Validation & Assay Performance Summary

LanthaScreen™-STAT1 U2OS

Cat. no. K1469

Modification Detected: Phosphorylation of Tyr701

LanthaScreen Cellular Kinase 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 / Assay Description

The JAK/STAT1 signaling pathway is known to be activated by type I/II interferons such as Interferon-γ (IFN–γ). In this pathway, binding of these cytokines to their respective cell surface receptors results in the activation of JAKs, which in turn phospho-activate STAT1 proteins at a specific tyrosine residue (Tyr-701). LanthaScreen™-STAT1 U2OS is a human cell line which constitutively expresses GFP-STAT1 fusion proteins. The JAK/STAT signaling pathway is known to be functionally intact in this cell line, therefore the GFP-STAT1 fusion protein serves as a substrate for the IFN–γ-inducible phosphorylation by JAKs. Using this cell line, a homogenous immuno-assay has been developed in which the phosphorylation state of GFP-STAT1 is detected in cell lysates using a terbium-labeled anti-pTyr-701-STAT1 antibody, in a time-resolved FRET (TR-FRET) readout.

The GFP-STAT1 DNA expression construct was transfected into U2OS cells using Lipofectamine™ LTX, followed by selection with Blasticidin. This cell line is a clonal population isolated by flow cytometry using GFP fluorescence as sorting marker. Using the lytic TR-FRET immuno-assay, this cell line is validated for EC_{50} and Z' under optimized conditions using IFN–γ as ligand for JAK-mediated GFP-STAT1 phosphorylation. This assay has also been tested for assay performance under variable experimental conditions, including cell plating density, stimulation time, DMSO tolerance and assay development time. Additional information using alternate stimuli and alternate assay protocol is also provided.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using LanthaScreen™ Tb-anti-STAT1 (pY701) antibody.

1. **Primary agonist dose response under optimized conditions (Average of 3 experiments)**

Z'-Factor (EC_{100})	= 0.70
Relative Response Ratio	= 2.9x
EC_{50} IFN-γ	= 0.39 ng/mL
Recommended cell no. cells/well	= 12000
Recommended [DMSO]	= 0.1%
Recommended Stim. Time	= 60 min
Recom. Assay incubation	= 60 min
Max. [Stimulation]	= ~20 ng/mL

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

Assay Testing Summary

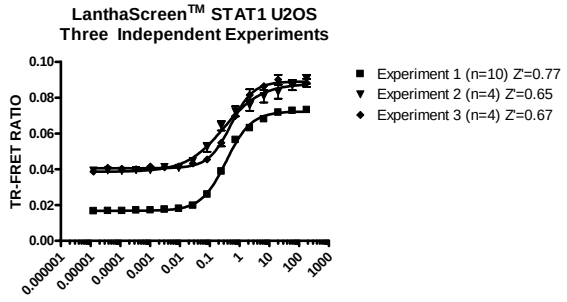
3. **Optimization of Assay Parameters**

4. **Alternate Agonist Profiles**

5. **Stealth RNAi™ Analysis**

Determination of maximum assay window

Figure 1 — IFN-γ induced STAT1 phosphorylation in LanthaScreen™-STAT1 U2OS cells under optimized conditions



	Experiment 1 (n=10) Z'=0.77	Experiment 2 (n=4) Z'=0.65	Experiment 3 (n=4) Z'=0.67
BOTTOM	0.01682	0.03859	0.04055
TOP	0.07228	0.08780	0.08898
LOGEC50	-0.4407	-0.5201	-0.2858
HILLSLOPE	1.035	0.6895	1.156
EC50	0.3625	0.3019	0.5179

LanthaScreen™-STAT1 U2OS (12000 cells/well in 32 µL of assay medium, 384-well format) were assayed on three separate days represented by the three dose response curves shown on the graph. Cells were plated the day prior to the assay. On the day of the assay, cells were pretreated with 4 µL of 1% DMSO before treatment with the indicated concentration of IFN-γ (4 µL addition) for 60 minutes. Cells were lysed by addition of 30 µl assay buffer (to 70 µL total volume), which included 5 nM of Tb-anti-pSTAT1 (pY701) antibody, and incubated for 60 min at room temperature. Fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. The 520/490 nm Emission Ratios are plotted for each experiment, with the number of replicates indicated.

