Validation & Assay Performance Summary

LanthaScreen™ ATF2 (19-106) A549

Cat. no. K1679

Modification Detected: Phosphorylation of Thr71

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.

Cell Line Description

LanthaScreen™ ATF2 (19-106) A549 contains a fusion protein consisting of GFP and a fragment encoding for AA 19-106 of ATF2 under the control of a CMV promotor. A549 is a lung carcinoma cell line which shows inducible activation of jnk by number of different ligands, such as TNF and EGF, which leads to the transient phosphorylation of GFP-ATF2 (19-106). This LanthaScreen™ cell line therefore allows the analysis of jnk activity using GFP-ATF2 phosphorylation as readout. The GFP-ATF2 (19-106) construct was transfected into A549 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 TNF as ligand for jnk mediated GFP-ATF2 (19-106) 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. Additional information using alternate stimuli is also provided.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using LanthaScreen™ Tb-anti-pATF2 (pThr71) antibody.

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

   Z'-Factor (EC_{100}) = 0.751
   Min emission ratio (untreated) = 0.101
   Max emission ratio (max stim.) = 0.327
   EC_{50} TNF = 26.52 pg/ml

   Recommended cell no. cells/well = 10000
   Recommended [DMSO] = 0.1%
   Recommended Stim. Time = 30 min
   Recom. Assay incubation = 60 min
   Max. [Stimulation] = 600 pg/ml

2. **Alternate Stimuli**
   Epidermal Growth Factor (EC_{50}) = 23.02 pg/ml
   Anisomysin (EC_{50}) = 16.85 nM

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

Assay Testing Summary

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

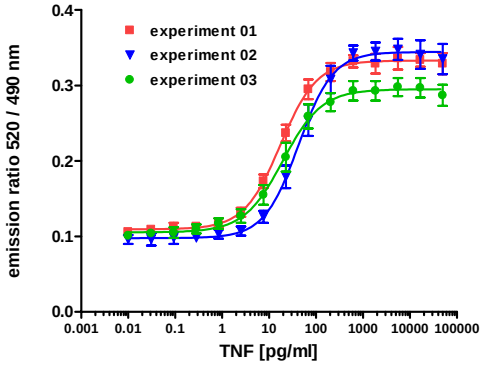
5. **Assay performance with variable stimulation time**

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

7. **Assay performance with variable assay incubation time**

Determination of maximum assay window

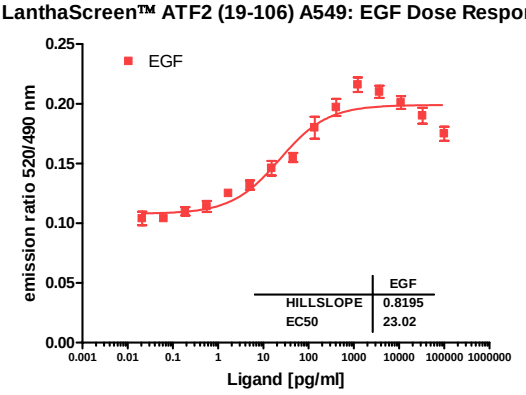
Figure 1 — TNF-α induced GFP-ATF2 (19-106) phosphorylation in LanthaScreen™ ATF2 (19-106) A549 cells under optimized conditions



LanthaScreen™ ATF2 (19-106) A549 (10,000 cells/well) 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 in a 384-well format and treated with the indicated concentration of TNF-α for 30 minutes. After removal of the assay medium by aspiration the cells were lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-pATF2 (pThr71) 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 (n=8 for each data point).

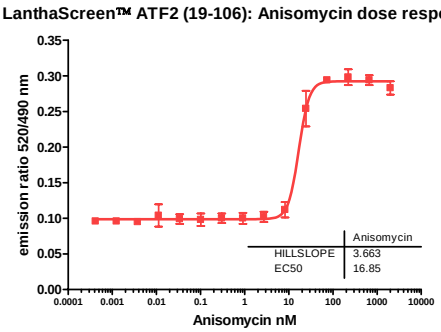
Alternate Agonist for LanthaScreen™ ATF2 (19-106) A549

Figure 2a – Treatment of LanthaScreen™ ATF2(19-106) A549 with EGF



LanthaScreen™ ATF2 (19-106) A549 cells were plated the day prior to the assay at 10,000 cells/well in a 384-well format in assay medium. 24 hours later,cells were treated for 30 min with the indicated concentration of either EGF or TNF-α. After removal of the assay medium by aspiration the cells were lysed by addition of 20 µl assay buffer, which included 2 nM of of Tb-anti-pATF2 (pThr71) 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 the indicated treatment (n=6 for each data point).

Figure 2b – Treatment of LanthaScreen™ ATF2 (19-106) A549 with Anisomycin



LanthaScreen™ ATF2 (19-106) A549 cells were plated the day prior to the assay at 10,000 cells/well in a 384-well format in assay medium. 24 hours later,cells were treated for 30 min with the indicated concentration of Anisomycin. After removal of the assay medium by aspiration the cells were lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-pATF2 (pThr71) 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 the indicated treatment (n=6 for 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 10% and 85% confluency. Do not allow cells to reach confluence.

