# Validation & Assay Performance Summary

## CellSensor® irf1-*bla* TF-1 Cell Line

Cat. no. K1657

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 Jak2/Stat5 pathway to be the 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* TF-1 cell line contains a beta-lactamase reporter gene under control of the interferon regulatory factor 1 (irf1) response element stably integrated into TF-1 cells. TF-1 cells are a human erythroleukemia cell line that is growth dependent on GM-CSF and have an intact GM-CSF–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 GM-CSF.

## Validation Summary

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

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

| | |
|---|---|
| GM-CSF $EC_{50}$ | = 0.6 ng/mL |
| Z'-Factor ($EC_{100}$) | = 0.85 |
| Response Ratio | = 14 |
| | |
| Optimum cell no. | = 60K cells/well |
| Optimum [DMSO] | = up to 1% |
| Optimum Stim. Time | = 5 hours |
| Max. [Stimulation] | = 3 ng/mL |

2. **Ligand Panel and Inhibitor Testing**
   *See Ligand Panel and Inhibitor Testing Section*

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 times**

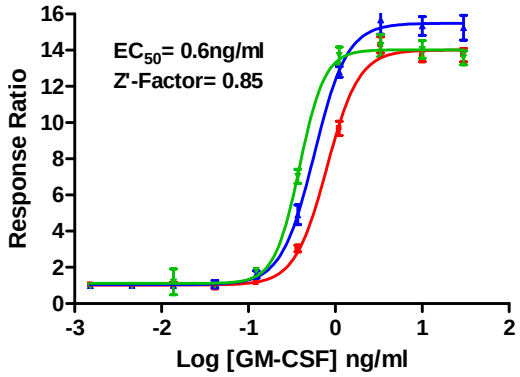
6. **Assay performance with variable substrate loading times**

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

8. **Cryopreserved cell testing**

## Primary Agonist Dose Response

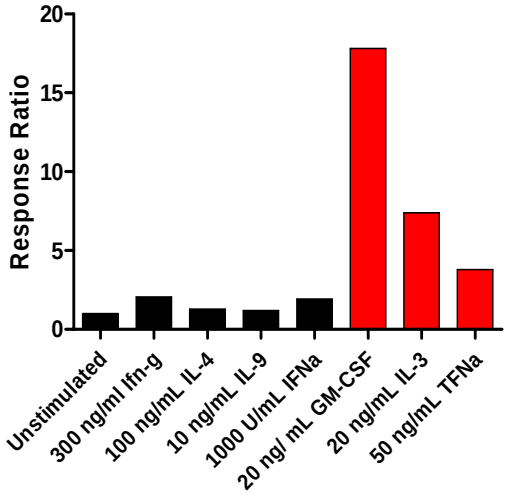
**Figure 1 –irf1-*bla* TF-1 dose response to GM-CSF under optimized conditions**




irf1-*bla* TF-1 cells (10,000 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 GM-CSF (Invitrogen # PHC2015) 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 GM-CSF (n=5 for each data point).

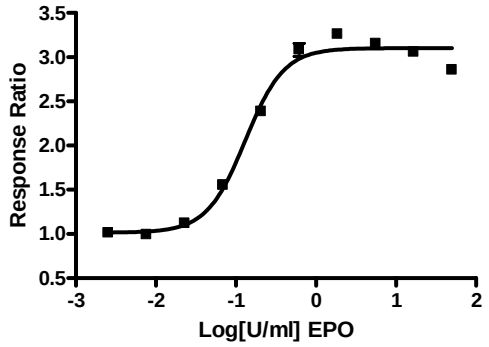
## Ligand Panel and Inhibitor Testing

**Figure 2 — irf1-*bla* TF-1 response to various ligands**




irf1-*bla* TF-1 cells (100,000 cells/well) were plated in a 96-well plate and stimulated with the listed compounds 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 each compound (n=5 for each data point).

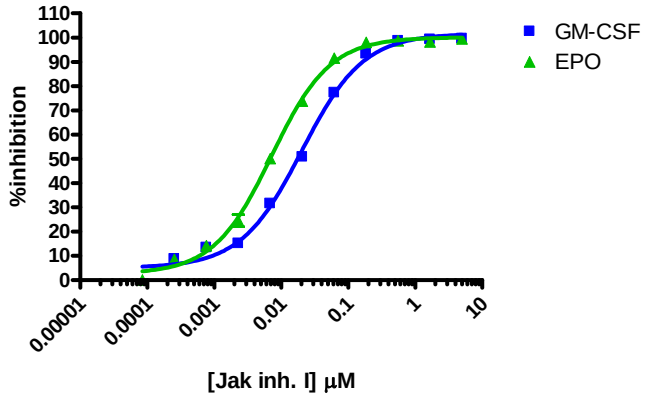
**Figure 3 — irf1-*bla* TF-1 response to EPO**




