Validation & Assay Performance Summary

LanthaScreen® IkB alpha GripTite® Cell Line

Cat. No. K1681

Cell Line Description

The LanthaScreen® IkB alpha GripTite® cell line contains a fusion protein consisting of the cDNA encoding for GFP and IkB alpha (GFP is fused to the N-terminus of IkB alpha under the control of a CMV promotor. GripTite® cells are a modified HEK293 human kidney cell line which shows inducible activation of the NFkB signaling pathway by a number of different ligands, such as TNF and IL-1. Activation of the NFkB pathway involves the activation of IKK resulting in the phosphorylation of its target protein IkB alpha. This LanthaScreen® cell line allows therefore the analysis of IKK activity using GFP-IkB alpha phosphorylation as readout. The GFP-IkB alpha construct was transfected into 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 TNF as ligand for IKK mediated GFP-IkB alpha 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-IkB alpha (pSer32) antibody.

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

   Z'-Factor (EC_{100}) = 0.706
   Min emission ratio (untreated) = 0.02
   Max emission ratio (max stim.) = 0.08
   EC_{50} TNF = 182 pg/ml

   Recommended cell no. cells/well = 20,000
   Recommended [DMSO] = 0.2%
   Recommended Stim. Time = 30 min
   Recom. Assay incubation = 60 min
   Max. [Stimulation] = 6.25 ng/ml

2. **Alternate Stimuli**
   Interleukin 1 (EC_{50}) = 716 pg/ml

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

Assay Testing Summary

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

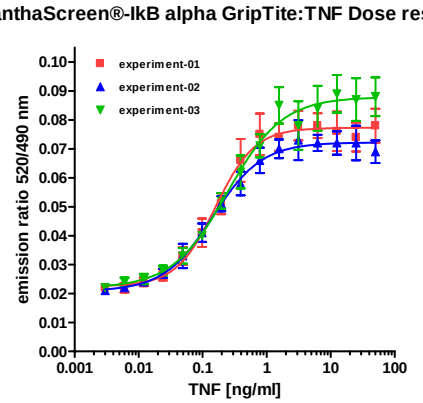
5. **Assay performance with variable stimulation time**

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

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

Determination of maximum assay window

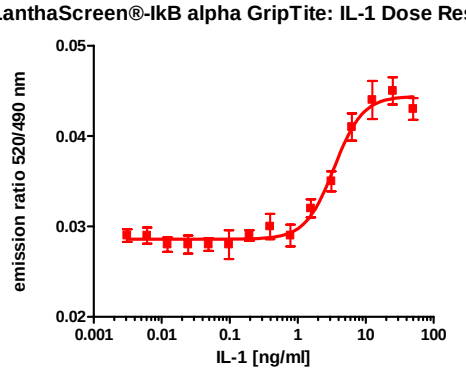
Figure 1 — TNF-alpha induced GFP-IkB alpha phosphorylation in LanthaScreen®-IkB alpha GripTite® cells under optimized conditions



LanthaScreen® IkB alpha GripTite® (20,000 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-alpha for 30 minutes. Cells were lysed by addition of 30 µl assay buffer, which included 3 nM of Tb-anti-pIkB alpha (pSer32) 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®-IkB GripTite® Cells

Figure 2 – Treatment of LanthaScreen®- IkB alpha GripTite® cells with IL-1



LanthaScreen® IkB alpha GripTite® cells were plated the day prior to the assay at 20,000 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 Il-1. Cells were lysed by addition of 30 µl assay buffer, which included 3 nM of Tb-anti-pIkB alpha (pSer32) 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=8 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.

