Validation & Assay Performance Summary

LanthaScreen™ c-Jun (1-79) HeLa

Cat. no.: K1680

Modification Detected: Phosphorylation of Ser73

LanthaScreen™ Cellular 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™ c-Jun (1-79) HeLa contains a fusion protein consisting of GFP and a fragment encoding for AA 1-79 of c-Jun under the control of a CMV promotor. HeLa is a cervical cancer 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-c-Jun (1-79). This LanthaScreen cell line therefore allows the analysis of JNK activity using GFP-c-Jun phosphorylation as readout. The GFP-c-Jun (1-79) construct was transfected into HeLa 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-c-Jun (1-79) 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-pc-Jun (pSer73) antibody.

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

   Z'-Factor (EC_{100}) = 0.692
   Min emission ratio (untreated) = 0.080
   Max emission ratio (max stim.) = 0.032
   EC_{50} TNF = 405 pg/ml

   Recommended cells/well = 10,000
   Recommended [DMSO] = 0.1%
   Recommended Stim. Time = 30 min
   Recom. Assay incubation = 60 min

2. **Alternate Stimuli**
   Epidermal Growth Factor (EC_{50}) = 250 pg/ml
   Anisomysin (EC_{50}) = 19.1 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**

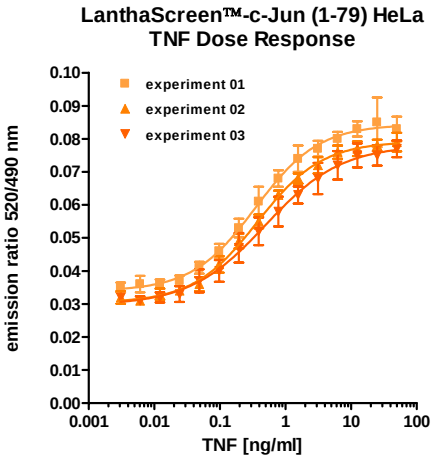
5. **Assay performance with variable stimulation time**

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-c-Jun (1-79) phosphorylation in LanthaScreen™ c-Jun (1-79) HeLa cells under optimized conditions

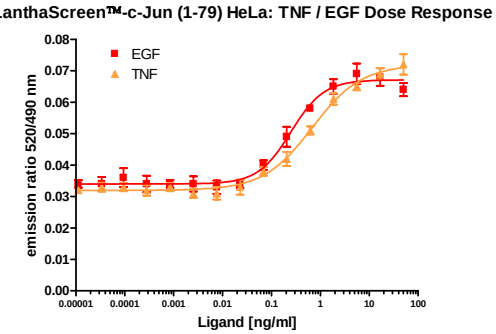


	experiment 01	experiment 02	experiment 03
HILLSLOPE	0.8970	0.8832	0.7507
EC50	0.3491	0.3723	0.4956

LanthaScreen™-c-Jun (1-79) HeLa (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-pc-Jun (pSer73) 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).

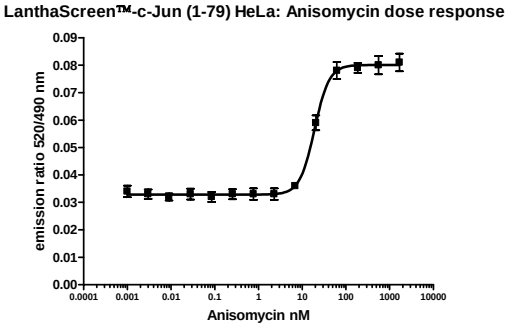
Alternate Agonists for LanthaScreen™ c-Jun (1-79) HeLa

Figure 2a – Treatment of LanthaScreen™ c-Jun (1-79) HeLa with TNF-α and EGF



LanthaScreen™ c-Jun (1-79) HeLa 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 Tb-anti-pc-Jun (pSer73) 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™-c-Jun (1-79) HeLa with Anisomycin



LanthaScreen™ c-Jun (1-79) HeLa 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-pc-Jun (pSer73) 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.

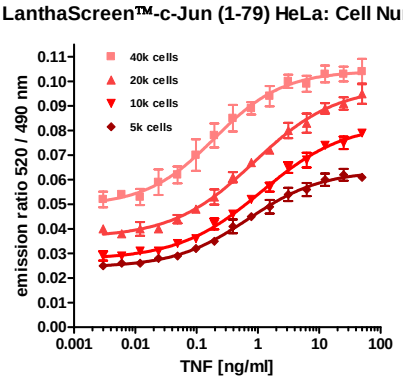
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

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

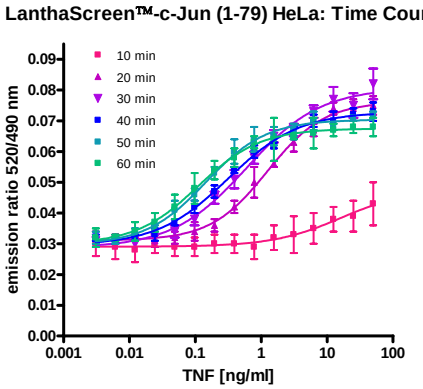
Figure 3 — TNF-α treatment of LanthaScreen™ c-Jun (1-79) HeLa cells with different plating cell numbers / well



LanthaScreen™ c-Jun (1-79) HeLa 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 Tb-anti-pc-Jun (pSer73) 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

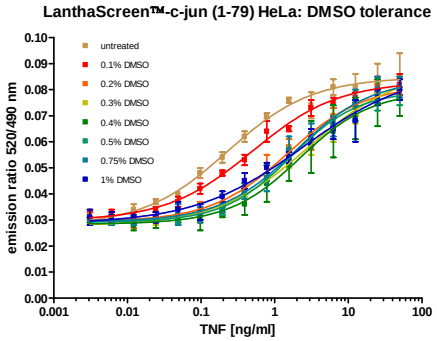
Figure 4 — Time Course of TNF-α treatment of LanthaScreen™ c-Jun (1-79) HeLa



LanthaScreen™-c-Jun (1-79) HeLa 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 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 Tb-anti-pc-Jun (pSer73) 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

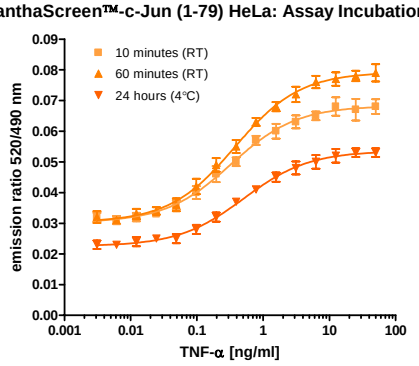
Figure 5 – TNF-α treatment of LanthaScreen™ c-Jun (1-79) HeLa cells in presence of variable DMSO concentrations



LanthaScreen™-c-Jun (1-79) HeLa 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 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 Tb-anti-pc-Jun (pSer73) 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=3 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™ c-Jun (1-79) HeLa treated with TNF-α



LanthaScreen™-c-Jun (1-79) HeLa 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 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-pc-Jun (pSer73) antibody, and incubated for 10 and 60 min at room temperature or 24 hrs at 4C. 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).