$EC_{50}$ = 0.13 U/ml
$EC_{80}$ = 0.28 U/ml
RR max = 3.2
Z' max = 0.7

The cells were washed of GM-CSF, resuspended in assay media without GM-CSF, and incubated for 16 hours. Cells were then plated onto a 384-well assay plate at a density of 50,000 cells/well and stimulated with different concentrations of EPO (R&D Systems, 287-TC) in the presence of 0.5% DMSO at 37°C/5%$CO_2$ for 5 hours. LiveBLAzer™-FRET B/G substrate was then loaded at room temperature for 2.5 hours. (n=16)

**Figure 4 — Jak inhibitor testing**




With GM-CSF:
RR at $EC_{80}$ = 10.5
Z' at $EC_{80}$ = 0.9
$IC_{50}$ (Jak Inhibitor I) = 20 nM

With EPO:
RR at $EC_{80}$ = 3.8
Z' at $EC_{80}$ = 0.88
$IC_{50}$ (Jak Inhibitor I) = 7.2 nM

The cells were washed of GM-CSF, resuspended in assay media without GM-CSF, and incubated for 16 hours. Cells were then plated onto a 384-well assay plate at a density of 50,000 cells/well and stimulated with different concentrations of Jak inhibitor 1 (Calbiochem 420099) at 37°C/5%$CO_2$ for 30 minutes prior to addition of GM-CSF or EPO. After the addition of GM-CSF (0.5 ng/ml) or EPO (0.3 U/ml) the plate was incubated at 37°C/5%$CO_2$ for 5 hours. LiveBLAzer™-FRET B/G substrate was then loaded at room temperature for 2.5 hours. (n=8)

## 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 at a density between 3 x $10^4$ and 5 x $10^5$ cells/ml. Do not allow cells to reach 7 x $10^5$ cells/ml.

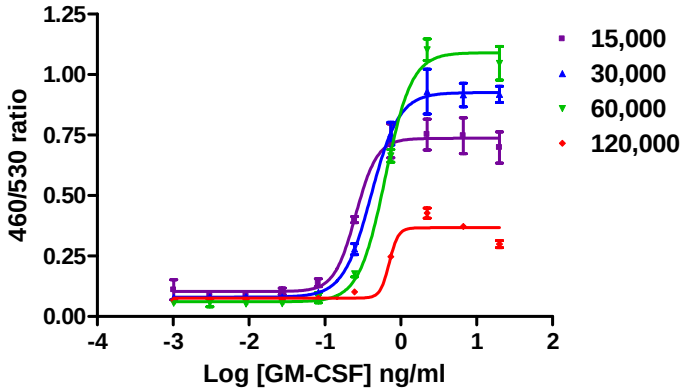
Note: For optimal cell line performance, use dialyzed FBS (Invitrogen # 26400-036).

**Table 1 – Cell Culture and Maintenance**

| Component | Growth Medium | Assay Medium | Freezing Medium |
|---|---|---|---|
| RPMI 1640 | 90% | — | — |
| Opti–MEM® | — | 97% | — |
| Dialyzed FBS **Do Not Substitute!** | 10% | 0.5% | — |
| NEAA | 0.1 mM | 0.1 mM | — |
| Sodium pyruvate | 1 mM | 1 mM | — |
| GM-CSF | 2 ng/ml | -- | -- |
| 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

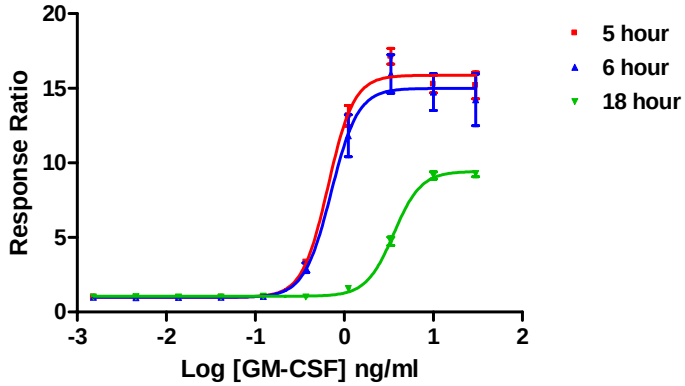
**Figure 5 — irf1-*bla* TF-1 dose response to GM-CSF using 15000, 30000, 60000 or 120,000K cells/well**




irf1-*bla* TF-1 cells were plated at 15, 30, 60 or 120K cells/well in a 384-well format. Cells were then stimulated with GM-CSF (Invitrogen # PHC2015) 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 460/530 Ratios plotted for each cell number against the indicated concentrations of GM-CSF.

