Validation & Assay Performance Summary

CellSensor® ISRE RA-1 Validated Assay

Cat. no. K1674

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.

Target Description

Toll-like receptors (TLRs) are responsible for coordinating the immune system's recognition of pathogens. There are 10 different TLRs in humans (TLRs 1-10). The various TLRs are expressed in different tissues, respond to different ligands, and play diverse roles in host defense. TLR3 plays a role in host defense against viruses. TLR3 recognizes dsRNA associated with viral infection, and induces the activation of NFkB and the production of type I interferons (4). The pathway involved in response to viral infection is distinct from the interferon signaling pathway (1). Following virus infection and TLR3 activation by dsRNA, IRF3 (interferon regulatory factor 3) is phosphorylated on specific Ser and Thr residues by the kinases TBK1 and IKK epsilon. Phosphorylation results in translocation of IRF3 from the cytoplasm to the nucleus and interaction with the co-activator CREB-binding protein (CBP)/p300. Once in the nucleus, IRF3 binds to a DNA sequence containing the interferon-stimulated response element (ISRE) and increases transcriptional activation of interferon-responsive genes (2,3,6,7,8). The CellSensor™ ISRE-*bla* RA-1 Cell Line contains the interferon-stimulated response element (ISRE) upstream of *bla* integrated into RA-1 cells, a human B cell lymphoma. ISRE-*bla* RA-1 cells are responsive to Poly I:C, a double-stranded RNA agonist for TLR3 mimicking viral infection.

Cell Line Description

The CellSensor™ ISRE RA-1 cells contain the beta-lactamase gene under the control of the interferon stimulated response element (ISRE). This cell line was engineered by transduction of the ISRE-*bla* construct into RA-1 cells by lentivirus. Flow cytometry was then used to isolate a pool of cells responsive to Poly I:C stimulation. ISRE RA-1 cells have been tested for assay performance using variable assay conditions, including DMSO concentration, cell number, stimulation time, substrate loading time and have been validated for Z' and EC_{50} concentrations of Polyinosinic–polycytidylic acid (Poly I:C). Additional testing data using alternate stimuli are also provided.

Validation Summary

Performance of this assay was validated under optimized conditions in 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **Primary agonist dose response under optimized conditions (n=3)**

   Poly I:C EC_{50} = 4.9 ug/ml
   Z'-Factor (EC_{100}) = 0.77
   Response Ratio = 15.7

   Optimum cell no. = 50K cells/well
   Optimum [DMSO] = up to 0.5%
   Stimulation Time = 16-18 hours for agonist assay, 5 hours for inhibitor assay
   Max. [Stimulation] = 25 ug/ml Poly I:C

2. **Alternate TLR ligand dose response**

   The ISRE RA-1 cell line is very specific for TLR3/Poly I:C, there was no response to other TLR ligands.

3. **Inhibitor dose response**
   See Inhibitor dose response section

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

Assay Testing Summary

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

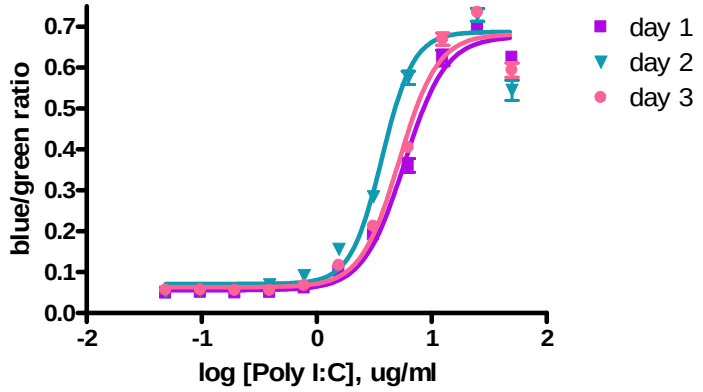
6. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

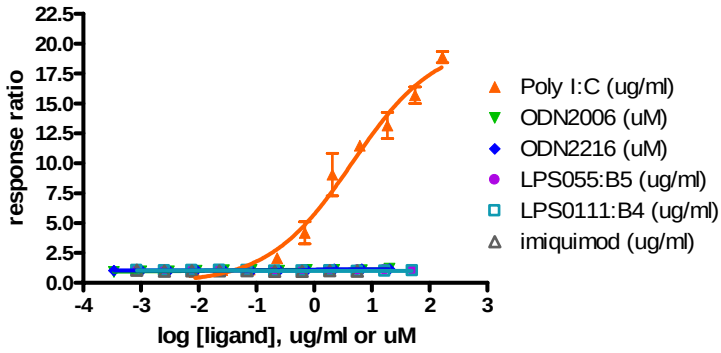
Figure 1 —Poly I:C dose response under optimized conditions



