Validation & Assay Performance Summary

LanthaScreen™-STAT3 GripTite™

Cat. no. K1488

Modification Detected: Phosphorylation of Tyr705

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/Cell Line Description

The JAK/STAT3 signaling pathway is known to be activated by cytokines such as IL-6. In this pathway, binding of IL-6 to its cell surface receptors results in the activation of JAKs, which in turn phospho-activate STAT3 proteins at a specific tyrosine residue (Tyr-705). LanthaScreen™-STAT3 GripTite™ is a human cell line which constitutively expresses a GFP-STAT3 fusion protein. The JAK/STAT signaling pathway is known to be functionally intact in this cell line, therefore the GFP-STAT3 fusion protein serves as a substrate for the IL-6-inducible phosphorylation by JAKs. Using this cell line, an assay format has been developed in which the phosphorylation state of GFP-STAT3 is detected in cell lysates using a terbium-labeled anti-pTyr-705-STAT3 antibody, in a time-resolved FRET (TR-FRET) readout.

The GFP-STAT3 DNA expression construct was transfected into HEK-293/GripTite™ cells using Lipofectamine™ 2000, followed by selection with Blasticidin. This cell line is a clonal population isolated by flow cytometry using GFP fluorescence as sorting marker. This cell line is validated for EC_{50} and Z' under optimized conditions using IL-6 as ligand for JAK-mediated GFP-STAT3 phosphorylation. This cell lines has also been tested for assay performance under variable experimental conditions, including cell plating density, stimulation time, DMSO tolerance and assay development time.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using LanthaScreen™ Tb-anti-STAT3 (pTyr-705) antibody.

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

Z'-Factor (EC_{100})	= 0.72
Relative Response Ratio	= 3.3
EC_{50} IL-6	= 2.2 ng/mL
Recommended cell no. cells/well	= 20000
Recommended [DMSO]	= 0.1%
Recommended Stim. Time	= 30 min
Recom. Assay incubation	= 60 min
Max. [Stimulation]	= 200 ng/mL

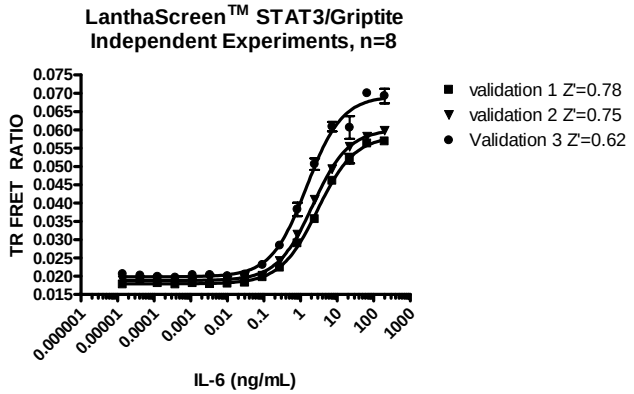
2. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

3. **Optimization of Assay Parameters**
4. **Alternate Agonist Profiles**
5. **Alternate Cell Lysis Protocol**
6. **Stealth RNAi™ Analysis**

Determination of maximum assay window

Figure 1 — IL-6 induced GFP-STAT3 phosphorylation in LanthaScreen™-Griptite™ cells under optimized conditions



	validation 1 Z'=0.78	validation 2 Z'=0.75	Validation 3 Z'=0.62
BOTTOM	0.01794	0.01882	0.01987
TOP	0.05830	0.06012	0.06920
LOGEC50	0.4696	0.3417	0.1743
HILLSLOPE	0.8629	0.8941	0.8985
EC50	2.949	2.196	1.494

LanthaScreen™-STAT3 Griptite™ (20000 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 IL-6 (4 µL addition) for 30 minutes. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 5 nM of Tb-anti-pSTAT3 (pTyr-705) 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.

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 30% and 90% confluency. Do not allow cells to reach confluence.

