Validation & Assay Performance Summary

CellSensor® NFAT-*bla* RA-1 Validated Assay

Cat. no. K1490

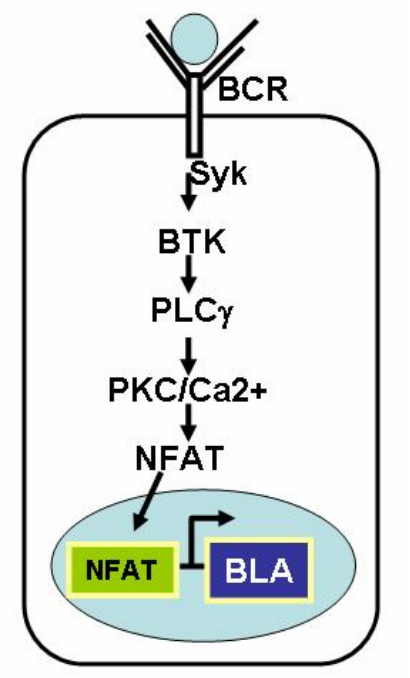
CellSensor® Cell-Based Assay Validation Packet

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

B cell receptor (BCR) mediated signaling pathways are important for B cell proliferation and differentiation. Abnormal B cell signaling has been linked to various diseases such as Lupus, lymphoma and various immune disorders.
Binding of antigen to the BCR promotes the activation of several protein tyrosine kinases (PTK) which leads to phosphorylation of the BCR complex, the recruitment and activation of the PTK Syk, which in turn promotes phosphorylation of PLCγ, Shc and Vav. Additionally, the Tec family member Btk is recruited to the plasma membrane where it is involved in activation of PLCγ. Initiation of B lymphocyte activation is dependent on the tyrosine phosphorylation-dependent formation of multi-molecular effector protein complexes that activate downstream signaling pathways including PKC/Ca2+/NFAT.

Cell Line Description



The CellSensor® NFAT RA-1 cells contain the beta-lactamase gene under the control of the Nuclear Factor of Activated T cells (NFAT) response element stably integrated into RA-1 cells. This cell line was engineered by transduction of the NFAT-*bla* construct into RA-1 cells by lentivirus. Flow cytometry was then used to isolate a pool of cells responsive to anti-IgM stimulation. NFAT 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 anti-IgM. 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)**

   Anti-IgM EC_{50} = 4.5 ug/ml
   Z'-Factor (EC_{100}) = 0.66
   Response Ratio = 8.7

   Optimum cell no. = 50K cells/well
   Optimum [DMSO] = up to 1%
   Stimulation Time = 5 hours
   Max. [Stimulation] = 25 μg/ml Anti-IgM

2. **Alternate ligands**
   The NFAT RA-1 cell line is very specific for Anti-IgM.

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

4. **Cryopreserved cell testing**

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

Assay Testing Summary

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

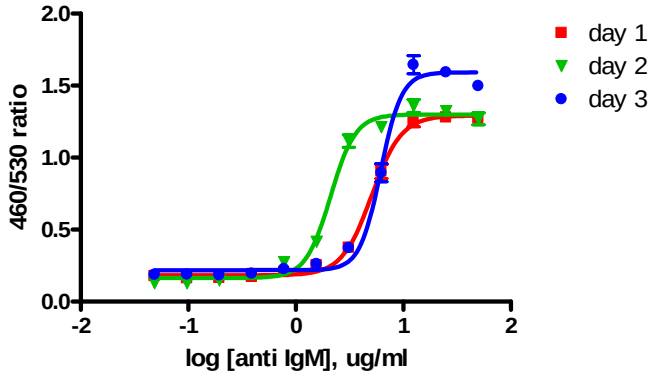
7. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

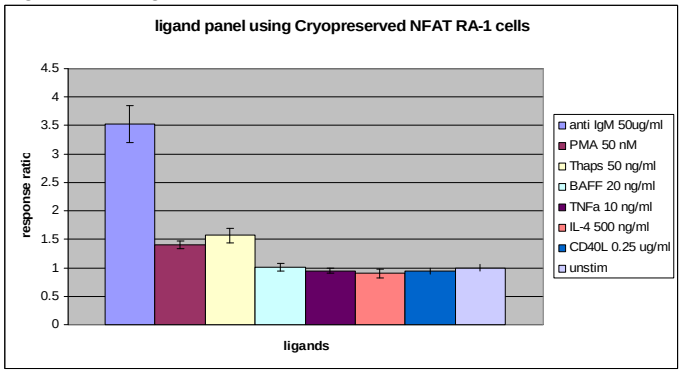
Figure 1 —Anti-IgM dose response under optimized conditions



NFAT RA-1 cells were assayed on three separate days. The cells were starved overnight in a flask and then plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate. Cells were stimulated with Anti-IgM (Invitrogen, #AHI0601) over the indicated concentration range in the presence of 0.1% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1μM final concentration of CCF4-AM) for 2 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 Anti-IgM (n= 16 for each data point).

