Validation & Assay Performance Summary

LanthaScreen™ STAT5 TF-1

Cat. no. K1598

Modification Detected: Phosphorylation of Tyr694/Tyr699

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

In hematopoeitic cells, the JAK2/STAT5 signaling pathway plays an essential role in blood cell formation in response to cytokines such as GM-CSF, IL-3, and EPO. In this pathway, binding of these cytokines to their respective cell surface receptors results in the activation of JAK2, which in turn phospho-activates STAT5 proteins at specific tyrosine residues (Tyr-694/699). LanthaScreen™-STAT5 TF-1 is a human hematopoietic cell line which constitutively expresses GFP-STAT5 fusion proteins. The JAK2/STAT5 signaling pathway is known to be functionally intact in this cell line, therefore the GFP-STAT5 fusion protein serves as a substrate for the inducible phosphorylation by JAK2. Using this cell line, a homogenous immuno-assay has been developed in which the phosphorylation state of GFP-STAT5 is detected in cell lysates using a LanthaScreen™ Terbium-anti-mouse and anti-phospho STAT5 [pTyr694/699] antibody pair, in a time-resolved FRET (TR-FRET) readout.

GFP-STAT5α Lentivirus was transduced into TF-1 cells followed by selection with Blasticidine. This cell line is a clonal population isolated by flow cytometry using GFP fluorescence as sorting marker, and has been screened for the constitutive expression of GFP-STAT5 fusion protein. Using a lytic TR-FRET immuno-assay, this cell line is validated for EC_{50} and Z' under optimized conditions using GM-CSF as an agonist for JAK2 -mediated GFP-STAT5 phosphorylation. This assay has also been tested for assay performance under variable experimental conditions, including cell plating density, stimulation time, DMSO tolerance and lysis/equilibration time.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using the LanthaScreen™ Tb-anti-mouse / anti-phospho STAT5 [Tyr 694/699] antibody pair.

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

Z'-Factor (EC_{100})	= 0.64
Relative Response Ratio	= 2.5x
EC_{50} GM-CSF	= 0.33 ng/mL
Recommended cell no. cells/well	= $1x10^5$
Recommended [DMSO]	= 0.1%
Recommended Stim. Time	= 30 minutes
Recom. Assay incubation	= 2 hours
Max. [Stimulation]	= ~5 ng/mL

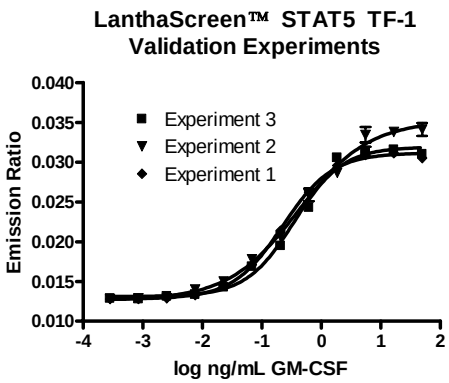
2. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

3. **Assay performance with variable cell number**
4. **Assay performance with variable stimulation time**
5. **Assay performance with variable DMSO concentration**
6. **Assay performance with variable cell lysis / equilibration time**
7. **Assay performance with Alternate Agonist**
8. **Assay performance with small molecule inhibitor**

Validation Experiments (3 Separate Days)

Figure 1 — GM-CSF-stimulated GFP-STAT5 phosphorylation in LanthaScreen™-STAT5 TF-1 cells under optimized conditions



	Experiment 3	Experiment 2	Experiment 1
BOTTOM	0.01309	0.01267	0.01280
TOP	0.03191	0.03519	0.03116
LOGEC50	-0.4550	-0.3810	-0.6478
HILLSLOPE	1.069	0.7381	1.041
EC50	0.3507	0.4159	0.2250

On the day prior to the assay, LanthaScreen™-STAT5 TF-1 were starved in assay medium overnight in Petri dishes. On the day of the experiment, cells were plated at 100000 cells/well in 16 µL of assay medium, 384-well format. Cells were then pretreated with 2 µL of assay medium containing 1% DMSO before treatment with the indicated concentration of GM-CSF (2 µL addition) for 30 minutes. Cells were lysed by addition of 15 µl lysis buffer, which included 5 nM anti-phospho-STAT5 [pTyr 694/699] + 10 nM LanthaScreen™ Tb-anti-mouse antibodies, and incubated for 120 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, and are represented by the average of at least 8 replicates at each data point.

