Validation & Assay Performance Summary

CellSensor® NFκB-*bla* ME-180 Cell Line

Cat. no. K1667

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 (NFKB) signaling regulates genes involved in apoptosis, viral defense, cancer, inflammation, and autoimmune disease. 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 NFKB inducible kinase (NIK). NIK phosporylates proteins that inhibit NFKB in the cytoplasm, thereby marking these inhibitory factors for degradation. NFκB is then free to enter the nucleus and regulate transcription.

Cell Line Description

The CellSensor® NFκB-*bla* ME-180 cell line contains a beta-lactamase reporter gene under control of the Nuclear Factor Kappa Beta (NFκB) response element stably integrated into ME-180 cells. This cell line is validated for EC_{50} and Z'-Factor under optimized conditions using Tumor Necrosis Factor Alpha (TNFα). This cell line has also been tested for assay performance under variable experimental conditions, including stimulation time, substrate loading time and DMSO concentration. Additional testing information using alternate stimuli 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. **Primary agonist dose response under optimized conditions(n=3)**

TNFα EC_{50}	= 0.4 ng/mL
Z'-Factor (EC_{100})	= 0.83
Response Ratio	= 31
Recommended cell no.	= 12K cells/well
Optimum [DMSO]	= up to 1%
Optimum Stim.Time	= 5 hours
Max. [Stimulation]	= ~100 ng/mL

2. **Alternate Agonist Dose Response**
   IL-1B EC_{50} = 0.6 ng/ml

3. **Small Molecule Inhibitor Testing**
   See small molecule inhibitor testing section

4. **Stealth™ RNAi Testing**
   See Stealth™ RNAi testing section

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

Assay Testing Summary

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

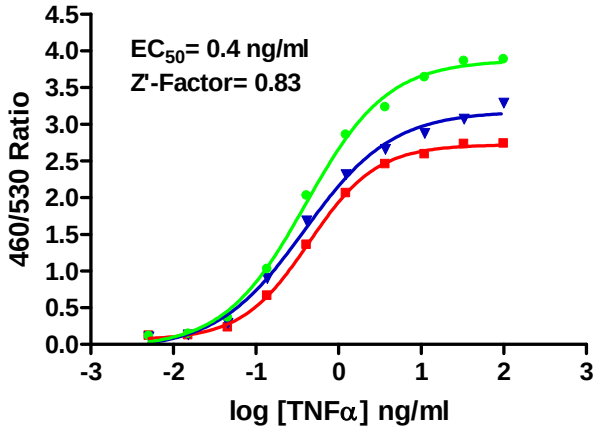
7. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

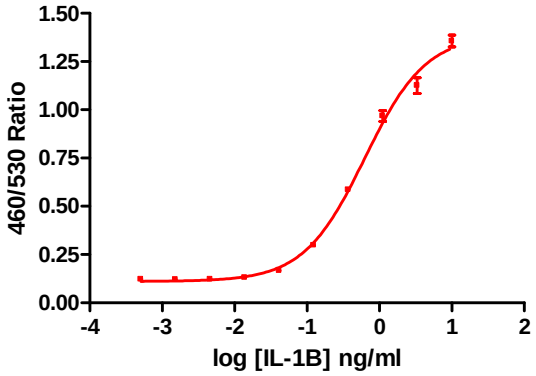
Figure 1 — NFκB-*bla* ME-180 dose response to Tumor Necrosis Factor alpha (TNFα) under optimized conditions



NFκB-*bla* ME-180 cells (12,000 cells/well) were assayed on three separate days represented by the three curves shown on the graph. Cells were plated the day before the assay in a 384-well format and stimulated with TNFα (R&D Systems #210-TA-010) 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 TNFα (n=16 for each data point).

Alternate Agonist Dose Response

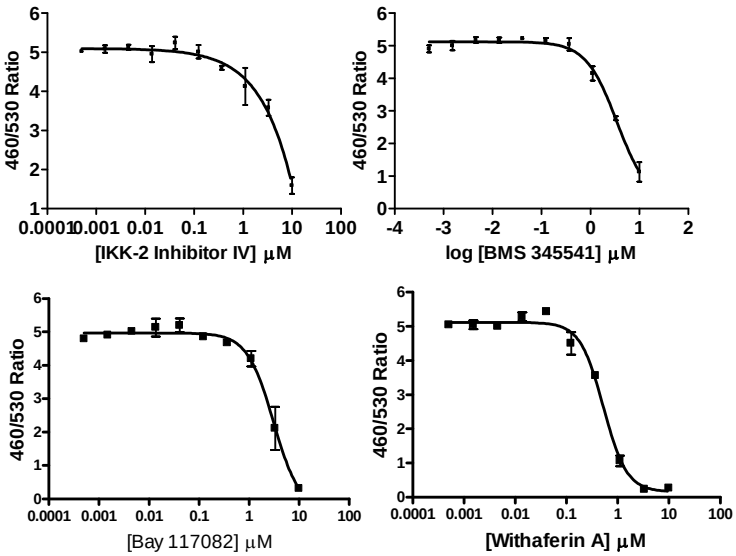
Figure 2 — NFκB-*bla* ME-180 dose response to IL-1B



