Validation & Assay Performance Summary

CellSensor® NFAT-*bla* Jurkat Cell Line

Cat. no. K1671

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

T cell receptor signaling plays an important role in T cell activation, proliferation and differentiation leading to the initiation of the adaptive immune response. The binding of T cell antigen receptors (TCRs) with their ligands, major histocompatibility molecule-peptide complex (MHC-peptide) initiates series tyrosine phosphorylation events that trigger multiple signaling pathways, one of which is the activation of Ca2+/protein kinase C pathways. The increased intracellular calcium levels and the activation of PKCs leads to the activation the nuclear factor of activator T cells (NFAT) transcription factor resulting in downstream gene expression. Cross-linking TCRs by anti-CD3/CD28, thapsigargin or ionomycin and PMA can be used to raise intracellular calcium levels, and activate PKC. This cell line can also be used as a parental cell line to build specific GPCR assays after transfection of additional genes of interest or to detect changes in intracellular calcium levels.

Cell Line Description

The CellSensor® NFAT-*bla* Jurkat cell line contains a beta-lactamase reporter gene under the control of the NFAT response element stably integrated into Jurkat cells. This cell line has been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time. Z' and EC_{50} concentrations have been determined using PMA and ionomycin as well as anti-CD3/CD28. 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 96-well format using LiveBLAzer™-FRET B/G Substrate.

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

   Anti-CD3/CD28 EC_{50} = 0.19 nM (29 ng/ml)
   Z'-Factor (EC_{100}) = 0.66
   Response Ratio = 4.3

   Optimum cell no. = 25,000 cells/well
   Optimum [DMSO] = 0.0-1.0%
   Optimum Stim. Time = 5 hrs
   Max. [Stimulation] = 2 nM

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

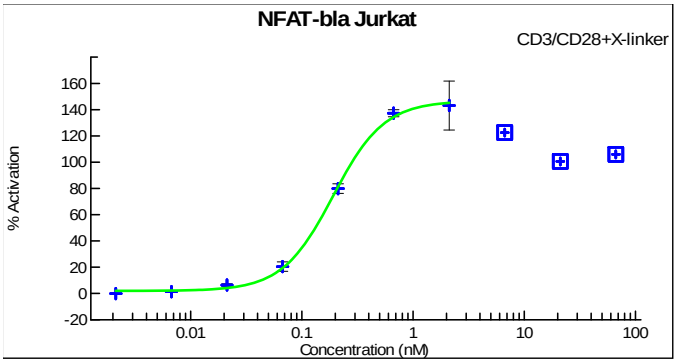
3. **Small molecule inhibitor Testing**
   See Compound Panel

4. **Assay performance with Cryopreserved cells**

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

Primary Agonist Dose Response

Figure 1 — Anti-CD3/CD28 dose response under optimized conditions

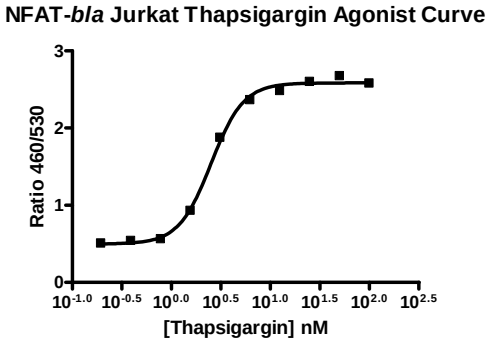


NFAT-*bla* Jurkat cells (25,000 cells/well) were plated in a 384-well format and were stimulated with anti-CD3/anti-CD28 (BD Pharmingen/555329 and 555725) plus cross-linker (Pierce/31160) over the indicated concentration range in the presence of 0.1% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the percent activation plotted for the indicated concentrations of antibodies (n=4 for each data point).

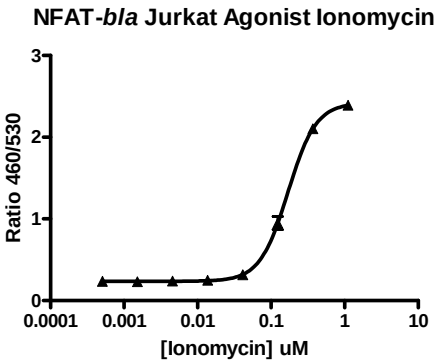
Alternate Stimuli

Figure 2 — NFAT-*bla* Jurkat cells respond to Thapsigargin, Ionomycin and PMA plus Ionomycin

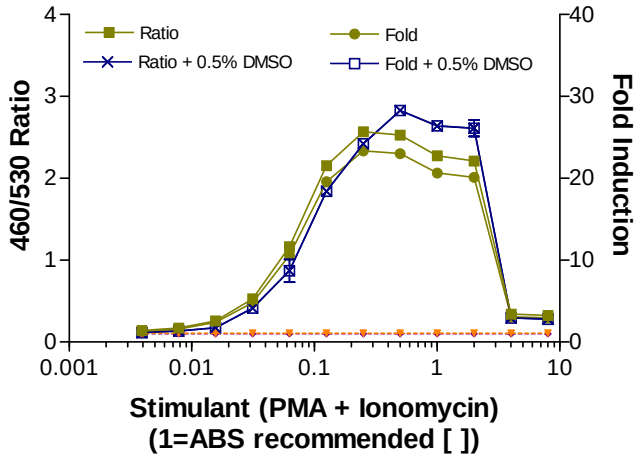
A.



B.



