Validation & Assay Performance Summary

invitrogen™

CellSensor® NFκB RAW264.7 Cell Line

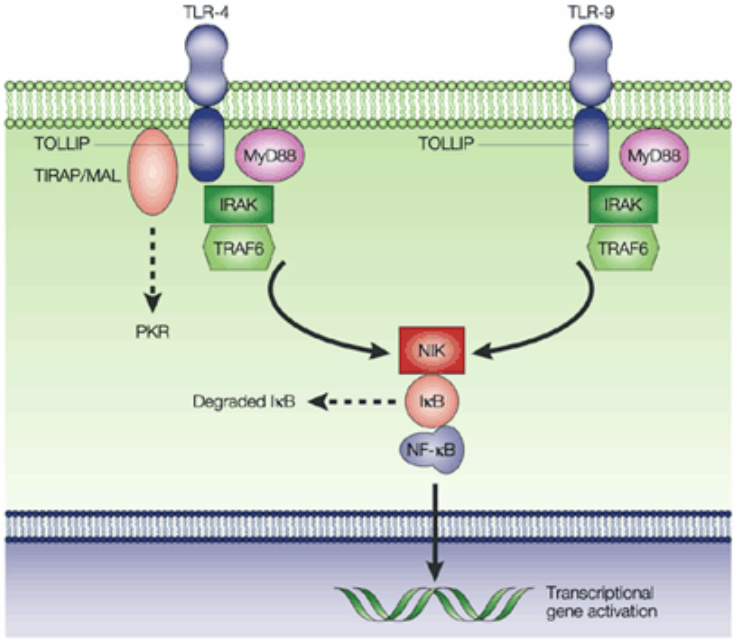
Cat. no. K1673

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

The molecules responsible for coordinating the immune system's recognition of pathogens are toll-like receptors (TLRs) of which there are currently 10 identified families in humans. TLRs are pattern recognition receptors (PRRs), binding to pathogen-associated molecular patterns (PAMPs), small molecular sequences consistently found on pathogens. This binding activates the transcription of immune genes and regulators such as cytokines, chemokines, and co-stimulatory molecules, thereby promoting the initial immune response of macrophages and neutrophils.



Nature Reviews | Drug Discovery

Cell Line Description

The CellSensor® NFκB-*bla* RAW264.7 cell line contains a beta-lactamase reporter gene under control of the NFκB response element stably integrated into mouse monocyte RAW 264.7 cells. This cell line is a clonal population isolated in response Lipopolysaccharide (a TLR4 ligand) 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 EC_{50} concentrations of TLR 7 ligand: Imiquimod. Additional testing information using known inhibitors or activators 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)**

   Imiquimod EC_{50} = 0.54 µg/ml
   Z'-Factor (EC_{100}) = 0.72
   Response Ratio = 5.3

   Optimum cell no. = 20,000 cells/well
   Optimum [DMSO] = 0.0-0.5%
   Optimum Stim. Time = 16 hrs
   Max. [Stimulation] = 5 µg/ml

2. **Alternate Stimuli**
   See Compound Panel Section

3. **Small molecule inhibitor Testing**
   See Compound Panel

4. **Receptor Knockdown by Stealth® RNAi**

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

Assay Testing Summary

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

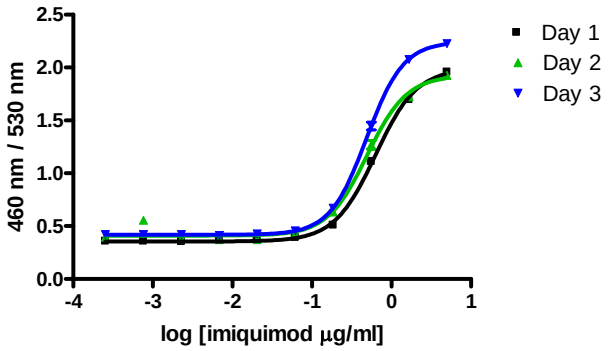
7. **Assay performance with variable stimulation time**

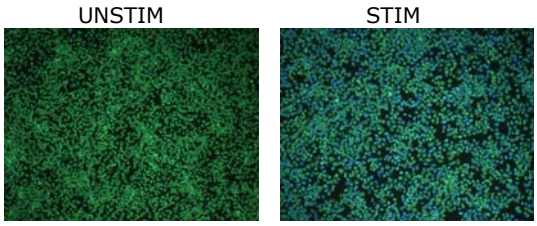
8. **Assay performance with variable substrate loading time**