NFκB-*bla* ME-180 cells (12,000 cells/well) were plated the day before the assay in a 384-well format and stimulated with IL-1B over the indicated concentration range 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 against the indicated concentrations of IL-1B (n=8 for each data point).

Small Molecule Inhibitor Testing

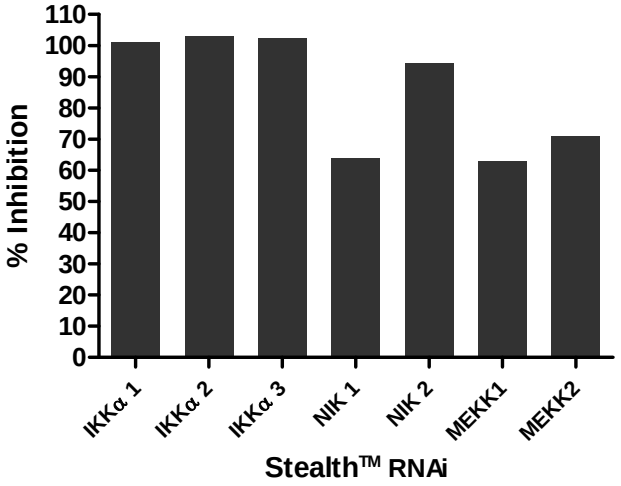
Figure 3 — NFκB-*bla* ME-180 dose response to various small molecule inhibitors



NFκB-*bla* ME-180 cells (12,000 cells/well) were plated in a 96-well format and treated with the listed small molecule inhibitors over the indicated concentration range in the presence of 0.5% DMSO for 30 min. Cells were then stimulated with TNFα for 5 hours, followed by loading of 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 emission ratios plotted against the indicated concentrations of inhibitor (BMS345541 is an IKK-2 III inhibitor).

Stealth™ RNAi Testing

Figure 4 — NFκB-*bla* ME-180 response to various RNAi oligos



NFκB-*bla* ME-180 cells (8,000 cells/well) were plated in a 96-well format and treated Lipofecatmine™ 2000 mixtures containing the listed Stealth™ RNAi oligos for 60 hrs. Following an Assay Media exchange, cells were then stimulated with TNFα for 5 hours, followed by loading of 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 converted to % inhibition.

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% CO_2 incubator. Maintain cells between 10% and 70% confluency. Do not allow cells to reach confluence.

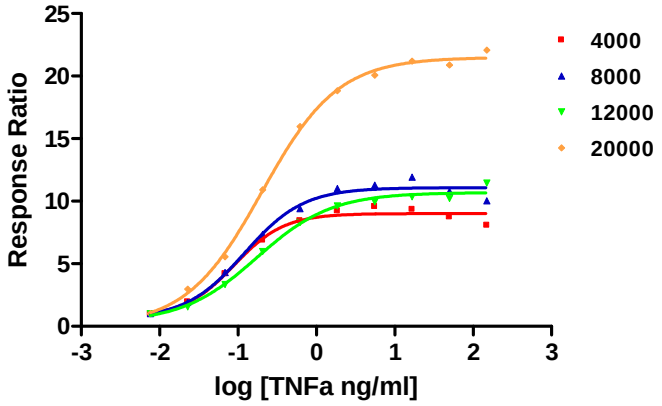
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For optimal cell line performance, use dialyzed FBS (Invitrogen# 26400-036). For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture Media Conditions

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM	90%	—	—
Opti–MEM®	—	99.5%	—
Dialyzed FBS **Do Not Substitute!**	10%	0.5%	—
NEAA	0.1 mM	0.1 mM	—
Sodium pyruvate	--	1 mM	—
HEPES (pH 7.3)	25 mM	—	—
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