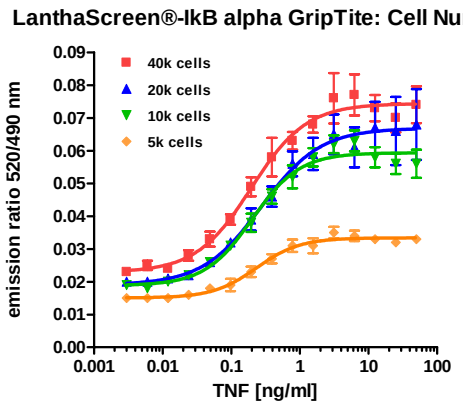
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	—	—
OPTIMEM	—	99%	—
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

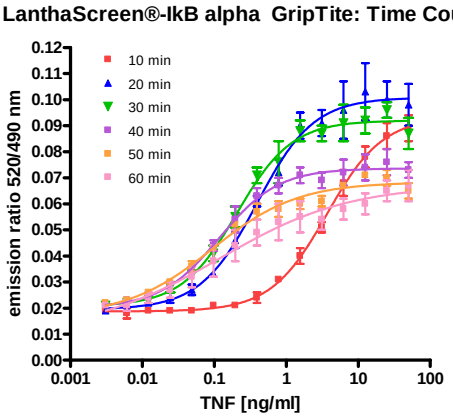
Figure 3 — TNF-alpha treatment of LanthaScreen®-IkB alpha GripTite® cells with different plating cell numbers / well



LanthaScreen® IkB alpha GripTite® 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-alpha for 30 min. Cells were lysed by addition of 30 µl assay buffer, which included 3 nM of Tb-anti-pIkB alpha (pSer32) 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

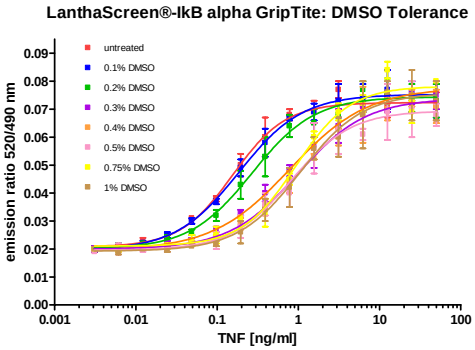
Figure 4 — Time Course of TNF-alpha treatment of LanthaScreen®- IkB alpha GripTite® cells



LanthaScreen®-IkB alpha GripTite® cells were plated the day prior to the assay at 20,000 cells/well in a 384-well format in assay medium. 24 hours later, cells were treated with the indicated concentration of TNF-alpha for the indicated period of time (10 - 60 min). Cells were lysed by addition of 30 µl assay buffer, which included 3 nM of Tb-anti-pIkB alpha (pSer32) 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-alpha treatment of LanthaScreen™ IkB alpha GripTite® cells cells in presence of variable DMSO concentrations

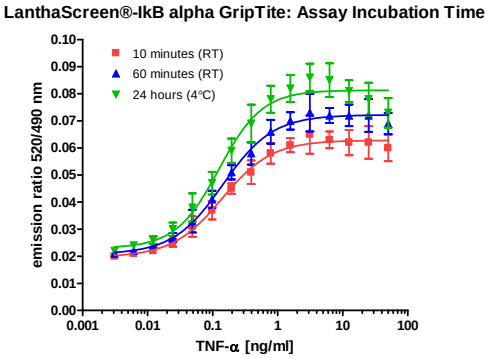


LanthaScreen®-IkB alpha GripTite® cells were plated the day prior to the assay at 20,000 cells/well in a 384-well format in assay medium. 24 hours later, cells were pretreated for 30 minutes with the indicated concentration DMSO followed by incubation with the indicated concentration of TNF-alpha for additional 30 minutes. Cells were lysed by addition of 30 µl assay buffer, which included 3 nM of Tb-anti-pIkB alpha (pSer32) 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 0.2%. The assay window remains unchanged.

Assay Performance with Variable Assay Incubation Time

Figure 6 —Variable Assay incubation time of LanthaScreen®- IkB alpha GripTite® cells treated with TNF- alpha



LanthaScreen®-IkB alpha GripTite® cells were plated the day prior to the assay at 20,000 cells/well in a 384-well format in assay medium. 24 hours later, cells were treated with the indicated concentration of TNF-alpha for 30 minutes. Cells were lysed by addition of 30 µl assay buffer, which included 3 nM of Tb-anti-pIkB alpha (pSer32) 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).