

Validation & Assay Performance Summary



CellSensor® STAT6-*bla* RA-1 Cell Line

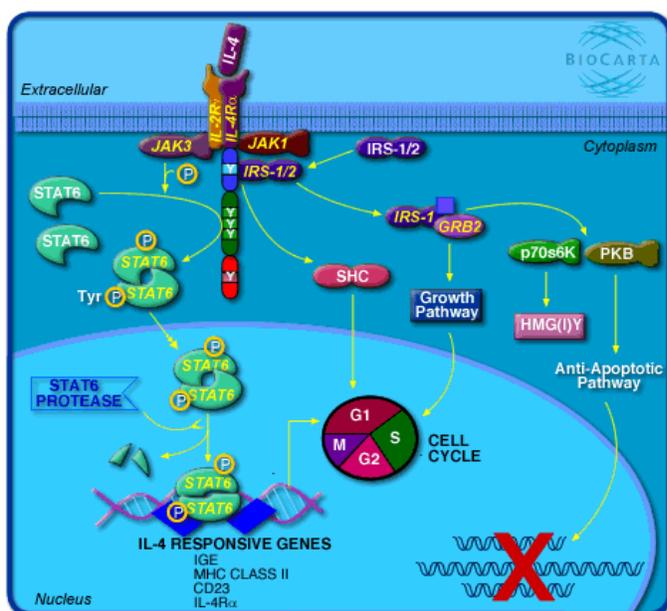
Cat. no. K1646

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

IL-4 is known to induce the growth and differentiation of B cells, T cells, myeloid cells, and hepatocytes. In B cells, IL-4 acts as a co-mitogen to induce the expression of Fc receptor for IgE and major histocompatibility complex (MHC) class II molecules and it stimulates the transcription of immunoglobulin heavy-chain germline Ige and Igg1 genes, leading to class switching. Interleukin-4 function is mediated by the IL-4 receptor complex which activates Jak1 and Jak3 tyrosine kinases. STAT6 is recruited to the IL-4R complex and is subsequently phosphorylated by the Jak kinases. This causes STAT6 to dimerize and translocate to the nucleus where it binds to specific sequences on IL-4 responsive genes. Optimal transcription response of IL-4-inducible promoters requires two Th2-mediated stimuli: CD40 Ligand and IL-4. CD40 Ligand is a protein that is expressed on the surface of T cells. It regulates B cell function by engaging CD40 on the B cell surface leading to signalling through p38 MAPK. (Silvennoinen, 2002)



Cell Line Description

The CellSensor® STAT6-*bla* RA-1 cell line contains a beta-lactamase reporter gene under control of the STAT6 response element stably integrated into Ramos-1 (RA-1) cells. This cell line is a clonal population isolated in response to IL-4 by flow cytometry. This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time and validated for Z' and EC₅₀ concentrations of IL-4. Additional testing information using known inhibitors or activator of the pathway are 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)

hIL-4 EC₅₀ = 0.53 ng/ml
Z'-Factor (EC₁₀₀) = 0.72
Response Ratio = 4.6

Recommended cell no. cells/well = 30,000
Recommended [DMSO] = 0.0-1.0%
Recommended Stim. Time = 5 hrs
Max. [Stimulation] = 100 ng/ml

2. Alternate Stimuli

See Compound Panel Section

3. Small molecule inhibitor Testing

See Compound Panel

4. Cell culture and maintenance

See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

5. Assay performance with variable cell number

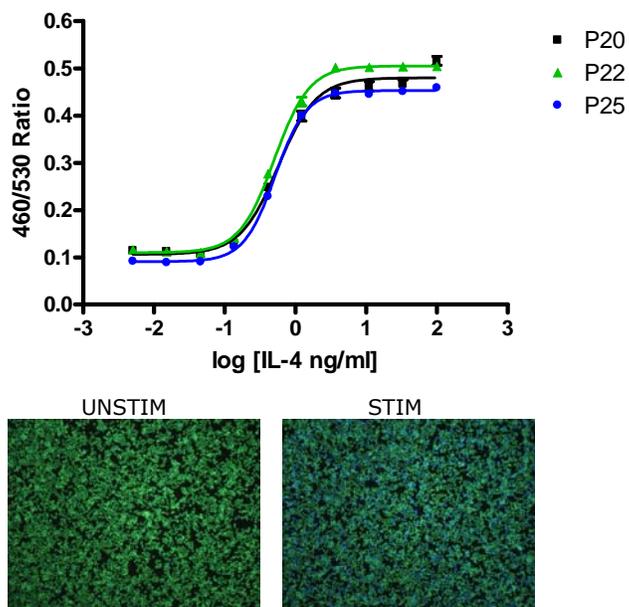
6. Assay performance with variable stimulation time