ISRE RA-1 cells were assayed on three separate days. The day of the assay, cells were plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate and stimulated with Poly I:C (Sigma #P9582) over the indicated concentration range in the presence of 0.1% DMSO for 18 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the ratios plotted against the indicated concentrations of Poly I:C (n= 16 for each data point).

TLR Ligand Panel Dose Response

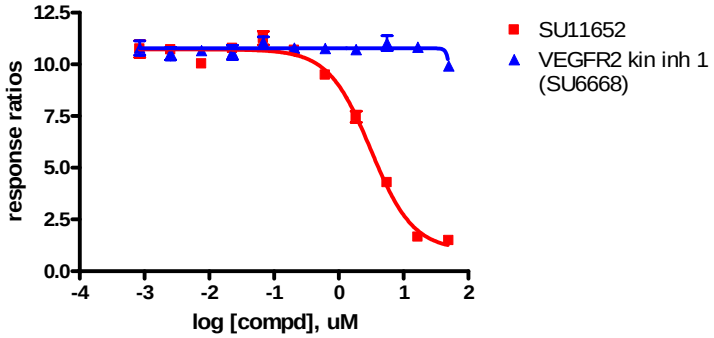
Figure 2 —TLR ligand panel dose response



ISRE RA-1 cells were plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate the day of the assay. Cells were stimulated with either Poly I:C (Sigma #P9582), ODN2006 or ODN2216 (custom CpG oligos from Invitrogen, ligands for TLR9), LPS055:B5 or LPS0111:B4 (Sigma #L6529 and# L4391, ligands for TLR4), or Imiquimod (EMD Biosciences #401020, ligand for TLR7/8) over the indicated concentration range for ~16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the ratios plotted against the indicated concentrations of the ligands (n= 3 for each data point).

Inhibitor Dose Response

Figure 3 — Inhibitor dose response



ISRE RA-1 cells were plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate. Cells were treated with inhibitors SU11652 (Calbiochem # 572660) or VEGF Receptor 2 Kinase Inhibitor 1 (SU6668) (Calbiochem #676480) and incubated at 37 degrees C for 30 min., followed by EC_{80} Poly I:C stimulation for 5 hours. Cells were then loaded for 3 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 ratios are shown plotted against the indicated concentrations of inhibitor. The IC_{50} of SU11652 is 3 uM. (n= 4 for each data point).

Cell Culture and Maintenance

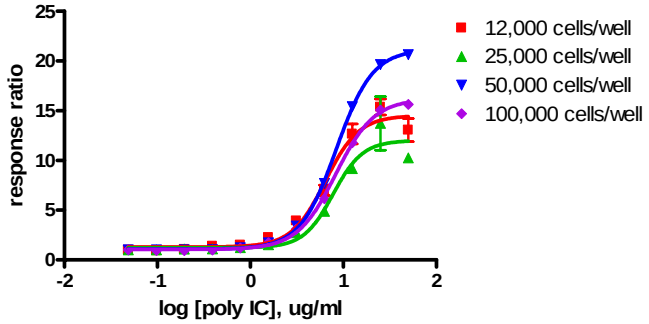
Cells should be maintained at between 0.2 and 1.5 million cells/mL in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split cells at least twice a week. Do not allow cells to exceed 1.5 million cells/mL.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium (–)	Growth Medium (+)	Assay Medium	Freeze Medium
RPMI 1640	90%	90%	90%	—
Heat-Inactivated FBS **Do not substitute!**	10%	10%	10%	—
NEAA	0.1 mM	0.1 mM	0.1 mM	—
Sodium Pyruvate	1 mM	1 mM	1 mM	—
Blasticidin	—	5 µg/mL	—	—
Penicillin	100 U/mL	100 U/mL	100 U/mL	—
Streptomycin	100 µg/mL	100 µg/mL	100 µg/mL	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