Note: The use of lysis buffer containing freshly added NaPP or $NaVO_4$ (phosphatase inhibitors), may result in lower TR-FRET response ratios (as seen above). However, the data quality (Z') and EC_{50} are not appreciably affected by these variables.

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 10% and 85% confluency. Do not allow cells to reach confluence.

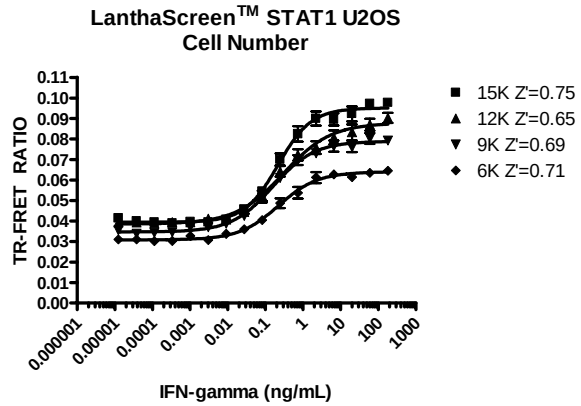
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
McCoy's5A w/ L-glutamine	90%	—	—
OPTI-MEM w/ Hepes/L-Glutamine, w/o Phenol Red	—	99%	—
Dialyzed FBS **Do Not Substitute!**	10%	—	—
FBS charcoal/dextran treated	—	1%	—
Non-Essential Amino Acids	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	—	—
Sodium Pyruvate	1 mM	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

Figure 2 — IFN-γ treatment of LanthaScreen™-STAT1 U2OS cells with different plating cell numbers/well

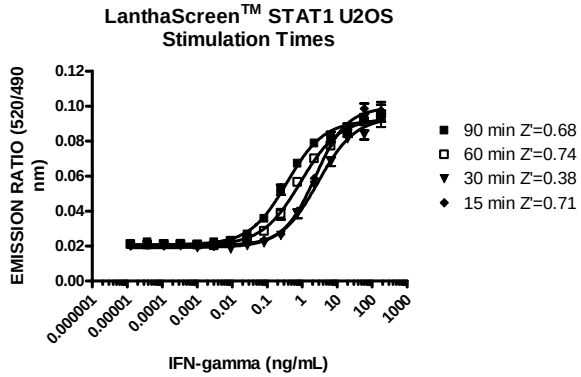


	15K Z'=0.75	12K Z'=0.65	9K Z'=0.69	6K Z'=0.71
BOTTOM	0.03947	0.03859	0.03470	0.03085
TOP	0.09509	0.08780	0.07887	0.06396
LOGEC50	-0.6627	-0.5201	-0.8007	-0.6557
HILLSLOPE	0.9785	0.6895	0.7956	0.8315
EC50	0.2174	0.3019	0.1582	0.2209

LanthaScreen™-STAT1 U2OS (15000, 12000, 9000, or 6000 cells/well) were plated the day prior to the assay in a 384-well format and treated with the indicated concentration of IFN-γ for 60 minutes. Cells were lysed by addition of 30 µl assay buffer, which included 5 nM of Tb-anti-pSTAT1 (pY701) antibody, and incubated for 60 min at room temperature. Fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. The 520/490 nm Emission Ratios are plotted for each experiment, with n=4 replicates per data point.