7. Assay performance with variable substrate loading time

8. Assay performance with variable DMSO concentration

Primary Agonist Dose Response

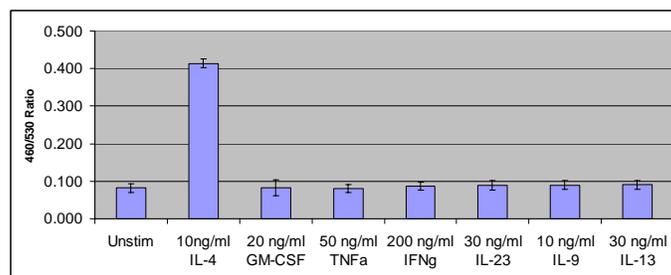
Figure 1 — hIL-4 dose response under optimized conditions



STAT6-*bla* RA-1 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 prior to the assay in a 384-well format and treated for 16 hours with 556 ng/ml CD40 Ligand (PHP0024). They were stimulated with hIL-4 (Invitrogen Corp. PHC0044) 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 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 hIL-4 (n=16 for each data point). Images of unstimulated and stimulated cells at 30,000 cells/well plating density are shown in the bottom.

Ligand Panel

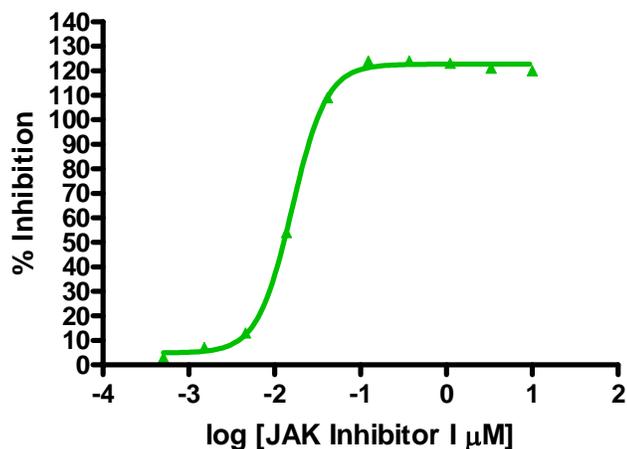
Figure 2 — STAT6-*bla* RA-1 response specific for IL-4



STAT6-*bla* RA-1 cells (30,000 cells/well) were plated the day prior to the assay in a 384-well format in assay medium and treated for 16 hours with 556 ng/ml CD40 Ligand (PHP0024). They were treated with a panel of ligands at the indicated concentrations for 5 hours and then loaded with LiveBLazer™-FRET B/G Substrate for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate

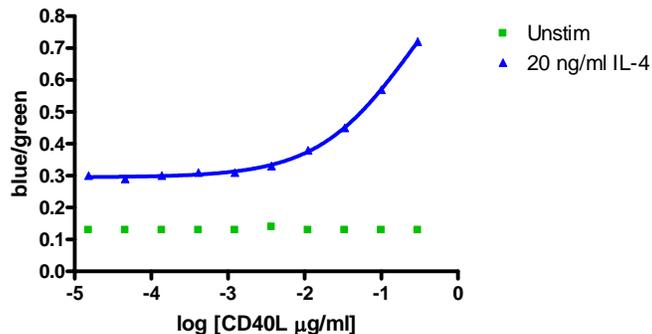
reader and the 460/530 Ratios plotted for each treatment (n=8 for each data point).

Figure 3 — STAT6-*bla* RA-1 response silenced by JAK Inhibitor



STAT6-*bla* RA-1 cells (30,000 cells/well) were plated the day prior to the assay in a 384-well format in assay medium containing 556 ng/ml CD40 Ligand (PHP0024). After 16 hours, they were treated with the indicated concentration of JAK Inhibitor I (EMD Biosciences, 420099) for 1 hour. The cells were stimulated with IL-4 (Invitrogen Corp. PHC0044) at the EC₈₀ for 5 hours and then loaded with LiveBLAZer™-FRET B/G Substrate for 2.5 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 Ratios plotted for each treatment (n=8 for each data point).

Figure 4 — STAT6-*bla* RA-1 response to IL-4 is amplified by CD40 Ligand.



STAT6-*bla* RA-1 cells (30,000 cells/well) were plated the day prior to the assay in a 384-well format and treated for 16 hours with indicated concentration range of CD40 Ligand (PHP0024). They were stimulated with 20 ng/ml hIL-4 (Invitrogen Corp. PHC0044) or left unstimulated for 5 hours. Cells were then loaded with LiveBLAZer™-FRET B/G Substrate for 2 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 CD40L (n=8 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% CO₂ incubator. Maintain cells between 2 x 10⁵ to 1 x 10⁶ cells/ml.

