

Validation & Assay Performance Summary



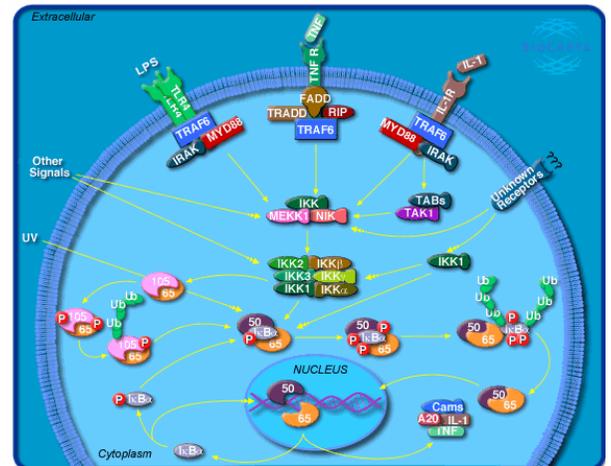
CellSensor[®] NFκB-*bla* RA-1 Cell Line

Cat. no. K1665

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

Nuclear factor κB is a nuclear transcription factor that regulates expression of several genes critical to the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. The activation of NFκB is involved in stress response that activated by a variety of stimuli including growth factors, cytokines, lymphokines, UV, pharmacological agents, and stress. When inactive, NFκB is found in the cytoplasm, bound by members of the IκB family of inhibitor proteins. The various stimuli that activate NFκB cause phosphorylation of IκB, followed by its ubiquitination and subsequent degradation. At this point the NFκB is free to translocate to the nucleus where it binds with a consensus sequence of various genes, thus activating their transcription.



Cell Line Description

The CellSensor[®] NFκB-*bla* RA-1 cell line contains a beta-lactamase reporter gene under control of the NFκB consensus binding sequence stably integrated into Ramos (RA-1) cells. This cell line has been validated for IC₅₀ and Z'-Factor under optimized conditions using TNFα. 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. Primary agonist dose response under optimized conditions(n=3)

TNF α IC ₅₀	= 0.27 ng/mL
Z'-Factor (EC ₁₀₀)	= 0.82
Response Ratio	= 3.66
Optimum cell no.	= 50K cells/well
Optimum [DMSO]	= 0.5%
Optimum Stim. Time	= 5 hours
Max. [Inhibition]	= 50 ng/mL

2. Cell culture and maintenance

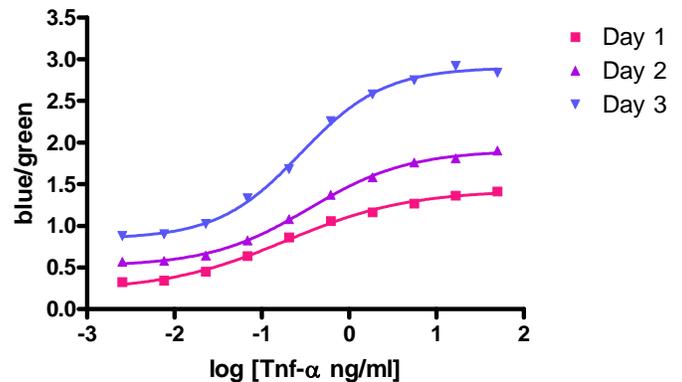
See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

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

Primary Agonist Dose Response

Figure 1 —NF κ B-*bla* ra-1 dose response to TNF α under optimized conditions



NF κ B-*bla* RA-1 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 TNF α 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 TNF α (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. Passage or feed cells at least twice a week and maintain them in a 37°C/5% CO₂ incubator. Maintain cells between 5% and 60% confluency. Do not allow cells to reach confluence.

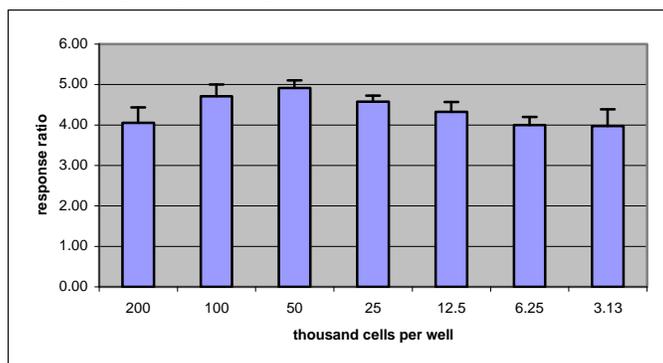
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. Freeze cells at 2x10⁶ cells/ml in Freezing Medium. For optimal cell line performance, use dialyzed FBS (Invitrogen #26400-010).