Assay Performance with Variable Stimulation Time

Figure 3 — Time Course of IFN-γ treatment of LanthaScreen™- STAT1 U2OS

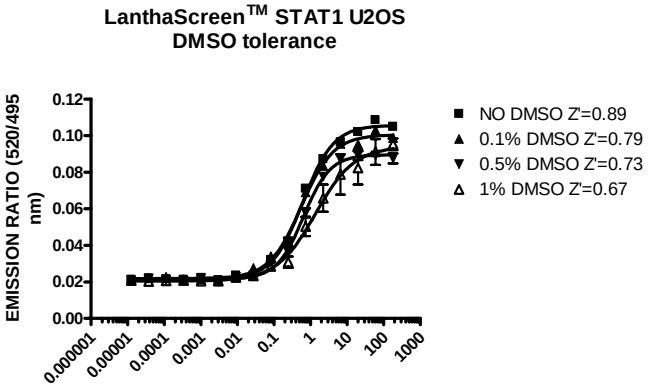


	90 min Z'=0.68	60 min Z'=0.74	30 min Z'=0.38	15 min Z'=0.71
BOTTOM	0.02078	0.02037	0.01915	0.02043
TOP	0.09190	0.09310	0.09371	0.09924
LOGEC50	-0.4324	-0.03851	0.4531	0.3812
HILLSLOPE	0.8190	0.7847	0.8184	0.9601
EC50	0.3695	0.9151	2.839	2.405

LanthaScreen™-STAT1 U2OS (12000 cells/well) were plated the day prior to the assay in a 384-well format and treated with the indicated concentration of IFN-γ for the time indicated. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-pSTAT1 (pY701) antibody, and incubated for 60 min at room temperature. Fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. The 520/490 nm Emission Ratios are plotted for each experiment, with n=4 replicates per data point.

Assay Performance with Variable DMSO Concentration

Figure 4 – IFN-γ treatment of LanthaScreen™-STAT1 U2OS cells in presence of variable DMSO concentrations



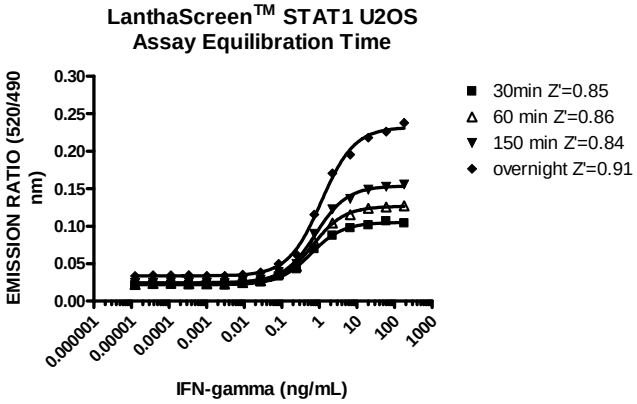
	NO DMSO Z'=0.89	0.1% DMSO Z'=0.79	0.5% DMSO Z'=0.73	1% DMSO Z'=0.67
BOTTOM	0.02144	0.02179	0.02153	0.02042
TOP	0.1056	0.1004	0.08955	0.09407
LOGEC50	-0.2098	-0.2522	-0.1911	0.1370
HILLSLOPE	1.037	0.9942	1.191	0.8158
EC50	0.6168	0.5594	0.6440	1.371

LanthaScreen™-STAT1 U2OS (12000 cells/well) were plated the day prior to the assay in a 384-well format and pretreated with the indicated concentration of DMSO for 1h, prior to IFN-γ stimulation. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-pSTAT1 (pY701) antibody, and incubated for 60 min at room temperature. Fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. The 520/490 nm Emission Ratios are plotted for each experiment, with n=4 replicates per data point.

Note: We observed significant changes in the EC50 values and data quality for IFN-γ at DMSO concentrations greater than 0.5%. The assay window remains unchanged, however.