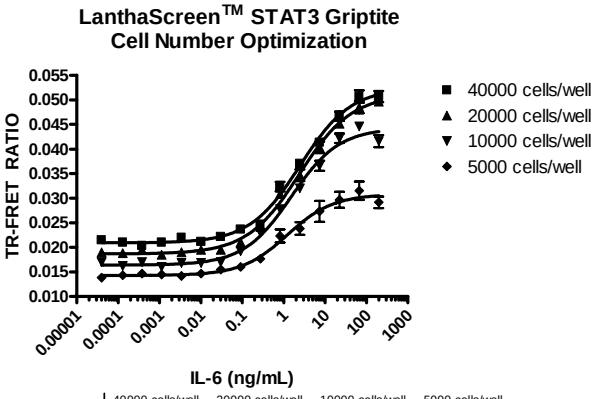
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	—	—
OPTIMEM	—	99%	—
Dialyzed FBS **Do Not Substitute!**	10%	—	—
FBS charcoal/dextran treated	—	1%	—
NEAA	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 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

Figure 2 — IL-6 treatment of LanthaScreen™- GFP-STAT3 GripTite™ cells with different cell numbers / well

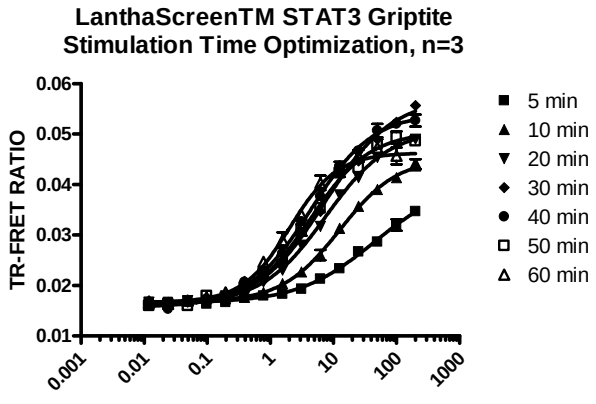


	40000 cells/well	20000 cells/well	10000 cells/well	5000 cells/well
BOTTOM	0.02091	0.01867	0.01639	0.01428
TOP	0.05242	0.05113	0.04423	0.03079
LOGEC50	0.3989	0.3819	0.1855	0.09008
HILLSLOPE	0.7111	0.6881	0.7538	0.7875
EC50	2.505	2.409	1.533	1.230

LanthaScreen™-STAT3 GripTite™ (cells/well indicated above in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were treated with the indicated concentration of IL-6 for 30 minutes. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 5 nM of Tb-anti-pSTAT3 (pTyr-705) 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.

Assay Performance with Variable Stimulation Time

Figure 3 — Time Course of IL-6 treatment of LanthaScreen™- GFP-STAT3 GripTite™

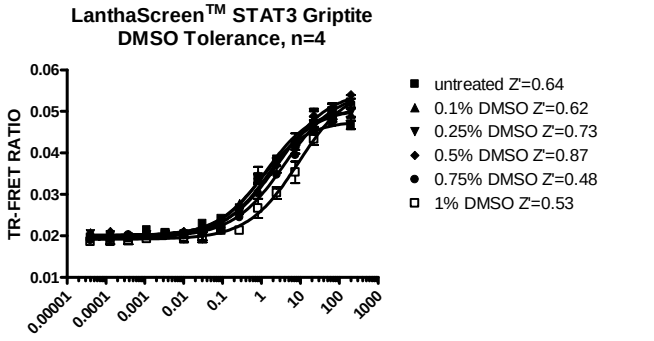


	5 min	10 min	20 min	30 min	40 min	50 min	60 min
BOTTOM	0.01651	0.01669	0.01627	0.01567	0.01582	0.01608	0.01666
TOP	0.04071	0.04532	0.05098	0.05717	0.05419	0.05044	0.04627
LOGEC50	1.650	1.101	0.8764	0.8617	0.6418	0.6395	0.3092
HILLSLOPE	0.7201	0.9409	0.8576	0.8078	0.8780	0.9252	1.156
EC50	44.69	12.63	7.524	7.273	4.384	4.360	2.038

LanthaScreen™-STAT3 GripTite™ (20000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were treated with the indicated concentration of IL-6, and incubated for the indicated time. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 5 nM of Tb-anti-pSTAT3 (pTyr-705) 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.