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blasticidin and culture them in Growth Medium with Blasticidin. These cells are dependent on 2 ng/mL GM-CSF for growth, and this reagent should be added at time of passage. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between $5x10^4$ and $1x10^6$/mL. Do not allow cells to exceed $1x10^6$/mL.

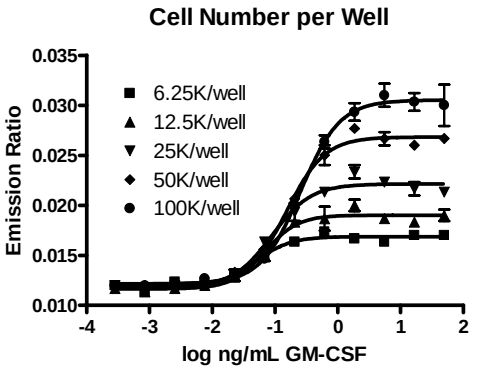
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	90%	—	—
Opti–MEM I®	—	99%	—
Dialyzed FBS **Do Not Substitute!**	10%	--	—
Charcoal/Dextran-Treated FCS	--	0.5%	
NEAA	0.1 mM	0.1 mM	—
Sodium pyruvate	1 mM	1 mM	—
GM-CSF	2 ng/mL	--	--
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 — GM-CSF-stimulated GFP-STAT5 phosphorylation in LanthaScreen™-STAT5 TF-1 cells under variable cell plating density

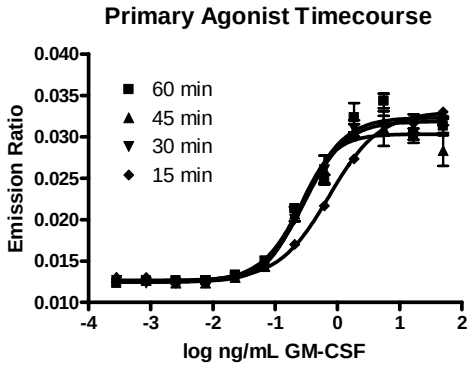


	6.25K/well	12.5K/well	25K/well	50K/well	100K/well
BOTTOM	0.01190	0.01165	0.01181	0.01210	0.01216
TOP	0.01688	0.01901	0.02213	0.02683	0.03052
LOGEC50	-1.308	-1.165	-1.049	-0.8082	-0.5951
HILLSLOPE	1.554	1.651	1.396	1.439	1.385
EC50	0.04918	0.06845	0.08923	0.1555	0.2540

On the day prior to the assay, LanthaScreen™-STAT5 TF-1 were starved overnight in assay medium in Petri dishes. On the day of the experiment, cells were plated at the indicated cells/well in assay medium, 384-well format. Cells were then stimulated with the indicated concentration of GM-CSF (to 20 µL total volume) for 30 minutes. Cells were lysed by addition of 15 µl lysis buffer, which included 5 nM anti-phospho-STAT5 [pTyr 694/699] + 10 nM LanthaScreen™ Tb-anti-mouse antibodies, and incubated for 120 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, and are represented by the average of at least 3 replicates at each data point.

Assay Performance with Variable Stimulation Time

Figure 3 — GM-CSF-stimulated GFP-STAT5 phosphorylation in LanthaScreen™-STAT5 TF-1 cells under variable stimulation times.