Assay Performance with Variable Assay Equilibration Time

Figure 5 —Variable Assay equilibration time of LanthaScreen™- STAT1 U2OS treated with IFN-γ

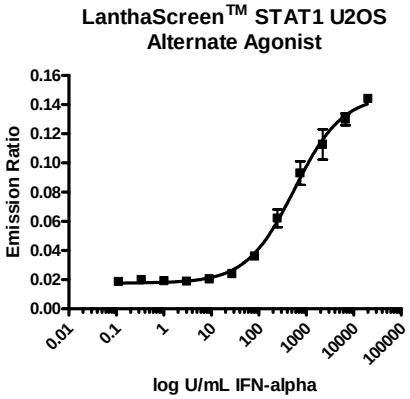


	Z'=0.85	Data Set-B	Data Set-C	Data Set-D
BOTTOM	0.02176	0.02309	0.02466	0.03353
TOP	0.1049	0.1264	0.1535	0.2319
LOGEC50	-0.2187	-0.1711	-0.1030	0.05408
HILLSLOPE	1.025	1.038	1.027	0.9836
EC50	0.6043	0.6744	0.7889	1.133

LanthaScreen™-STAT1 U2OS (12000 cells/well) were plated the day prior to the assay in a 384-well format and treated with the indicated concentration of IFN-γ for 60 minutes. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-pSTAT1 (pY701) antibody, and allowed to equilibrate at room temperature for the time indicated. Fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. The 520/490 nm Emission Ratios are plotted for each experiment, with n=10 replicates per data point.

Alternate Agonist for LanthaScreen™-STAT1 U2OS

Figure 6 – Treatment of LanthaScreen™-STAT1 U2OS with IFN-α

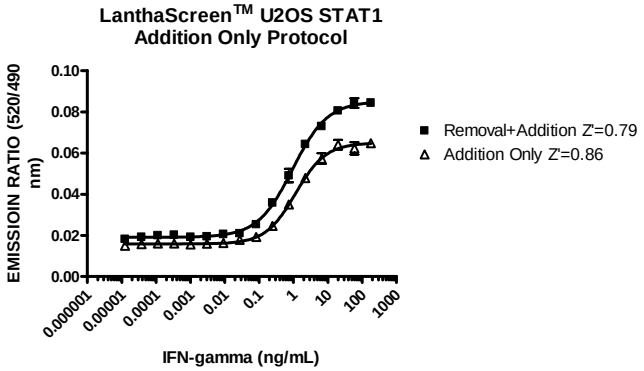


	STAT1 U2OS tyr 701
BOTTOM	0.01759
TOP	0.1462
LOGEC50	2.748
HILLSLOPE	0.8615
EC50	559.8

LanthaScreen™-STAT1 U2OS (12000 cells/well) were plated the day prior to the assay in a 384-well format and treated with the indicated concentration of IFN-α for 60 minutes. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-pSTAT1 (pY701) antibody, and incubated for 60 min at room temperature. Fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. The 520/490 nm Emission Ratios are plotted for each experiment, with n=3 replicates per data point.

Alternate Addition Only Protocol for LanthaScreen™-STAT1 U2OS

Figure 7 —Alternate vs. Standard Protocol for LanthaScreen™- STAT1 U2OS



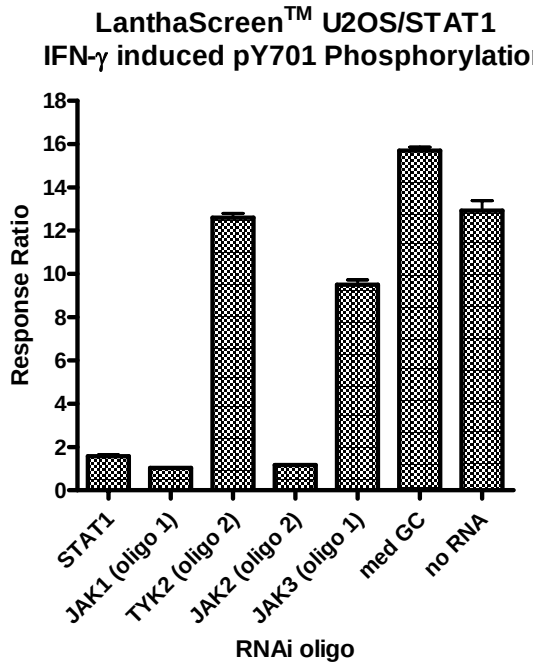
	removal+addition Z'=0.79	addition only Z'=0.83
BOTTOM	0.01908	0.01581
TOP	0.08509	0.06488
LOGEC50	-0.02472	0.06371
HILLSLOPE	0.8548	0.9896
EC50	0.9447	1.158