Response to Alternate Ligands

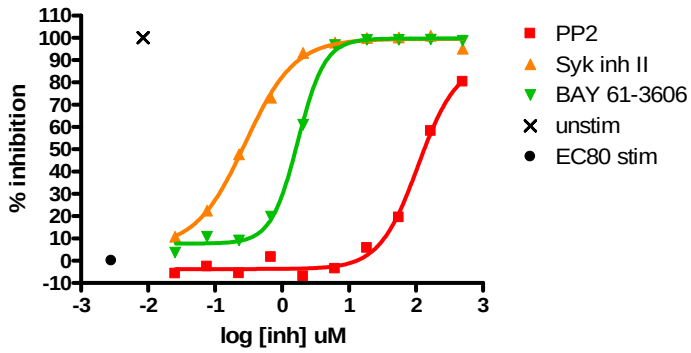
Figure 2 —Ligand Panel



Cryopreserved NFAT RA-1 cells were thawed from liquid nitrogen, plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate, and starved overnight in the assay plate. Cells were stimulated with either Anti-IgM (Invitrogen, #AHI0601), PMA (Sigma, #P1585), Thapsigargin (Sigma, #T9033), BAFF (R&D Systems, #2149-BF), TNFa (Invitrogen, #PHC3011), IL-4 (Invitrogen, #PCH0044), or CD40L (Invitrogen, #PHP0025) at the indicated concentrations for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1μM final concentration of CCF4-AM) 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 the ligands (n= 8 for each data point).

Inhibitor Dose Response

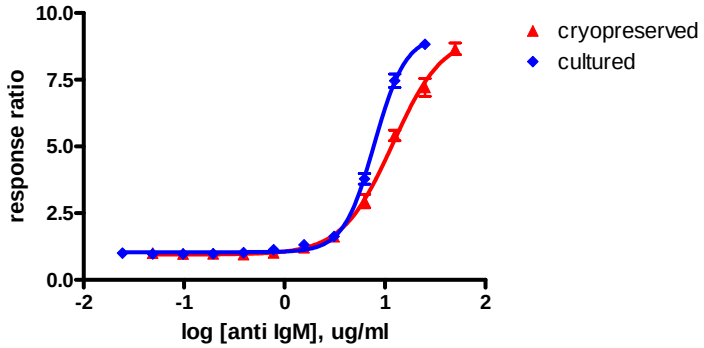
Figure 3 — Inhibitor dose response



NFAT RA-1 cells were starved overnight in a flask and then plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate. Cells were treated with inhibitors PP2 (Calbiochem # 529573), Syk inhibitor II (Calbiochem # 574712), or BAY 61-3606 (Sigma # B9685)and incubated at 37 degrees C for 30 min., followed by EC_{80} Anti-IgM stimulation for 5 hours. 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 % inhibition plotted against the indicated concentrations of inhibitor. The IC_{50} values of PP2, Syk inhibitor II, and BAY 61-3606 are 108 uM, 0.28 uM, and 1.7 uM respectively. (n= 4 for each data point).

Cryopreserved cells

Figure 4 — Cryopreserved vs. cultured cells



Cryopreserved NFAT RA-1 cells were thawed from liquid nitrogen, plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate, and starved overnight in the assay plate. Cultured NFAT RA-1 cells were starved overnight in a flask, and then plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate. Cells were stimulated with Anti-IgM (Invitrogen #AHI0601) over the indicated concentration range in the presence of 0.1% DMSO for 5 hours. 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 plotted against the indicated concentrations of inhibitor. (n= 8 for each data point).

