Validation & Assay Performance Summary

LanthaScreen™ STAT5 (JAK2 V617F) U2OS Cell-Based Assay

Cat. no. K1588

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

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. The recent discovery of an activating mutation in JAK2 (V617F) present in high percentage of myeloproliferative disease (MPD) patients suggests that this mutant JAK2 activity is a potential therapeutic target for certain forms of MPD. The assay described in this summary makes use of a cell line engineered for expression of the constitutively-active mutant kinase JAK2 V617F. By co-expressing GFP-STAT5 in this background, the phosphorylation state of STAT5 (specifically residue Tyr 694/699) can be modulated with JAK2 V617F inhibitors and analyzed in cell lysates using an anti-STAT5 [pTyr 694/699] and LanthaScreen™ terbium-anti-mouse antibody pair.

GFP-STAT5α Lentivirus was transduced into U2OS cells followed by selection with Blasticidine. The selected pool was then transfected with a GST-JAK2 V617F construct using Lipofectamine™ LTX, followed by selection with Geneticin. 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 and GST-JAK2 V617F. Using a lytic TR-FRET immuno-assay, this cell line is validated for IC_{50} and Z' under optimized conditions using JAK Inhibitor I (Pyridone 6) as a small molecule inhibitor for JAK2 V617F-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})	= 0.59
Relative Response Ratio	= 2.1x
IC_{50} JAK Inhibitor I	= 87 nM
Recommended cell no. cells/well	= 12000
Recommended [DMSO]	= 0.1%
Recommended Stim. Time	= 2 hours
Recom. Assay incubation	= 2 hours
Max. [Inhibition]	= ~1 nM

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

Assay Testing Summary

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

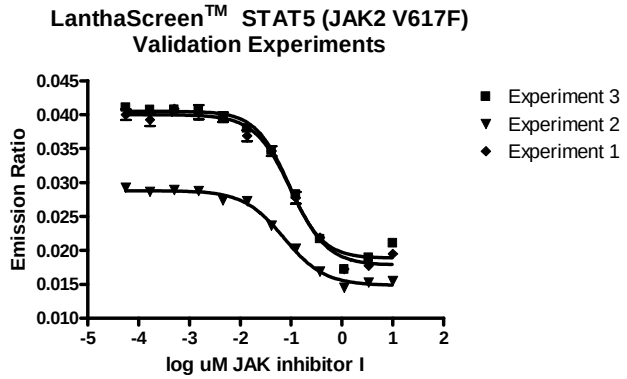
4. **Assay performance with variable antagonist incubation time**

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

6. **Assay performance with variable cell lysis / equilibration time**

Validation Experiments (3 Separate Days)

Figure 1 — JAK Inhibitor I-modulated GFP-STAT5 phosphorylation in LanthaScreen™-U2OS STAT5 (JAK2 V617F) cells under optimized conditions



	Experiment 3	Experiment 2	Experiment 1
BOTTOM	0.01885	0.01485	0.01783
TOP	0.04050	0.02882	0.03999
LOGEC50	-1.042	-1.141	-1.002
HILLSLOPE	-1.318	-1.094	-1.215
EC50	0.09071	0.07234	0.09944

LanthaScreen™-STAT5 (JAK2 V617F) 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 5 minutes with 4 µL of assay medium before treatment with the indicated concentration of JAK inhibitor I (4 µL addition) for 120 minutes. Cells were aspirated and lysed by addition of 20 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. 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 95% confluency. Do not allow cells to reach confluence.

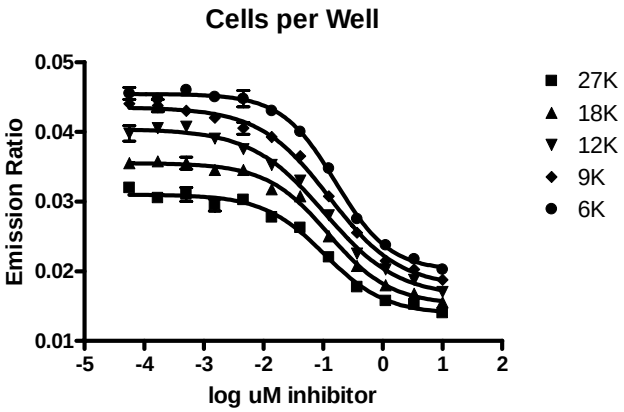
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
McCoy's5A w/ L-glutamine	90%	—	—
OPTI-MEM I 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	—	—
Geneticin (antibiotic)	500 µg/ml		
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

Figure 2 — JAK Inhibitor I-modulated GFP-STAT5 phosphorylation in LanthaScreen™-U2OS STAT5 (JAK2 V617F) cells using variable cell density

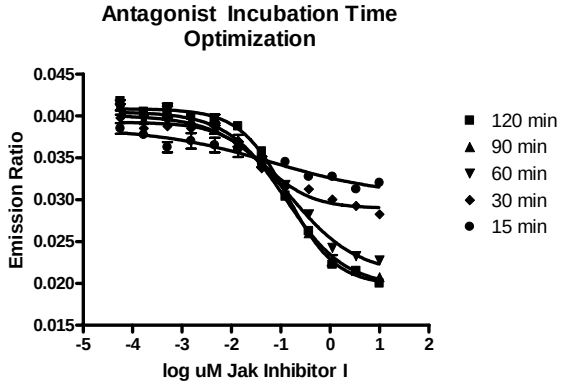


	27K	18K	12K	9K	6K
BOTTOM	0.01385	0.01520	0.01630	0.01772	0.02007
TOP	0.03099	0.03551	0.04041	0.04349	0.04542
LOGEC50	-0.9786	-0.9120	-0.9767	-0.8979	-0.7901
HILLSLOPE	-0.8467	-0.8356	-0.7049	-0.7390	-0.9564
EC50	0.1051	0.1225	0.1055	0.1265	0.1621

LanthaScreen™-STAT5 (JAK2 V617F) U2OS (cells/well indicated above- in 32 µL of assay medium, 384-well format). Cells were plated the day prior to the assay. On the day of the assay, cells were pretreated 5 minutes with 4 µL of assay medium before treatment with the indicated concentration of JAK inhibitor I (4 µL addition) for 120 minutes. Cells were aspirated and lysed by addition of 20 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 4 replicates at each data point.