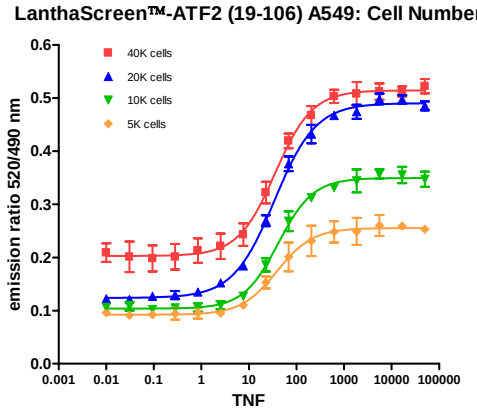
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	—	—
OPTIMEM	—	99%	—
FCS **Do Not Substitute!**	10%	—	—
FCS 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

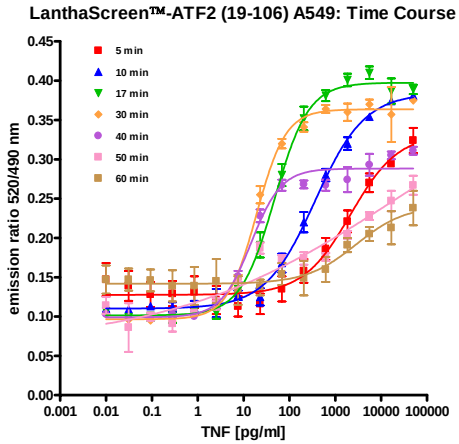
Figure 3 — TNF-α treatment of LanthaScreen™ ATF2 (19-106) A549 cells with different cell numbers/well



LanthaScreen™ ATF2 (19-106) A549 cells were plated the day prior to the assay at the indicated number of cells/well in a 384-well format in assay medium. 24 hours later cells were treated with the indicated concentration of TNF-α for 30 min. After removal of the assay medium by aspiration the cells were lysed by addition of 20 µl assay buffer, which included 2 nM of of Tb-anti-pATF2 (pThr71) 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 the indicated cell number (n=6 for each data point).

Assay Performance with Variable Stimulation Time

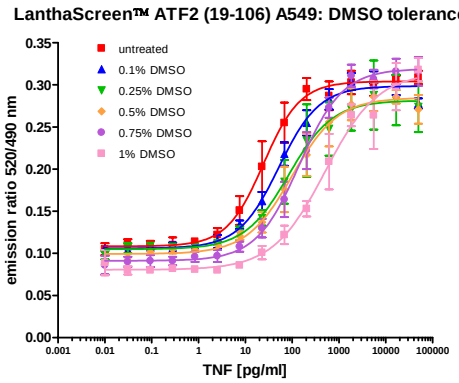
Figure 4 — Time Course of TNF-α treatment of LanthaScreen™ ATF2 (1-79) A549



LanthaScreen™ ATF2 (19-106) A549 cells were plated the day prior to the assay at 10000 cells/well in a 384-well format in assay medium. 24 hours later, cells were treated with the indicated concentration of TNF-α for the indicated period of time (10 - 60 min). After removal of the assay medium by aspiration the cells were lysed by addition of 20 µl assay buffer, which included 2 nM of of Tb-anti-pATF2 (pThr71) 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 the indicated incubation time (n=4 for each data point).

Assay Performance with Variable DMSO Concentration

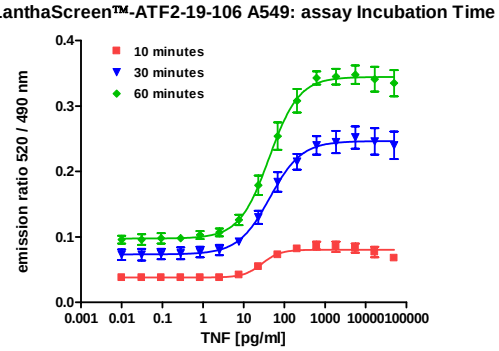
Figure 5 – TNF-α treatment of LanthaScreen™ ATF2 (19-106) A549 cells in presence of variable DMSO concentrations



LanthaScreen™ ATF2 (19-106) A549 cells were plated the day prior to the assay at 10000 cells/well in a 384-well format in assay medium. 24 hours later, cells were pretreated for 30 minutes with the indicated concentration DMSO followed by incubation with the indicated concentration of TNF-α for additional 30 minutes. After removal of the assay medium by aspiration the cells were lysed by addition of 20 µl assay buffer, which included 2 nM of of Tb-anti-pATF2 (pThr71) 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 the indicated DMSO concentrations (n=4 for each data point).
Note: We observed significant changes in the EC50 values for TNF at DMSO concentrations greater than 0.1%. The assay window remains unchanged.

Assay Performance with Variable Assay Incubation Time

Figure 6 —Variable Assay incubation time of LanthaScreen™ ATF2 (19-106) A549 treated with TNF-α



LanthaScreen™ ATF2 (19-106) A549 cells were plated the day prior to the assay at 10000 cells/well in a 384-well format in assay medium. 24 hours later, cells were treated with the indicated concentration of TNF-α for 30 minutes. After removal of the assay medium by aspiration the cells were lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-pATF2 (pThr71) antibody, and incubated for 10, 30 and 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 the indicated incubation time (n=8 for each data point).