Cell Culture and Maintenance

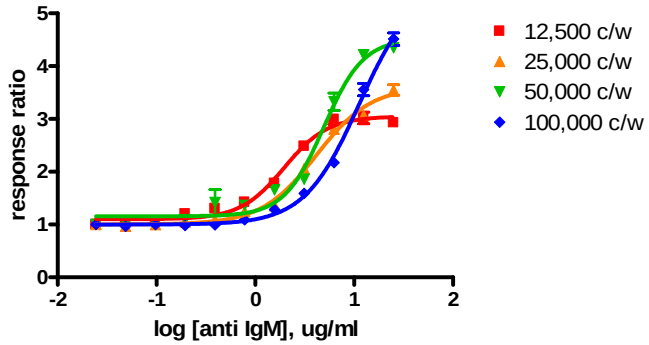
Cells should be maintained at between 0.2 and 1.5 million cells/mL in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split cells at least twice a week. Do not allow cells to exceed 1.5 million cells/mL.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium (–)	Growth Medium (+)	Assay Medium	Freeze Medium
RPMI 1640	90%	90%	—	—
Opti-MEM	—	—	99.5%	—
Heat-Inactivated FBS **Do not substitute!**	10%	10%	—	—
Dialyzed FBS	—	—	0.5%	—
NEAA	0.1 mM	0.1 mM	0.1 mM	—
Sodium Pyruvate	1 mM	1 mM	1 mM	—
HEPES	—	—	10mM	
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

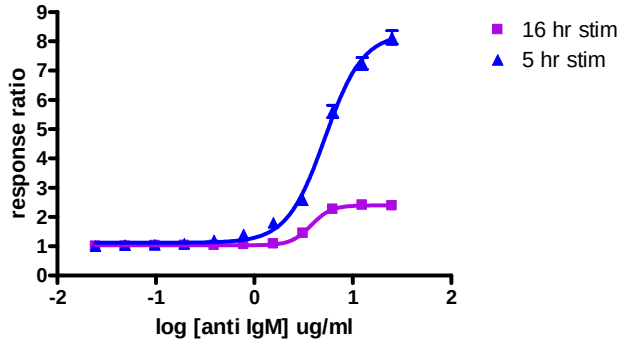
Figure 5— Anti-IgM dose response with 12, 25, 50, and 100K cells/well



NFAT RA-1 cells were starved overnight in a flask and then 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 Anti-IgM (Invitrogen #AHI0601) in the absence of DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 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 Anti-IgM (n=8 for each data point).

Assay performance with Variable Stimulation Time

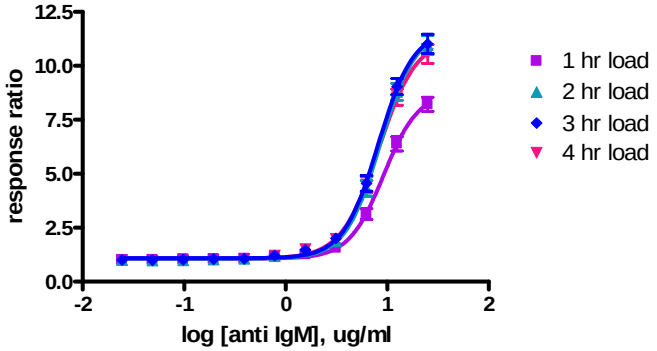
Figure 6 – Anti-IgM dose response with 5 and 16 hour stimulation times



NFAT RA-1 cells were starved overnight in a flask and then plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate the day of the assay. Anti-IgM (Invitrogen #AHI0601) was then added to the plate over the indicated concentration range for 5 or 16 hours in the absence of DMSO and then loaded for 2 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

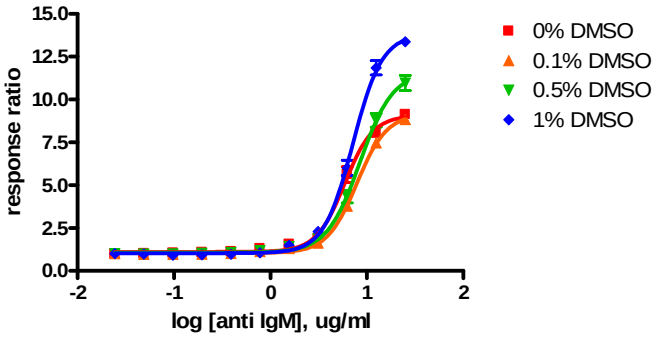
Figure 7 – Anti-IgM dose response with 1, 2, 3, and 4 hour loading times



NFAT RA-1 cells were starved overnight in a flask and then 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 Anti-IgM (Invitrogen #AHI0601) in the presence of 0.5% DMSO for 5 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 Anti-IgM (n=8 for each data point).

Assay Performance with variable DMSO concentration

Figure 8 – Anti-IgM dose response with 0, 0.1, 0.5 and 1% DMSO.



NFAT RA-1 cells were starved overnight in a flask and then plated at 50,000 cells/well in a 384-well black-walled tissue culture assay plate the day of the assay. Anti-IgM (Invitrogen #AHI0601) 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 5 hrs with agonist and loaded for 2 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 Anti-IgM (n=8 for each data point).