 0.1%
Optimum Stim. Time	= 16 hours
Max. [Inhibition]	= 3µM

2. **Parental and irf-1 *bla* HEL Cell Proliferation Assay**

Jak Inhibitor I IC_{50}	= 0.2µM

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

Assay Testing Summary

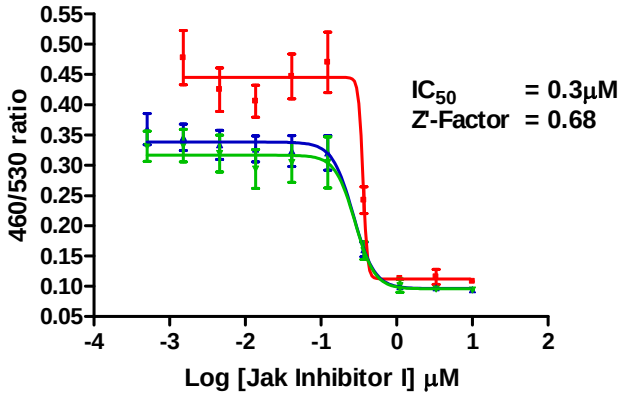
4. **Assay performance with variable cell number**

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

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

Primary Agonist Dose Response

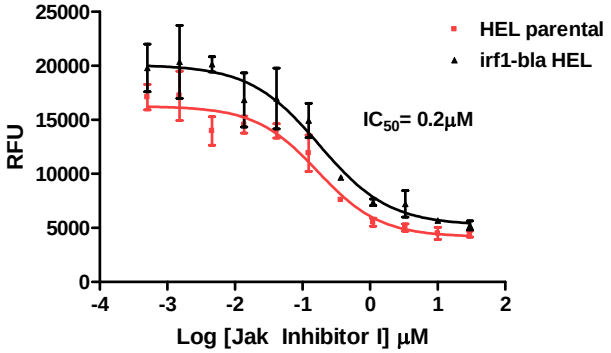
Figure 1 —irf1-*bla* HEL dose response to JAK Inhibitor I under optimized conditions



irf1-*bla* HEL cells (30,000 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 Jak Inhibitor I (EMD-Calbiochem # 420099) over the indicated concentration range in the presence of 0.1% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 4 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 Jak Inhibitor I (n=16 for each data point).

Parental and irf-1 *bla* HEL Cell Proliferation Assay

Figure 2 — Parental and irf1-*bla* HEL dose response to JAK Inhibitor quantified with Resazurin



irf1-*bla* HEL and parental HEL cells (8,000 cells/well) were plated the day of the assay in a 96-well format and stimulated with Jak Inhibitor I (EMD-Calbiochem # 420099) over the indicated concentration range in the presence of 0.1% DMSO for 72 hours. Cells were then loaded with 10x resazurin for 1.5 hours. Relative Fluorescence Units were obtained using a standard fluorescence plate reader and plotted for the indicated concentrations of Jak Inhibitor I.

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 2x105 and 1x106 cells/ml density. Freeze cells at 5 x 106 cells/mL in Freezing Medium.

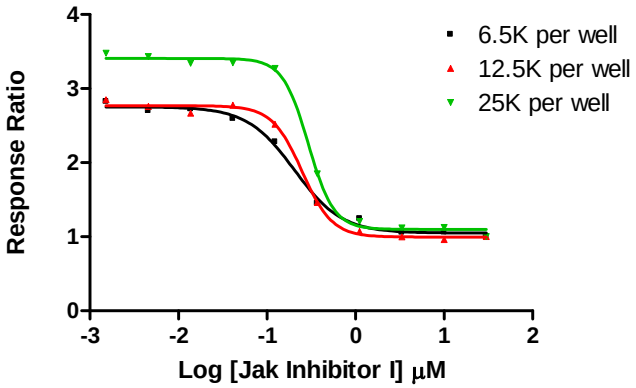
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For proper cell line performance, use dialyzed FBS (Invitrogen# 26400-036). For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	90%	90%	—
Dialyzed FBS **Do not substitute!**	10%	10%	—
NEAA	0.1 mM	0.1 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)	2 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

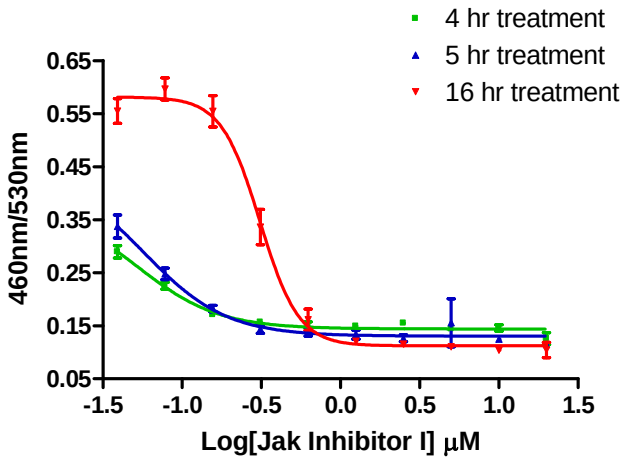
Figure5 — irf1-*bla* HEL dose response to Jak Inhibitor I with 6.5K, 12.5K and 25K cells/well



irf1-*bla* HEL cells were plated the day of the assay at 6,500 12,500 or 25,000 cells/well in a 384-well format. Cells were treated with Jak Inhibitor I (EMD-Calbiochem # 420099) in the presence of 0.1% 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 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 Jak Inhibitor I (n=4 for each data point).

Assay Performance with Variable Stimulation Time

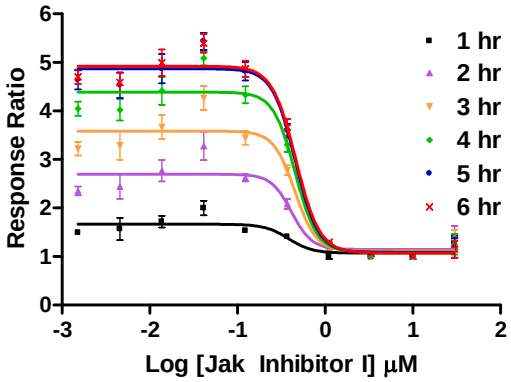
Figure 6 – irf1-*bla* HEL dose response to Jak Inhibitor I with 4, 5 and 16 hour stimulation times



irf1-*bla* HEL cells (50,000 cells/well) were plated the day of the assay in a 384-well assay plate. Jak Inhibitor I (EMD-Calbiochem # 420099) was then added to the plate over the indicated concentration range. Plates were treated for 4, 5 or 16 hrs with Jak Inhibitor I in 0.1% DMSO and then loaded for 4 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 Jak Inhibitor I (n=5 for each data point).

Assay Performance with Variable Substrate Loading Time

Figure 7 — irf1-*bla* HEL dose response to Jak Inhibitor I with 1, 2, 3, 4, 5, and 6 hour substrate loading times



irf1-*bla* HEL cells were plated the day of the assay at 25,000 cells/well in a 384-well format. Cells were treated with Jak Inhibitor I (EMD-Calbiochem # 420099) over the indicated concentration range in the presence of 0.1% DMSO for 16hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1, 2, 3, 4, 5, or 6 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 Jak Inhibitor I (n=4 for each data point).