LanthaScreen™-STAT1 U2OS (12000 cells/well) were plated the day prior to the assay in a 384-well format and treated with the indicated concentration of IFN-α for 60 minutes. For the addition only protocol, 30 µL lysis buffer containing 5 nM Tb-anti-STAT1 pY701 was added directly to the stimulated cells. As a control, the two step protocol is included for reference (cells were aspirated and lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-pSTAT1 (pY701) antibody). After lysis buffer addition, the assay was incubated for 60 min at room temperature. Fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. The 520/490 nm Emission Ratios are plotted for each experiment, with n=4 replicates per data point.

Note: We recommend a final [Tb-pY701 STAT1 antibody] of no less than 1 nM (2 nM optimally). Therefore, the addition only protocol will likely require the use of more Tb-pY701 STAT1 antibody than the two-step protocol.

Pathway Analysis using Stealth RNAi™

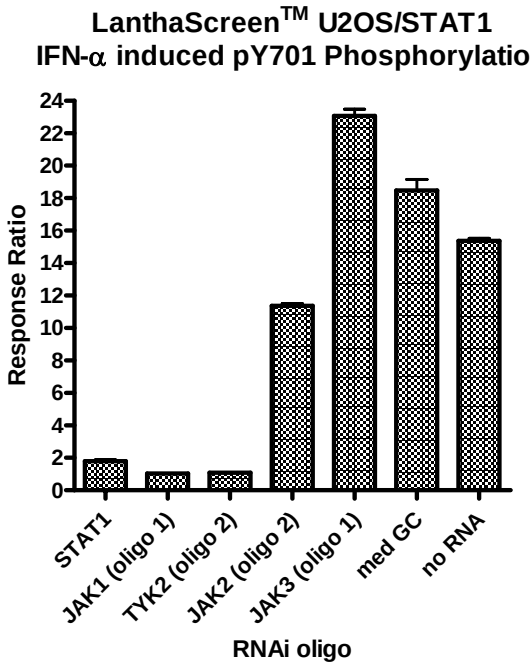
Figure 8 —LanthaScreen™-STAT1 U2OS treated with IFN-gamma



LanthaScreen™-STAT1 U2OS (10000 cells/well) were plated in 96-well format in complete growth medium and treated with oligo:RNAi Max (40 nM) complexes for 60 hours. On the day of the experiment, growth medium/RNAi complexes were removed and replaced with assay medium. After starvation, cells were stimulated +/- 10 ng/mL IFN-gamma for 60 minutes. Assay media were aspirated and cells lysed in the presence of 2 nM Tb-anti-STAT1 pY701 for 1 hour. Cell lysates were then transferred to 384-well plates and fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. Response ratios were calculated by normalizing the TR-FRET values for each RNAi oligo against the untreated control in the presence of that oligo (n=4 replicates per data point).

Pathway Analysis using Stealth RNAi™

Figure 9 —LanthaScreen™-STAT1 U2OS treated with IFN-alpha



LanthaScreen™-STAT1 U2OS (10000 cells/well) were plated in 96-well format in complete growth medium and treated with oligo:RNAi Max (40 nM) complexes for 60 hours. On the day of the experiment, growth medium/RNAi complexes were removed and replaced with assay medium. After starvation, cells were stimulated +/- 5000 U/mL IFN-alpha for 60 minutes. Assay media were aspirated and cells lysed in the presence of 2 nM Tb-anti-STAT1 pY701 for 1 hour. Cell lysates were then transferred to 384-well plates and fluorescence emission values at 520 nm and 490 nm were obtained using a BMG Pherastar plate reader set to TR-FRET mode. Response ratios were calculated by normalizing the TR-FRET values for each RNAi oligo against the untreated control in the presence of that oligo (n=4 replicates per data point).