Assay Performance with Variable DMSO Concentration

Figure 4 – IL-6 treatment of LanthaScreen™- GFP-STAT3 GripTite™ cells in presence of variable DMSO concentrations

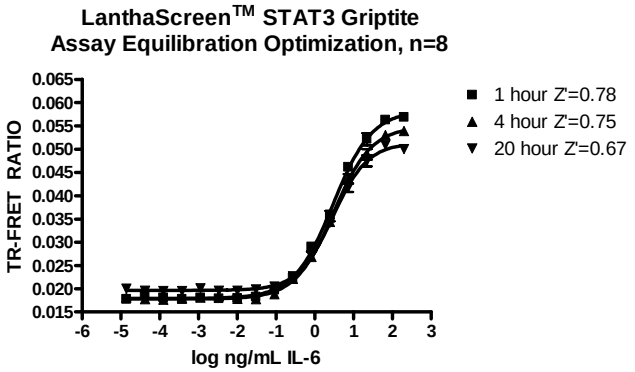


	untreated Z'=0.64	0.1% DMSO Z'=0.62	0.25% DMSO Z'=0.73	0.5% DMSO Z'=0.87	0.75% DMSO Z'=0.48	1% DMSO Z'=0.53
BOTTOM	0.02011	0.02012	0.01975	0.01991	0.01969	0.01918
TOP	0.04775	0.05064	0.05069	0.05509	0.05493	0.05620
LOGEC50	-0.001052	0.09943	0.2038	0.4364	0.5955	0.9521
HILLSLOPE	0.7352	0.7305	0.7264	0.6528	0.6540	0.6683
EC50	0.9976	1.257	1.599	2.732	3.940	8.957

LanthaScreen™-STAT3 GripTite™ (20000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were pretreated with various concentrations of DMSO prior to stimulation with the indicated concentration of IL-6 (30 minute stimulation). Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 5 nM of Tb-anti-pSTAT3 (pTyr-705) 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.

Assay Performance with Variable Assay Equilibration Time

Figure 5 —Variable Assay equilibration time of LanthaScreen™- GFP-STAT3 Griptite™ treated with IL-6

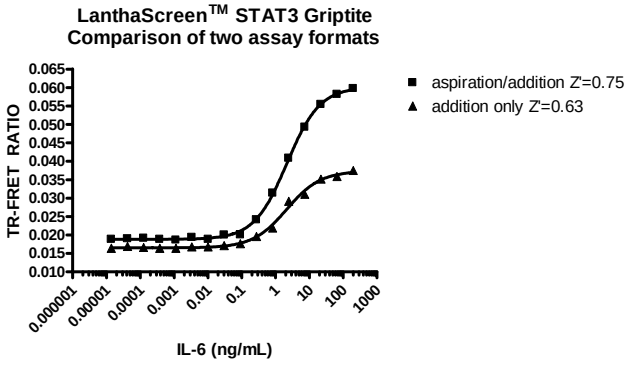


	1 hour Z'=0.78	4 hour Z'=0.75	20 hour Z'=0.67
BOTTOM	0.01794	0.01780	0.01966
TOP	0.05830	0.05486	0.05134
LOGEC50	0.4696	0.4823	0.4673
HILLSLOPE	0.8629	0.8841	0.9685
EC50	2.949	3.036	2.933

LanthaScreen™-STAT3 GripTite™ (20000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were treated with the indicated concentration of IL-6, and incubated for 30 minutes. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 5 nM of Tb-anti-pSTAT3 (pTyr-705) antibody, and the assay was allowed to equilibrate for the indicated time at room temperature prior to reading the plate. 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.

Alternate Addition Only Protocol for LanthaScreen™-STAT3 Griptite™

Figure 6 —Alternate vs. Standard Protocol

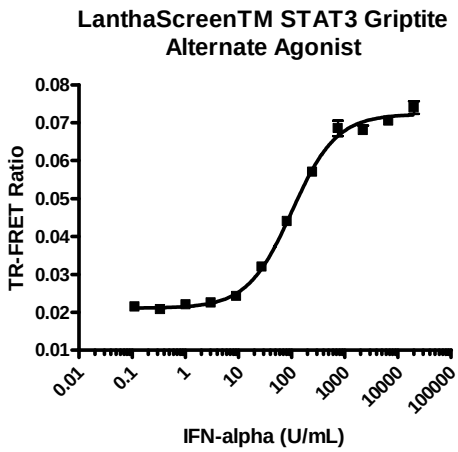


	aspiration/addition Z'=0.75	addition only Z'=0.63
BOTTOM	0.01882	0.01652
TOP	0.06012	0.03735
LOGEC50	0.3417	0.3315
HILLSLOPE	0.8941	0.8698
EC50	2.196	2.145

LanthaScreen™-STAT3 GripTite™ (20000 cells/well) were plated the day prior to the assay in a 384-well format and treated with the indicated concentration of IL-6 for 30 minutes. For the addition only protocol, 30 µL lysis buffer containing 5 nM Tb-anti-STAT3 pTyr-705 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 5 nM of Tb-anti-pSTAT3 (pTyr-705) 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.