Assay Performance with Variable Stimulation Time

Figure 3 — JAK Inhibitor I-modulated GFP-STAT5 phosphorylation in LanthaScreen™-U2OS STAT5 (JAK2 V617F) cells using variable antagonist treatment times.

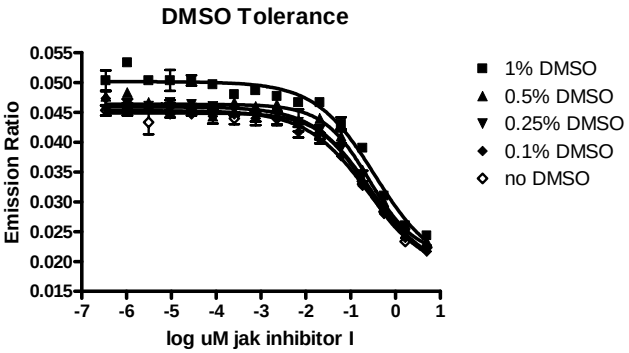


	120 min	90 min	60 min	30 min	15 min
BOTTOM	0.01981	0.01968	0.02105	0.02896	0.03035
TOP	0.04083	0.04047	0.04007	0.03922	0.03855
LOGEC50	-0.8598	-0.9009	-0.8355	-1.336	-1.180
HILLSLOPE	-0.8908	-0.7276	-0.6301	-0.8839	-0.3627
EC50	0.1381	0.1256	0.1461	0.04613	0.06604

LanthaScreen™-STAT5 (JAK2 V617F) U2OS (12000 cells/well in 32 µL of assay medium, 384-well format. Cells were plated the day prior to the assay. On the day of the assay, cells were pretreated 5 minutes with 4 µL of assay medium before treatment with the indicated concentration of JAK inhibitor I (4 µL addition) for the time indicated. Cells were aspirated and lysed by addition of 20 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 4 replicates at each data point.

Assay Performance with Variable DMSO Concentration

Figure 4 – JAK Inhibitor I-modulated GFP-STAT5 phosphorylation in LanthaScreen™-U2OS STAT5 (JAK2 V617F) cells using variable DMSO concentrations.

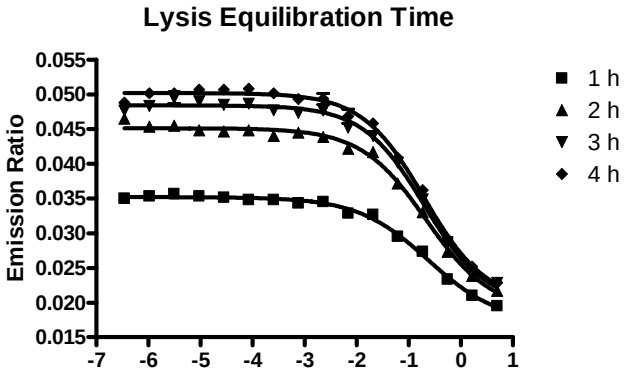


	1% DMSO	0.5% DMSO	0.25% DMSO	0.1% DMSO	no DMSO
BOTTOM	0.01872	0.02098	0.01854	0.01776	0.01999
TOP	0.05012	0.04635	0.04591	0.04530	0.04489
LOGEC50	-0.4547	-0.5838	-0.5675	-0.5951	-0.6492
HILLSLOPE	-0.6548	-0.8454	-0.6840	-0.6304	-0.8104
EC50	0.3510	0.2607	0.2707	0.2541	0.2243

LanthaScreen™-STAT5 (JAK2 V617F) U2OS (12000 cells/well in 32 µL of assay medium, 384-well format). Cells were plated the day prior to the assay. On the day of the assay, cells were pretreated with 4 µL of assay medium + indicated concentration of DMSO for 30 minutes before treatment with the indicated concentration of JAK inhibitor I (4 µL addition) for 120 minutes. Cells were aspirated and lysed by addition of 20 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 — JAK Inhibitor I-modulated GFP-STAT5 phosphorylation in LanthaScreen™-U2OS STAT5 (JAK2 V617F) cells using variable cell lysis/equilibration times.



	1 h	2 h	3 h	4 h
BOTTOM	0.01708	0.01880	0.01976	0.02000
TOP	0.03518	0.04511	0.04841	0.05019
LOGEC50	-0.6252	-0.6668	-0.7052	-0.7025
HILLSLOPE	-0.6402	-0.6948	-0.7371	-0.7286
EC50	0.2370	0.2154	0.1972	0.1984

LanthaScreen™-STAT5 (JAK2 V617F) U2OS (12000 cells/well in 32 µL of assay medium, 384-well format. Cells were plated the day prior to the assay. On the day of the assay, cells were pretreated 5 minutes with 4 µL of assay medium before treatment with the indicated concentration of JAK inhibitor I (4 µL addition) for 120 minutes. Cells were aspirated and lysed by addition of 20 µl lysis buffer, which included 5 nM anti-phospho-STAT5 [pTyr 694/699] + 10 nM LanthaScreen™ Tb-anti-mouse antibodies, and incubated for the time indicated 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.