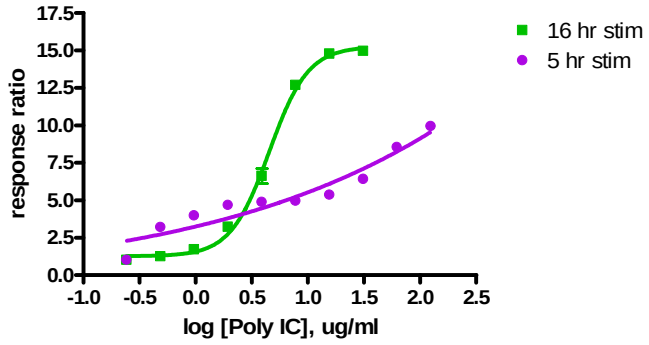
Figure 5— Poly I:C dose response with 12, 25, 50, and 100K cells/well



ISRE RA-1 cells were plated at 12,000, 25,000, 50,000, or 100,000 cells/well in a 384-well black-walled tissue culture assay plate the day of the assay. Cells were stimulated with Poly I:C (Sigma #P9582) in the presence of 0.5% DMSO for ~18 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) 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 against the indicated concentrations of Poly I:C (n=8 for each data point).

Assay performance with Variable Stimulation Time

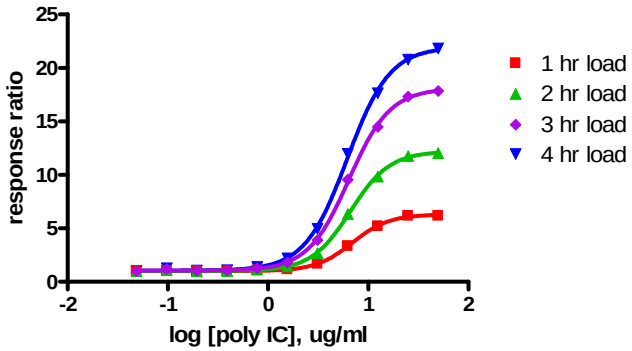
Figure 6 – Poly I:C dose response with 5 and 16 hour stimulation times



ISRE RA-1 cells were plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate the day of the assay. Poly I:C (Sigma #P9582) was then added to the plate over the indicated concentration range for 5 or 16 hours in 0.5% DMSO and then loaded for 3 hours with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios plotted (n=8 for each data point).

Assay performance with Variable Substrate Loading Time

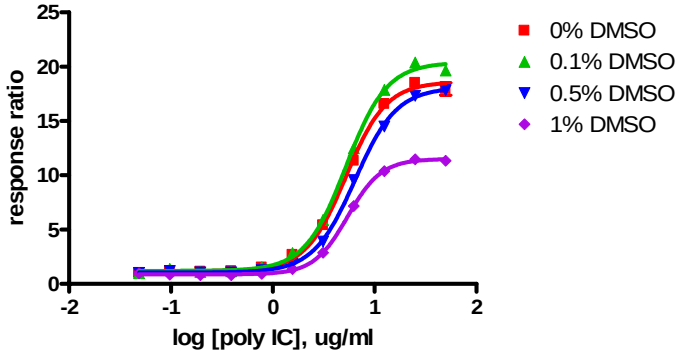
Figure 7 – Poly I:C dose response with 1, 2, 3, and 4 hour loading times



ISRE RA-1 cells were plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate the day of the assay. Cells were stimulated with Poly I:C (Sigma #P9582) in the presence of 0.5% DMSO for 17 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for either 1, 2, 3, or 4 hours. Fluorescence emission values at 460 nm and 530 nm for the various loading times were obtained using a standard fluorescence plate reader and the Response Ratios plotted against the indicated concentrations of Poly I:C (n=8 for each data point).

Assay Performance with variable DMSO concentration

Figure 8 – Poly I:C dose response with 0, 0.1, 0.5 and 1% DMSO.



ISRE RA-1 cells were plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate the day of the assay. Poly I:C (Sigma #P9582) was then added to the plate over the indicated concentration range. DMSO was added to the assay at concentrations from 0% to 1%. Cells were stimulated for 17 hrs with agonist and loaded for 3 hours with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios are shown plotted for each DMSO concentration against the indicated concentrations of Poly I:C (n=8 for each data point).

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