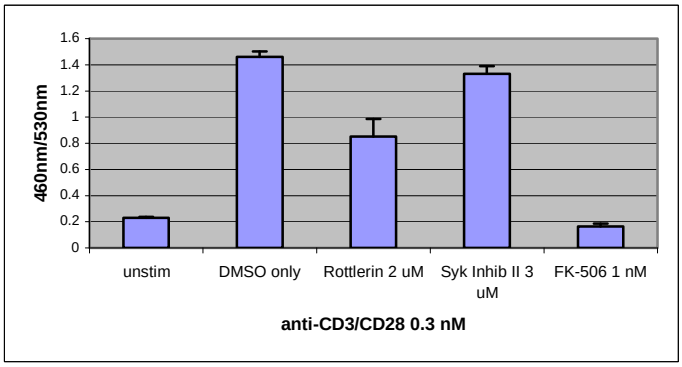
C.



NFAT-*bla* Jurkat cells (25,000 cells/well) were plated in a 384-well format in assay medium. They were treated for 5 hours with the indicated concentrations of thapsigargin (A, Sigma/T9033), Ionomycin (B) and PMA plus Ionomycin (C) 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 460nm/530nm ratio is plotted for each treatment (n=8 for each data point).

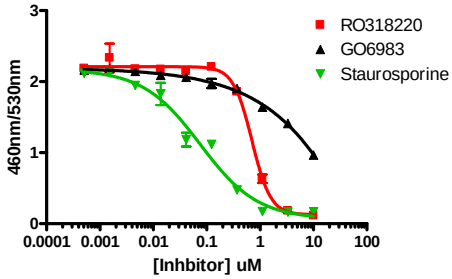
Inhibitor Testing

Figure 3 — Inhibition of anti-CD3/CD28-induced NFAT-*bla* Jurkat by Rottlerin, RO-31-8220 and FK506



NFAT-*bla* Jurkat cells (25,000 cells/well) were plated in a 384-well format in assay medium. They were treated with indicated inhibitors for 30 min and then with EC80 (0.4 nM) concentrations of anti-CD3/CD28 with cross-linker for 5 hours before cells were 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 460nm/530nm ratio is plotted for each treatment (n=4 for each data point).

Figure 4 — Inhibition of Thapsigargin-induced NFAT-*bla* Jurkat by RO-31-8220, Staurosporine and GO6983



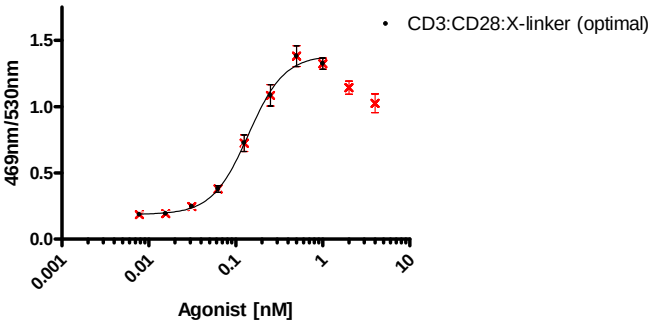
	RO318220	GO6983	Staurosporine
Sigmoidal dose-response (variable slope)			
Best-fit values			
BOTTOM	0.1261	-6.712	0.06472
TOP	2.211	2.190	2.173
LOGEC50	-0.1518	2.843	-1.122
HILLSLOPE	-2.481	-0.4338	-0.8081
EC50	0.7050	697.1	0.07544

NFAT-*bla* Jurkat cells (25,000 cells/well) were plated in a 384-well format in assay medium. They were treated with indicated concentrations of inhibitors for 30 min and then with EC80 (8 nM) concentration of thapsigargin (Sigma/T9033) for 5 hours before cells were 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 460nm/530nm ratio is plotted for each treatment (n=4 for each data point).

Cryopreserved Cell Testing

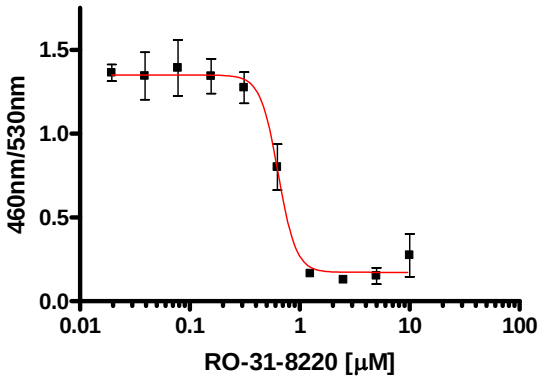
Figure 5 — Inhibition of anti-CD3/CD28-induced NFAT-*bla* Jurkat by RO-31-8220 using cryopreserved cells

A.



	CD3:CD28:X-linker
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.1860
TOP	1.388
LOGEC50	-0.8561
HILLSLOPE	2.094
EC50	0.1393

B.



	RO-31-8220
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.1720
TOP	1.349
LOGEC50	-0.1957
HILLSLOPE	-5.351
EC50	0.6372

Cryopreserved NFAT-*bla* Jurkat cells were thaw and resuspended in Assay medium directly and were plated (25,000 cells/well) in a 384-well format and were stimulated with anti-CD3/anti-CD28 (BD Pharmingen/555329 and 555725) plus cross-linker (Pierce/31160) 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 for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the percent activation plotted for the indicated concentrations of antibodies (n=4 for each data point) and shown in **A**. For **B**, cells were pretreated with indicated concentrations of RO-31-8220 for 30 minutes before stimulation.

Cell Culture and Maintenance

Thaw cells in Growth Medium without zeocin and culture them in Growth Medium with zeocin. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 0.5 x 10^5 and 1 x 10^6 cells/ml.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	90%	--	—
OptiMEM	--	99.5%	
Dialyzed FBS **(do not substitute!)**	10%	0.5%	—
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	—
Zeocin (antibiotic)	50 μg/ml	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%