Validation & Assay Performance Summary

LanthaScreen™ ERK2 U2OS Cellular Assay

Cat. no. K1587

Modification Detected: Phosphorylation of Thr185/Thr187

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 Map Kinase (MapK) signal transduction cascade is activated by growth factors such as EGF, PDGF, and HGF. Binding of these factors to their respective cell surface receptors results in the initiation of receptor tyrosine kinase activity, which leads to the sequential phospho-activation of downstream kinases such as Raf, MEK, and Erk1/2. Activated MEK phosphorylates ERK2 (Mapk1) proteins at a specific Thr-Tyr motif (Thr/Tyr 185/187). LanthaScreen™ ERK2 U2OS is a human osteosarcoma cell line which constitutively expresses GFP-ERK2. The MAPK signaling pathway is functionally intact in this cell line, therefore the GFP-ERK2 fusion protein serves as a substrate for the growth-factor-inducible phosphorylation by MEK. Using this cell line, a homogenous immuno-assay has been developed in which the phosphorylation state of GFP-ERK2 is detected in cell lysates using a terbium-labeled anti-ERK2 [pThr/pTyr 185/187] antibody, in a time-resolved FRET (TR-FRET) readout.

GFP-ERK2 lentivirus was transduced into U2OS cells, followed by selection with Blasticidin. This cell line is a clonal population isolated by flow cytometry using GFP fluorescence as sorting marker. Using the lytic TR-FRET immuno-assay, this cell line is validated for EC_{50} and Z' under optimized conditions using EGF as a ligand for GFP-Erk2 phosphorylation. This assay has also been tested for assay performance under variable experimental conditions, including cell plating density, stimulation time, DMSO tolerance and cell lysis/equilibration time. Additional information using alternate stimuli and small molecule inhibitor is also provided.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using LanthaScreen™ Tb-anti-ERK2 (pThr/pTyr 185/187) antibody.

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

Z'-Factor (EC_{100})	= 0.77
Relative Response Ratio	= 6.0x
EC_{50} EGF	= 0.26 ng/mL
Recommended cell no. cells/well	= 12000
Recommended [DMSO]	= 0.1%
Recommended Stim. Time	= 6 min
Recom. Lysis/Equil. Time	= 2 hours
Max. [Stimulation]	= ~10 ng/mL

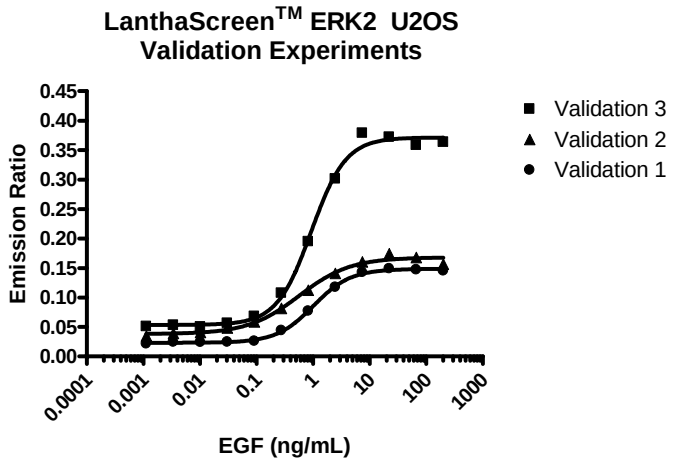
2. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

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 lysis / equilibration time**
7. **Assay performance with alternate agonists**
8. **Assay performance with known small molecule inhibitor**

Validation Experiments (3 Separate Days)

Figure 1 — EGF induced GFP-ERK2 phosphorylation in LanthaScreen™-ERK2 U2OS cells under optimized conditions



LanthaScreen™ Erk2 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 with 4 µL of 1% DMSO before treatment with the indicated concentration of EGF (4 µL addition) for 6 minutes. Media were aspirated and lysed by addition of 20 µl assay buffer, which included 2 nM of Tb-anti-phospho-Erk2 (pTpY 185/187) 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, representing the average of at least eight 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 90% confluency. Do not allow cells to reach confluence.