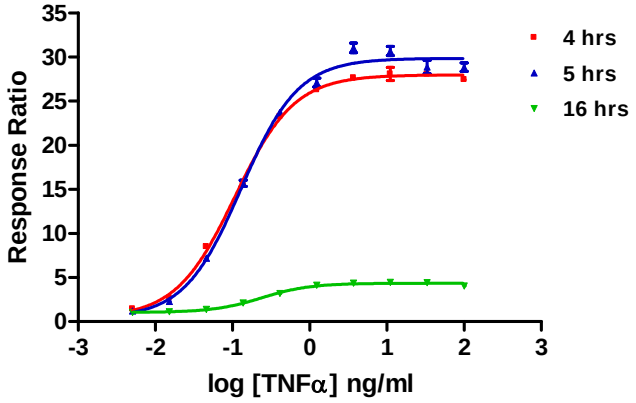
Figure 3 – NFκB-*bla* ME-180 dose response to TNFα with variable cells/well



NFκB-*bla* ME-180 cells were plated the day before the assay in a 384-well assay plate at 4000, 8000, 12000 or 20,000 cells/well. TNFα (R&D Systems #210-TA-010) was then added to the plate over the indicated concentration range and incubated for 5 hrs in 0.5% DMSO. Cells were 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 of each cell number plotted against the indicated concentrations of TNFα (n=8 for each data point).

Assay Performance with Variable Stimulation Time

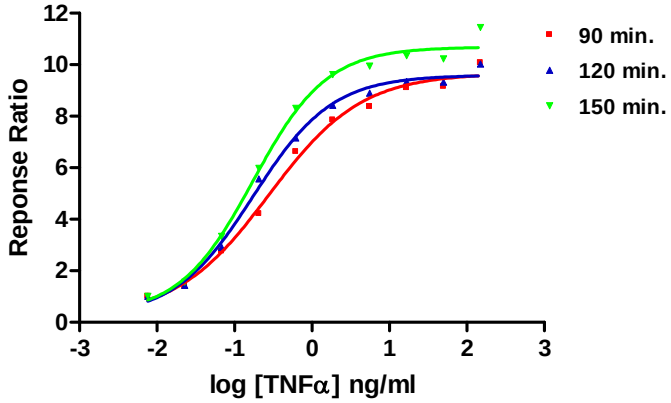
Figure 3 – NFκB-*bla* ME-180 dose response to TNFα with 4, 5and 16 hour stimulation times



NFκB-*bla* ME-180 cells (12,000 cells/well) were plated the day before the assay in a 384-well assay plate. TNFα (R&D Systems #210-TA-010) was then added to the plate over the indicated concentration range. Plates were treated for 4, 5 or 16 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 against the indicated concentrations of TNFα (n=8 for each data point).

Assay Performance with Variable Substrate Loading Time

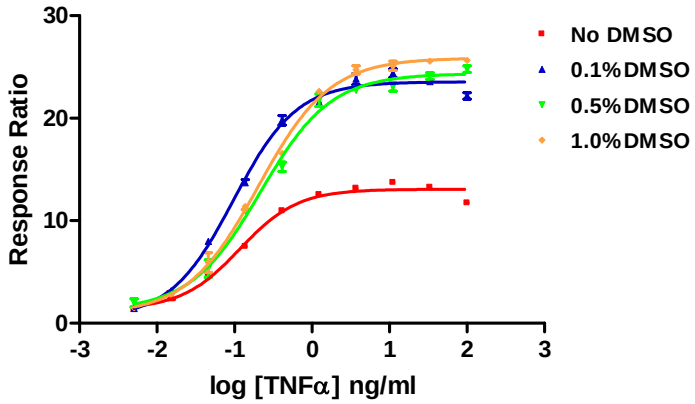
Figure 4 — NFκB-*bla* ME-180 dose response to TNFα with 1, 1.5 and 2 hour substrate loading times



NFκB-*bla* ME-180 cells were plated the day before the assay at 12,000 cells/well in a 384-well format. Cells were treated with TNFα (R&D Systems #210-TA-010) 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 either 90, 120 or 150 minutes. 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]

Figure 5 – NFκB-*bla* ME-180 dose response to TNFα with 0, 0.25, 0.5 and 1% DMSO



NFκB-*bla* ME-180 cells (12,000 cells/well) were plated the day before the assay in a 384-well assay plate. TNFα (R&D Systems #210-TA-010) was then added to the plate over the indicated concentration range with 0, 0.25, 0.5 or 1% final DMSO concentrations. Cells were 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 for each DMSO concentration were plotted against the indicated concentrations of TNFα(n=6 for each data point).