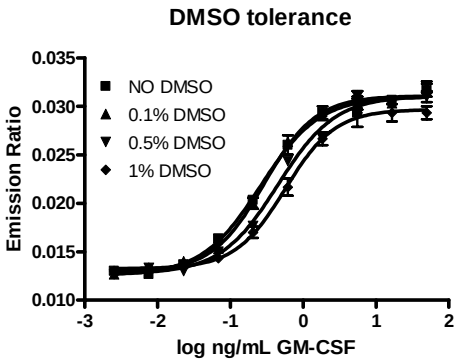


	60 min	45 min	30 min	15 min
BOTTOM	0.01255	0.01254	0.01245	0.01265
TOP	0.03233	0.03033	0.03187	0.03303
LOGEC50	-0.5392	-0.6066	-0.5258	-0.1331
HILLSLOPE	1.289	1.564	1.312	1.073
EC50	0.2889	0.2474	0.2980	0.7361

On the day prior to the assay, LanthaScreen™-STAT5 TF-1 were starved overnight in assay medium in Petri dishes. On the day of the experiment, cells were plated at 100000 cells/well in assay medium, 384-well format. Cells were then treated with the indicated concentration of GM-CSF (to 20 µL total volume) for the time indicated. Cells were lysed by addition of 15 µl lysis buffer, which included 5 nM anti-phospho-STAT5 [pTyr 694/699] + 10 nM LanthaScreen™ Tb-anti-mouse antibodies, and incubated for 120 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, and are represented by the average of at least 3 replicates at each data point.

Assay Performance with Variable DMSO Concentration

Figure 4 – GM-CSF-stimulated GFP-STAT5 phosphorylation in LanthaScreen™-STAT5 TF-1 cells with variable DMSO concentrations

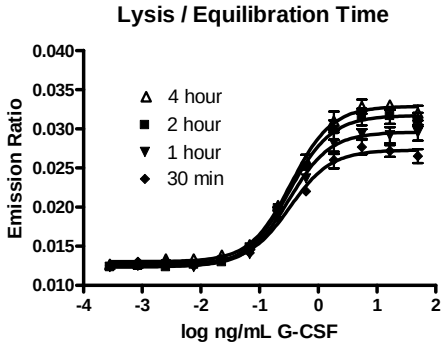


	NO DMSO	0.1% DMSO	0.5% DMSO	1% DMSO
BOTTOM	0.01256	0.01290	0.01272	0.01324
TOP	0.03099	0.03108	0.03107	0.02968
LOGEC50	-0.5837	-0.5380	-0.3460	-0.2464
HILLSLOPE	1.079	1.217	1.101	1.265
EC50	0.2608	0.2897	0.4508	0.5670

On the day prior to the assay, LanthaScreen™-STAT5 TF-1 were starved overnight in assay medium in Petri dishes. On the day of the experiment, cells were plated at 100000 cells/well in assay medium, 384-well format. Cells were pretreated with the indicated concentration of DMSO before stimulation with GM-CSF (to 20 µL total volume) for 30 minutes. Cells were lysed by addition of 15 µl lysis buffer, which included 5 nM anti-phospho-STAT5 [pTyr 694/699] + 10 nM LanthaScreen™ Tb-anti-mouse antibodies, and incubated for 120 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, and are represented by the average of at least 3 replicates at each data point.

Assay Performance with Variable Assay Equilibration Time

Figure 5 — GM-CSF-stimulated GFP-STAT5 phosphorylation in LanthaScreen™-STAT5 TF-1 cells under variable lysis / equilibration times