Assay Performance with Alternate Agonist

Figure 7 —LanthaScreen™- GFP-STAT3 Griptite™ treated with IFN-alpha

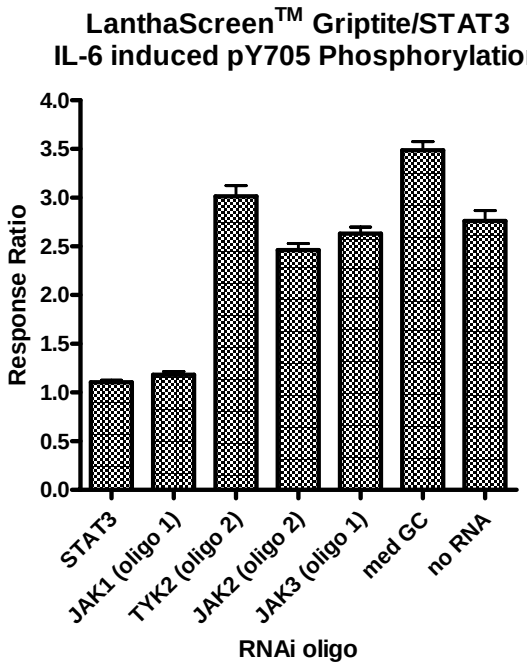


	STAT3 griptite tyr 705
BOTTOM	0.02111
TOP	0.07226
LOGEC50	2.006
HILLSLOPE	1.039
EC50	101.4

LanthaScreen™-STAT3 GripTite™ (20000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were treated with the indicated concentration of IFN-alpha, and incubated for 30 minutes. Cells were aspirated and lysed by addition of 20 µl assay buffer, which included 5 nM of Tb-anti-pSTAT3 (pTyr-705) antibody, and the assay was allowed to equilibrate for 1h at room temperature prior to reading the plate. 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.

Pathway Analysis using Stealth RNAi™

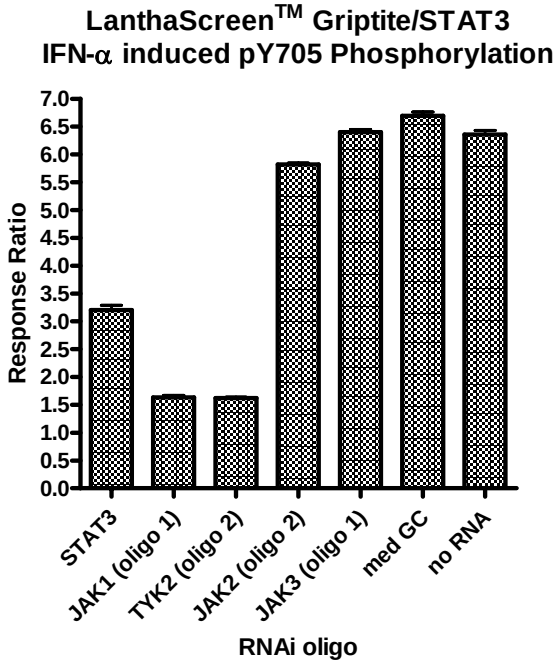
Figure 8 —LanthaScreen™- GFP-STAT3 Griptite™ treated with IL-6



LanthaScreen™-STAT3 Griptite (20000 cells/well) were plated in a 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 +/- 50 ng/mL IL-6 for 30 minutes. Assay media were aspirated and cells lysed in the presence of 5 nM Tb-anti-STAT3 pY705 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™- GFP-STAT3 Griptite™ treated with IFN-alpha



LanthaScreen™-STAT3 Griptite (20000 cells/well) were plated in a 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 30 minutes. Assay media were aspirated and cells lysed in the presence of 5 nM Tb-anti-STAT3 pY705 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).