## Assay Performance with Variable Stimulation Time

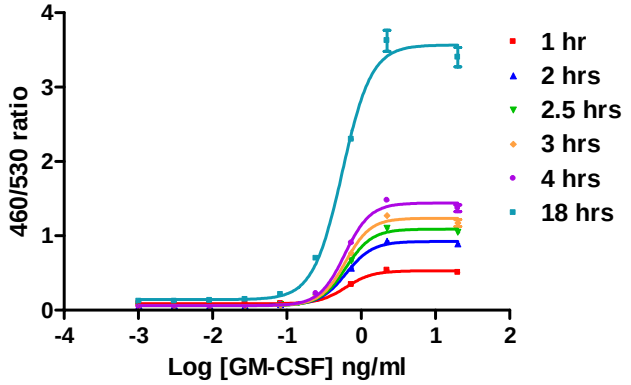
**Figure 6 – irf1-*bla* TF-1 dose response to GM-CSF using 5, 6 and 18 hour stimulation times.**




irf1-*bla* TF-1 cells (60,000 cells/well) were plated the in a 384-well assay plate. GM-CSF (Invitrogen # IL-6) was then added to the plate at over the indicated concentration range. Plates were stimulated for 5, 6 or 18 hrs with GM-CSF 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 of each stimulation time plotted for the indicated concentrations of GM-CSF.

## Assay Performance with Variable Substrate Loading Time

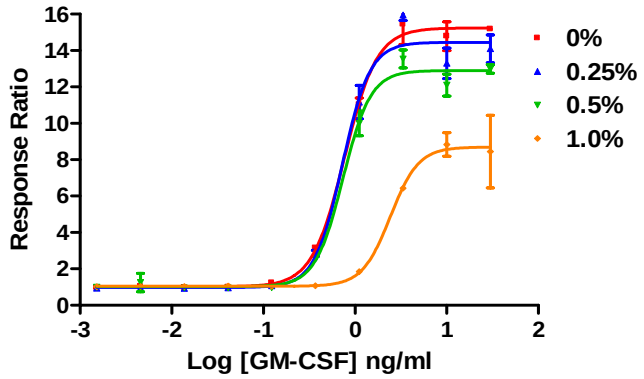
**Figure 7 — irf1-*bla* TF-1 dose response to GM-CSF with 1, 2, 2.5, 3, 4 and 18 hour substrate loading times**




irf1-*bla* TF-1 cells were plated at 60,000 cells/well in a 384-well format. Cells were stimulated with GM-CSF (Invitrogen # PHC2015) in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1, 2, 2.5, 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 for each substrate loading time plotted against the indicated concentrations of GM-CSF.

## Assay Performance with Variable DMSO Concentration

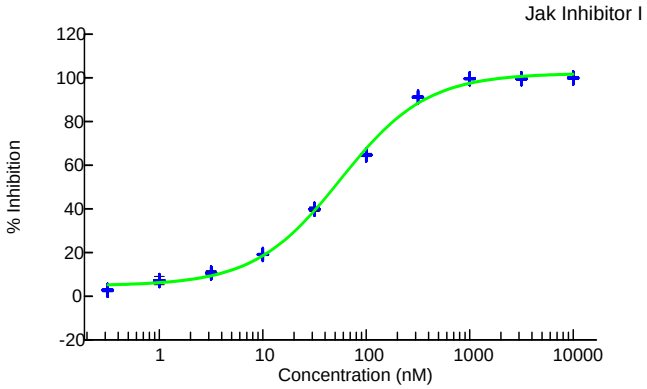
**Figure 8 – irf1-*bla* TF-1 dose response to GM-CSF using 0, 0.25, 0.5 and 1% DMSO**




irf1-*bla* TF-1 cells (60,000 cells/well) were plated in a 384-well plate and treated with the indicated concentrations of GM-CSF (Invitrogen # IL-6) 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 of each DMSO concentration plotted against the indicated concentrations of GM-CSF.

## Cryopreserved cells testing

**Figure 9 – Cryopreserved irf1-*bla* TF-1 cell testing**




Cryopreserved irf1-*bla* TF-1 cells were thawed and resuspended directly in assay medium and were plated in a 384-well plate (50,000 cells/well) overnight. Cells were then treated with the indicated concentrations of Jak Inhibitor with final DMSO concentration of 0.1% for 30 min. Plates were stimulated with GM-CSF for 5 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 fluorescence plate reader (n=2).