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	90%	90%	85%
Heat Inactivated FBS Do not substitute!	10%	10%	10%
Sodium Pyruvate	1 mM	1 mM	1 mM
Penicillin	100 U/mL	100 U/mL	100 U/mL
Streptomycin	100 µg/mL	100 µg/mL	100 µg/mL
Blasticidin (antibiotic)	5 µg/mL	—	—
DMSO	—	—	5%
Non-essential amino acids	0.1 mM	0.1 mM	0.1 mM

Assay Performance with Variable Cell Number

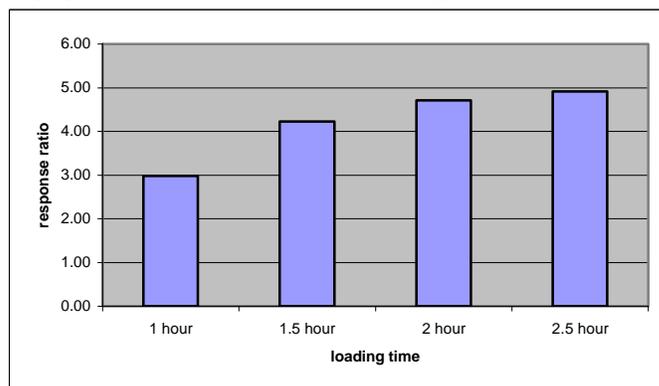
Figure 2 – NFκB-*bla* RA-1 response to TNFα with 200K, 100K, 50K, 25K, 12.5K, 6.25K and 3.13K cells/well



NFκB-*bla* RA-1 cells were plated the day of the assay at 200,000, 100,000, 50,000, 25,000, 12,500, 6,250 or 3,130 cells/well in a 384-well format. Cells were treated with TNFα (50 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 indicated concentrations of TNFα (n=4 for each data point).

Assay Performance with Variable Substrate Loading Time

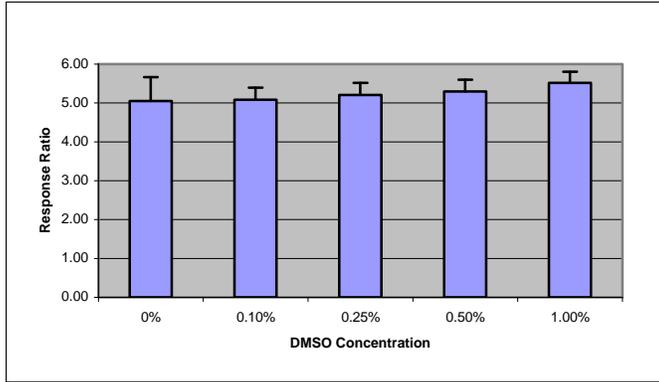
Figure 4 – NFκB-*bla* RA-1 dose response to TNFα with 1, 1.5, 2, and 2.5 hour substrate loading times



NFκB-*bla* RA-1 cells were plated the day of the assay at 50,000 cells/well in a 384-well format. Cells were treated with TNFα (50 ng/mL) in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1, 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 each substrate loading time against the indicated concentrations of TNFα (n=4 for each data point).

Assay Performance with Variable DMSO Concentration

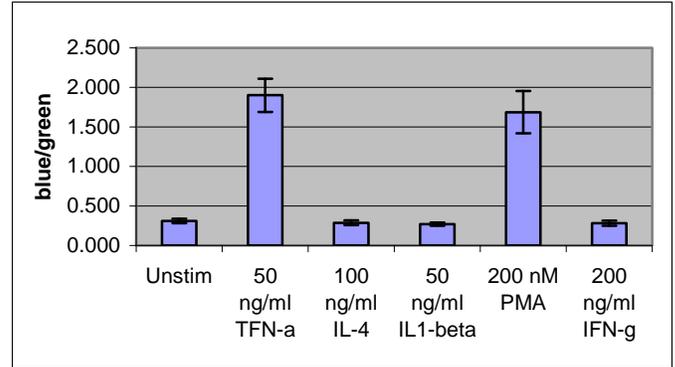
Figure 5 — NFkB-*bla* RA-1 response to TNF α with 0%, 0.1%, 0.25%, 0.5% and 1.0% DMSO



NFkB-*bla* RA-1 cells (50,000 cells/well) were plated the day of the assay in a 384-well assay plate. Plates were treated for 5 hrs with TNF α (50 ng/mL) in 0.0%, 0.1%, 0.25%, 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 TNF α (n=6 for each data point).

Ligand Panel

Figure 6 — NFkB-*bla* RA-1 response to various stimulants



NFkB-*bla* RA-1 cells (50,000 cells/well) were plated the day of the assay in a 384-well assay plate. 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. (n=4 for each data point).