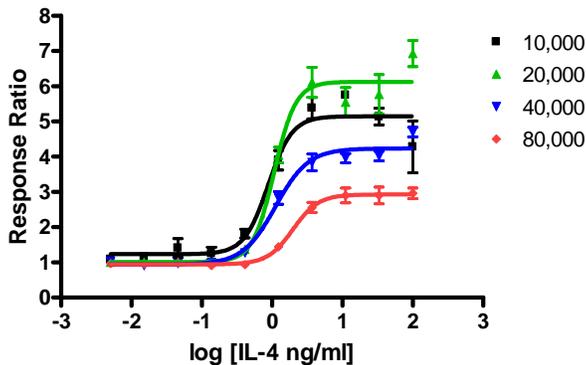
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	87%	--	—
OPTI-MEM	--	96.5	—
Heat Inactivated FBS	10%	0.5%	—
NEAA	0.1 mM	0.1 mM	—
Sodium Pyruvate	1.0mM	1.0mM	—
Penicillin (antibiotic)	100 U/ml	100 U/ml	—
Streptomycin (antibiotic)	100 μg/ml	100 μg/ml	—
Blasticidin (antibiotic)	7.5 μg/ml	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

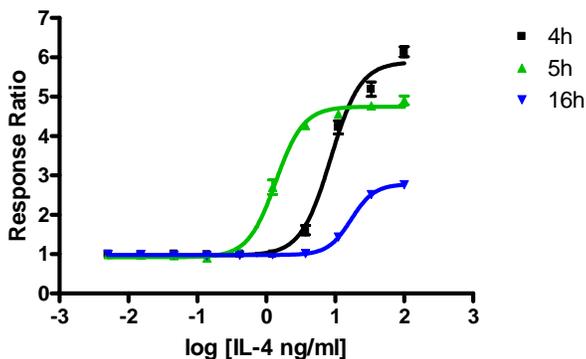
Figure 4 — hIL-4 dose response with different plating cell numbers/well



STAT6-*bla* RA-1 cells were plated the day prior to the assay at indicated number of cells/well in a 384-well format in assay medium containing 556 ng/ml CD40 Ligand (PHP0024). 16 hours later, cells were stimulated with hIL-4 (Invitrogen Corp. PHC0044) 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. (n=4 for each data point).

Assay Performance with Variable Stimulation Time

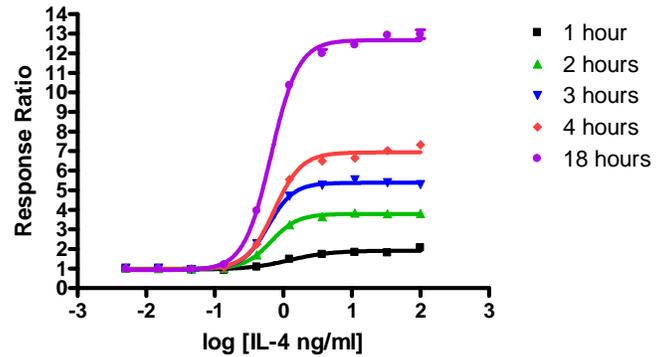
Figure 5 – hIL-4 dose response with 4 hour, 5 hour, and 16 hour stimulation times



STAT6-*bla* RA-1 cells were plated the day prior to the assay at 30,000 cells/well in a 384-well format in assay medium containing 556 ng/ml CD40 Ligand (PHP0024). "16 hour" cells were immediately treated with hIL-4 (Invitrogen Corp. PHC0044) for 16 hours. "5 hour" cells were incubated for 16 hours then stimulated with hIL-4 for 5 hours. "4 hour" cells were incubated for 17 hours and then stimulated with hIL-4 for 4 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 hIL-4 (n=6 for each data point).

Assay Performance with Variable Substrate Loading Time

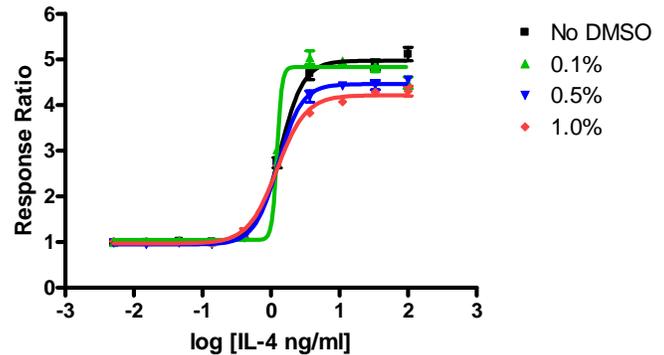
Figure 6 — hIL-4 dose response with various substrate loading times



STAT6-*bla* RA-1 cells were plated the day prior to the assay at 30,000 cells/well in a 384-well format in assay medium containing 556 ng/ml CD40 Ligand (PHP0024). 16 hours later, cells were stimulated with hIL-4 (Invitrogen Corp. PHC0044) in assay medium for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for indicated 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 hIL-4 (n=16 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 7 – hIL-4 dose response with 0, 0.1, 0.5 and 1% DMSO



STAT6-*bla* RA-1 cells were plated the day prior to the assay at 30,000 cells/well in a 384-well format in assay medium containing 556 ng/ml CD40 Ligand (PHP0024). 16 hours later, cells were stimulated with hIL-4 (Invitrogen Corp. PHC0044) in the presence of indicated amount of 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 for each DMSO concentration were plotted against the indicated concentrations of hIL-4 (n=6 for each data point).

References:

Pesu, M., Aittomaki, S., Takaluoma, K., Lagerstedt, A., and Silvennoinen, O. (2002) *J.Biol. Chem.* **277**, 38254-38261