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

Primary Agonist Dose Response

Figure 1 — Imiquimod dose response under optimized conditions

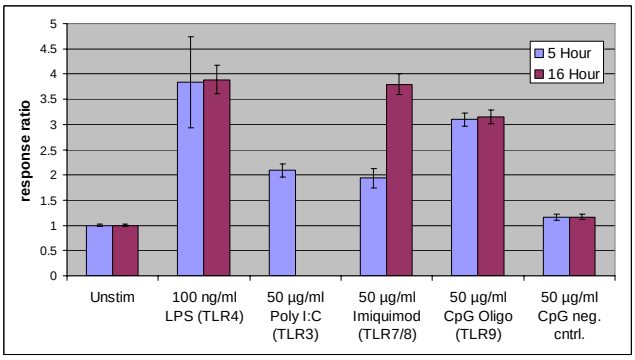




NFκB-*bla* RAW264.7 cells (20,000 cells/well) were assayed on three separate days represented by the three curves shown on the graph. Cells were plated in a 384-well format and were stimulated with imiquimod (EMD Biosciences Cat.No. 401020) over the indicated concentration range in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460nm/530nm ratio plotted for the indicated concentrations of imiquimod (n=16 for each data point). Images of unstimulated and stimulated cells are shown.

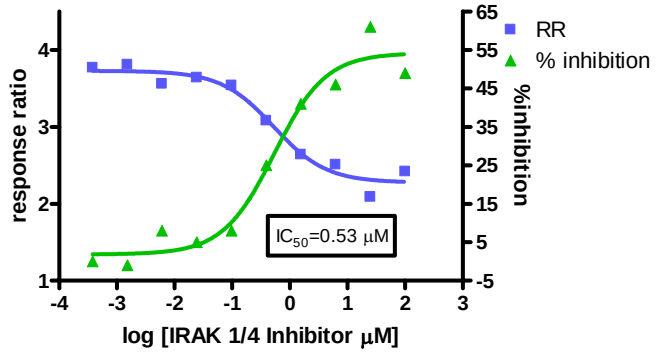
Ligand Panel

Figure 2 — NFκB-*bla* RAW264.7 responds to ligands of various Toll-like Receptors



NFκB-*bla* RAW264.7 cells (20,000 cells/well) were plated in a 384-well format in assay medium. They were treated for 5 or 16 hours with a panel of ligands at the indicated concentrations, 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 Response Ratios plotted for each treatment (n=8 for each data point).

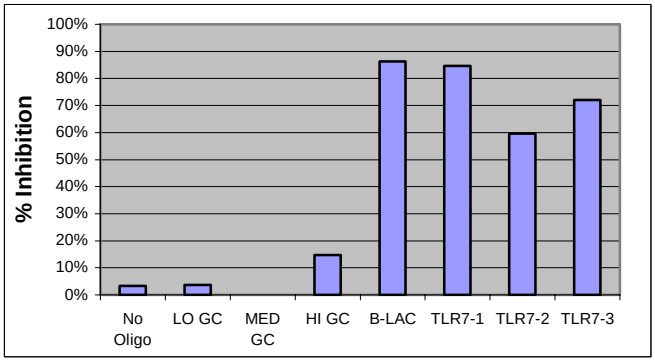
Figure 3 — NFκB-*bla* RAW264.7 response silenced by IRAK 1/4 Inhibitor



NFκB-*bla* RAW264.7 cells (20,000 cells/well) were plated the day prior to the assay in a 384-well format in assay medium They were treated with the indicated concentration of IRAK ¼ Inhibitor (Calbiochem, 407601) and then stimulated with imiquimod (EMD Biosciences Cat.No. 401020)) at the EC_{80} for 16 hours. The cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios plotted for each treatment (n=14 for each data point).

Receptor Knockdown by Stealth® RNAi

Figure 4 — NFκB-*bla* RAW264.7 response silenced by knocking down TLR7 expression with Stealth ® RNAi



NFκB-*bla* RAW264.7 cells (20,000 cells/well) were plated in a 96-well format in assay medium. They were transfected with either no oligo, BlockIT fluorescent transfection-efficiency indicator, one of three different GC-content negative controls (Lo, Med, and Hi), β-lactamase positive control, or with one of three *Mus musculus* TLR7-specific Stealth® RNAi oligos (Invitrogen, 1320003). They were transfected using Lipofectamine 2000 (and incubated for 52 hours. They were then stimulated with imiquimod (EMD Biosciences Cat.No. 401020)) at the EC_{80} for 16 hours. The cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the % Inhibition plotted for each treatment (n=14 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% CO_2 incubator. Maintain cells between 20 and 80% confluence.

