Validation & Assay Performance Summary

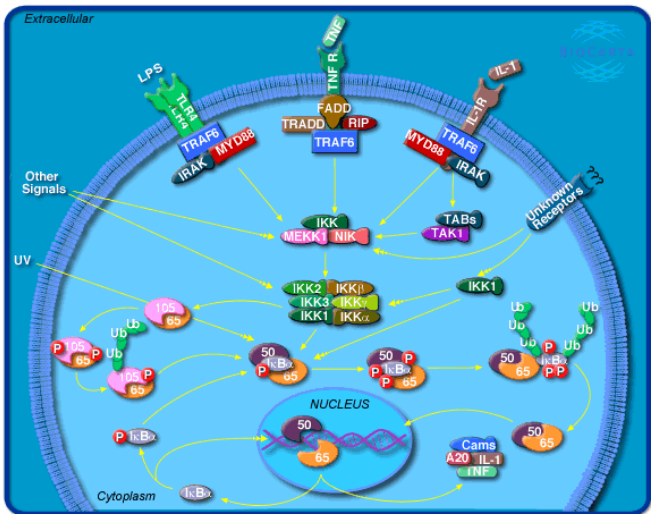
CellSensor® NFκB-*bla* FreeStyle™ 293F Cell Line

Cat. no. K1663

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 kappa B (NFκB) signaling regulates genes involved in apoptosis, viral defense, cancer, inflammation, and autoimmune disease. This cell line is responsive to Tumor Necrosis Factor alpha (TNF-alpha) can be used to probe the NFκB signaling pathway. TNF alpha binds its receptor, which recruits a protein called TNF receptor death domain (TRADD). TRADD binds TNF receptor associated factor 2 (TRAF-2) which in turn activates NFκB inducible kinase (NIK). NIK phosphorylates proteins which inhibit NFκB in the cytoplasm. This marks these inhibitory factors for degradation. NFκB is then free to enter the nucleus and regulate transcription.



Cell Line Description

The CellSensor® NFκB-*bla* FreeStyle™ 293F cell line contains a beta-lactamase reporter gene under control of the NFκB response element stably integrated into FreeStyle™ 293F cells. This cell line is a clonal population isolated in response to TNFα by flow cytometry. This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, substrate loading time, and validated for Z' and EC_{50} concentrations of TNFα.

Validation Summary

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

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

TNFα EC_{50}	= 0.103 ng/ml
Z'-Factor (EC_{100})	= 0.90
Response Ratio	= 24.5
Optimum cell no.	= 20K cells/well
Optimum [DMSO]	= 0.5%
Optimum Stim. Time	= 5 hours
Max. [Stimulation]	= 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**

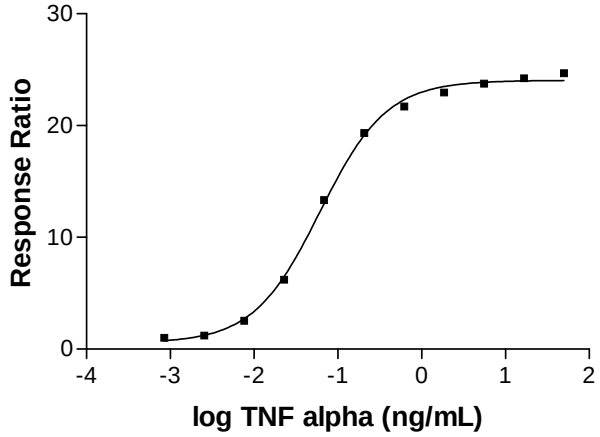
4. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

Figure 1 –NFκB-*bla* FreeStyle™ 293F dose response to TNFα under optimized conditions



NFκB-*bla* FreeStyle™ 293F cells (20,000 cells/well) were plated in a 384-well plate and stimulated with TNFα 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 Response Ratios plotted for the indicated concentrations of TNFα (data is average of data collected on three separate days).

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 2 x 10^5 cells/ml and 2 x 10^6 cells/ml. 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 5 x 10^6 cells/ml in Freezing Medium.