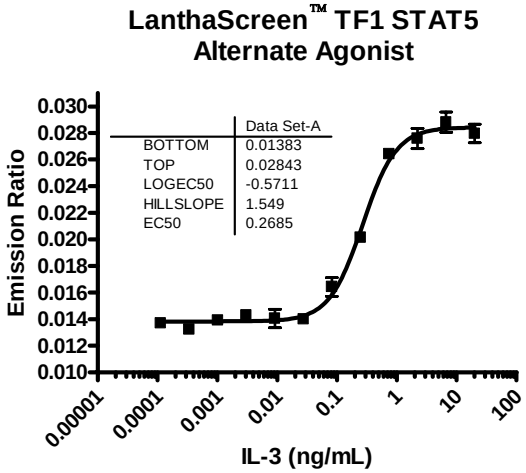


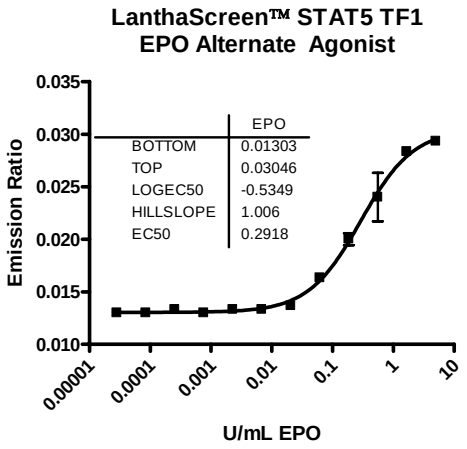
	2 hour	1 hour	30 min	4 hour
BOTTOM	0.01236	0.01239	0.01271	0.01302
TOP	0.03171	0.02956	0.02727	0.03287
LOGEC50	-0.4909	-0.4936	-0.4804	-0.4650
HILLSLOPE	1.204	1.245	1.245	1.256
EC50	0.3230	0.3209	0.3308	0.3428

On the day prior to the assay, LanthaScreen™-STAT5 TF-1 were starved overnight in assay medium in Petri dishes. On the day of the experiment, cells were plated at 100000 cells/well in assay medium, 384-well format. Cells were then treated with the indicated concentration of GM-CSF (to 20 µL total volume) for 30 minutes. Cells were lysed by addition of 15 µl lysis buffer, which included 5 nM anti-phospho-STAT5 [pTyr 694/699] + 10 nM LanthaScreen™ Tb-anti-mouse antibodies, and allowed to equilibrate for the time indicated at room temperature before being measured. 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, and are represented by the average of at least 3 replicates at each data point.

Assay Performance with Alternate Agonist

Figure 6 —IL3/EPO-stimulated GFP-STAT5 phosphorylation in LanthaScreen™-STAT5 TF-1 cells.

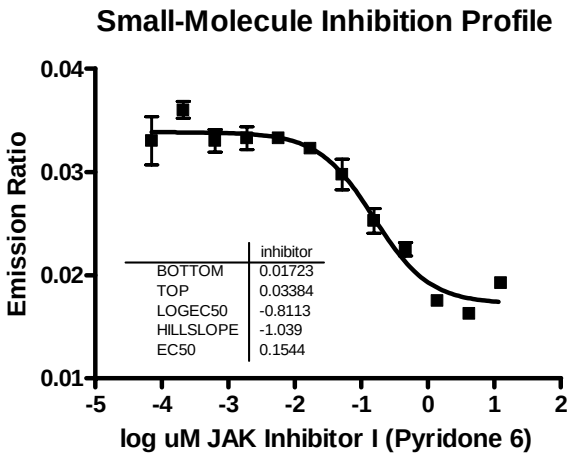




On the day prior to the assay, LanthaScreen™-STAT5 TF-1 were starved overnight in assay medium in Petri dishes. On the day of the experiment, cells were plated at 100000 cells/well in assay medium, 384-well format. Cells were then treated with the indicated concentration of IL-3 or EPO (to 20 μL total volume) for the 30 minutes. Cells were lysed by addition of 15 μl lysis buffer, which included 5 nM anti-phospho-STAT5 [pTyr 694/699] + 10 nM LanthaScreen™ Tb-anti-mouse antibodies, and incubated for 120 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, and are represented by the average of at least 3 replicates at each data point.

Assay Performance with Small Molecule Inhibitor

Figure 7 — Inhibition of JAK-mediated phosphorylation in LanthaScreen™-STAT5 TF-1 cells.



On the day prior to the assay, LanthaScreen™-STAT5 TF-1 were starved overnight in assay medium in Petri dishes. On the day of the experiment, cells were plated at 100000 cells/well in assay medium, 384-well format. Cells were pretreated with the indicated concentration of JAK Inhibitor I before stimulation with 2.5 ng/mL GM-CSF (to 20 μL total volume) for 30 minutes. Cells were lysed by addition of 15 μL lysis buffer, which included 5 nM anti-phospho-STAT5 [pTyr 694/699] + 10 nM LanthaScreen™ Tb-anti-mouse antibodies, and incubated for 120 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, and are represented by the average of at least 3 replicates at each data point.