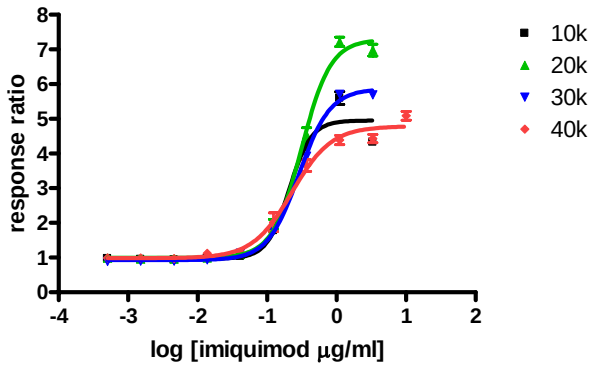
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to the customer protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM w/ GlutaMAX™	90%	--	—
OptiMEM	--	99.5%	
Dialyzed FBS **(do not substitute!)**	10%	0.5%	—
HEPES	25 mM	25 mM	
NEAA	0.1 mM	0.1 mM	—
Sodium Pyruvate	--	1 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

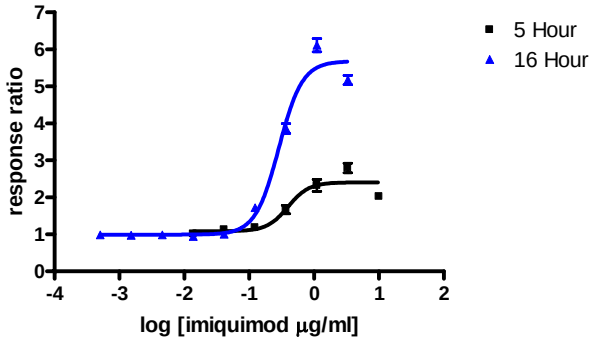
Figure4 — Imiquimod dose response with different plating cell numbers/well



NFκB-*bla* RAW264.7 cells were plated with indicated number of cells/well in a 384-well format in assay medium. They were stimulated with indicated concentration of imiquimod (EMD Biosciences Cat.No. 401020) for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 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=8 for each data point).

Assay Performance with Variable Stimulation Time

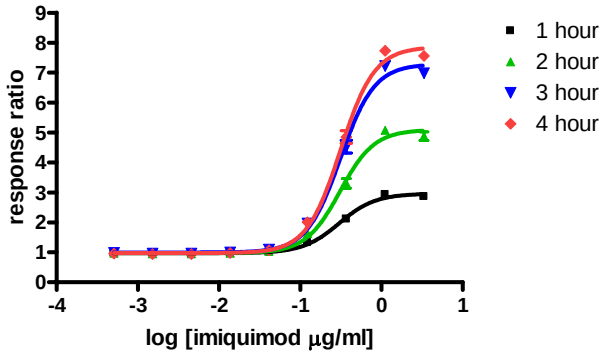
Figure 5 – Imiquimod dose response with 5 hour and 16 hour stimulation times



NFκB-*bla* RAW264.7 cells were plated at 20,000 cells/well in a 384-well format in assay medium. "16 hour" cells were immediately treated with imiquimod (EMD Biosciences Cat.No. 401020) for 16 hours. "5 hour" cells were incubated for 16 hours then stimulated with imiquimod for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 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 imiquimod (n=8 for each data point).

Assay Performance with Variable Substrate Loading Time

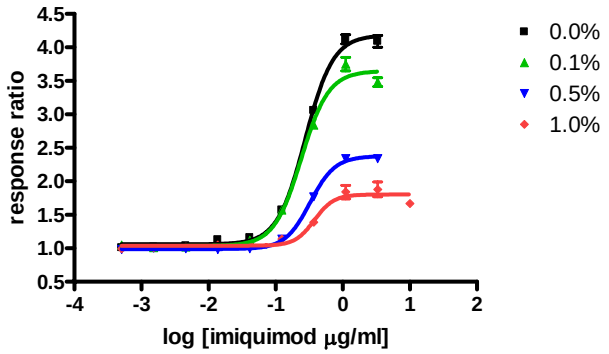
Figure 6 — Imiquimod dose response with various substrate loading times



NFκB-*bla* RAW264.7 cells were plated at 20,000 cells/well in a 384-well format in assay medium. They were stimulated with indicated concentration of imiquimod (EMD Biosciences Cat.No. 401020) for 16 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 imiquimod (n=8 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 7 – Imiquimod dose response with 0, 0.1, 0.5 and 1% DMSO



NFκB-*bla* RAW264.7 cells were plated at 20,000 cells/well in a 384-well format in assay medium. They were stimulated with imiquimod (EMD Biosciences Cat.No. 401020) in the presence of indicated amount of DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 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 imiquimod (n=8 for each data point).

References

Zuany-Amorim, C., Hastewell, J., Walker, C. Toll-like Receptors as Potential Therapeutic Targets for Multiple Diseases. *Nature Reviews Drug Discovery*. **1,** 797-807 (2002).

Krieg, Arthur. CpG motifs: the active ingredient in bacterial extracts? *Nature Medicine*. **9,** 831-835 (2003).

Chow, Jesse *et al.* Toll-like Receptor-4 Mediates Lipopolysaccharide-induced Signal Transduction. *J.of Biological Chemistry*. **274**, 10689-10692 (1999).