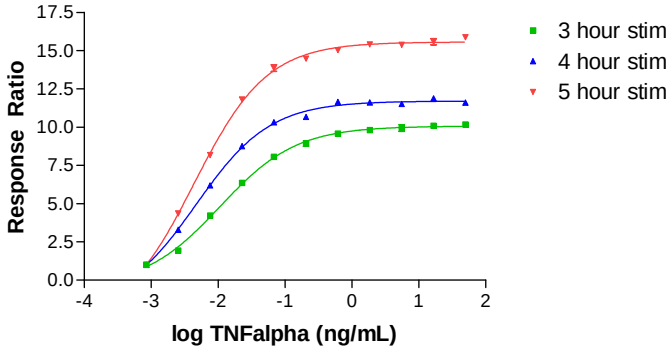
Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
FreeStyle™ 293 Expression Medium	100%	100%	45%
FreeStyle™ 293 Expression Medium (conditioned*)	—	—	45%
Blasticidin (antibiotic)	0.5 μg/ml (do not thaw with Blasticidin)	—	—
DMSO	—	—	10%

*Conditioned medium is medium cells have been grown in for 3 days

Assay Performance with Variable Stimulation Time

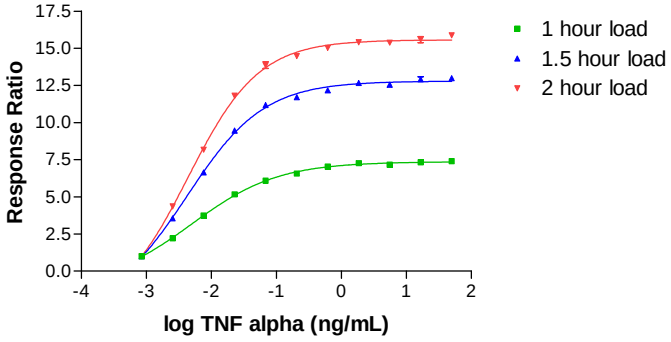
Figure 3 – NFκB-*bla* FreeStyle™ 293F dose response to Forskolin using 3, 4 and 5 hour stimulation times



NFκB-*bla* FreeStyle™ 293F cells (20,000 cells/well) were plated the in a 384-well assay plate. Plates were stimulated for 3, 4 or 5 hrs with TNFα in 0.5% DMSO and then 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 and the Response Ratios plotted for each stimulation time.

Assay Performance with Variable Substrate Loading Time

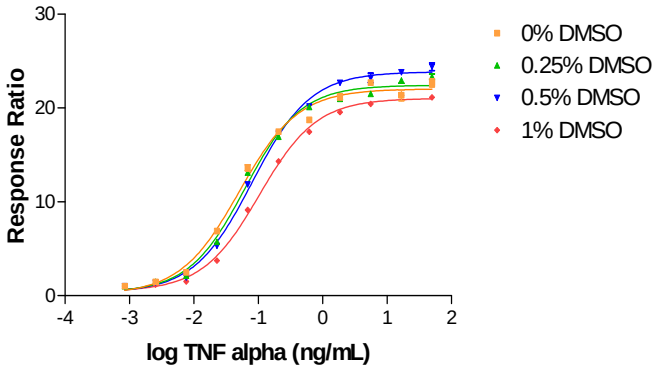
Figure 4 — NFκB-*bla* FreeStyle™ 293F response to forskolin with 1, 1.5, and 2 hour substrate loading times



NFκB-*bla* FreeStyle™ cells were plated at 20,000 cells/well in a 384-well format. Cells were stimulated with TNFα at various concentrations 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 or 2 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 5 – NFκB-*bla* FreeStyle™ 293F response to forskolin using 0, 0.25, 0.5 and 1% DMSO



NFκB-*bla* FreeStyle™ 293F cells (20,000 cells/well) were plated in a 384-well plate and treated with the indicated concentrations of TNFα with final DMSO concentrations ranging from 0% to 1%. Plates were stimulated 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 and the Response Ratios plotted for each TNFα concentration for each DMSO concentration.