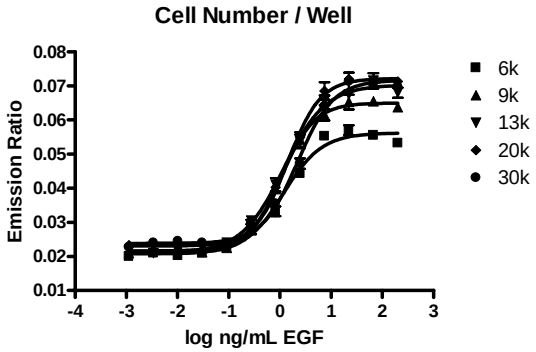
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
McCoy's5A w/ L-glutamine	90%	—	—
OPTI-MEM w/ Hepes/L-Glutamine, w/o Phenol Red	—	99%	—
Dialyzed FBS **Do Not Substitute!**	10%	—	—
FBS charcoal/dextran treated	—	0.5%	—
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	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

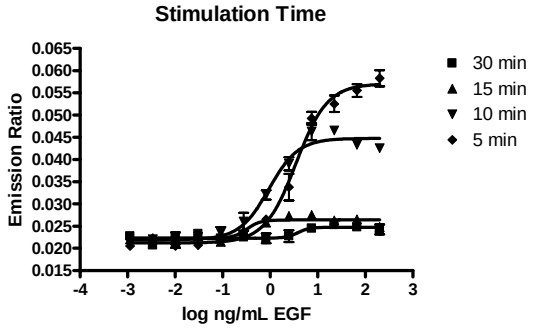
Figure 2 — EGF induced GFP-ERK2 phosphorylation in LanthaScreen™-ERK2 U2OS cells with variable cell number / well



LanthaScreen™-Erk2 U2OS (indicated cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were pretreated with 4 µL of 1% DMSO before treatment with the indicated concentration of EGF (4 µL addition) for 6 minutes +/- 1 minute. Media were aspirated and lysed by addition of 20 µl assay buffer, which included Tb-anti-phospho-Erk2 (pTpY 185/187) 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, representing the average of at least three replicates at each data point.

Assay Performance with Variable Stimulation Time

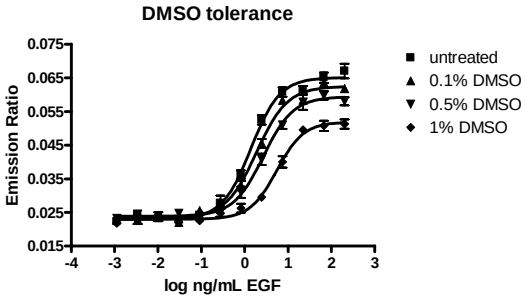
Figure 3 — EGF induced GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 U2OS cells with variable stimulation time



LanthaScreen™-Erk2 U2OS (12000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were pretreated with 4 µL of 1% DMSO before treatment with the indicated concentration of EGF (4 µL addition) for the time indicated. Media were aspirated and lysed by addition of 20 µl assay buffer, which included Tb-anti-phospho-Erk2 (pTpY 185/187) 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, representing the average of at least three replicates at each data point.

Assay Performance with Variable DMSO Concentration

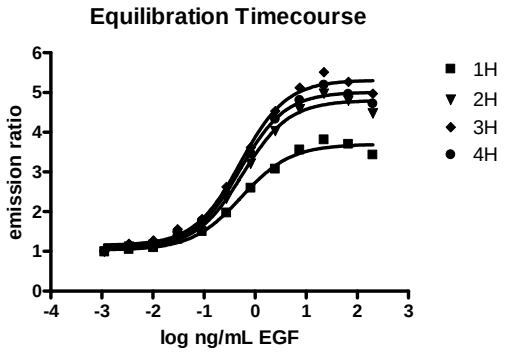
Figure 4 – EGF induced GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 U2OS cells with variable DMSO concentrations



LanthaScreen™-Erk2 U2OS (12000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were pretreated with the indicated concentration of DMSO before treatment with the indicated concentration of EGF (4 µL addition) for 6 minutes +/- 1 minute. Media were aspirated and lysed by addition of 20 µl assay buffer, which included Tb-anti-phospho-Erk2 (pTpY 185/187) 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, representing the average of at least three replicates at each data point.

Assay Performance with Variable Assay Lysis / Equilibration Time

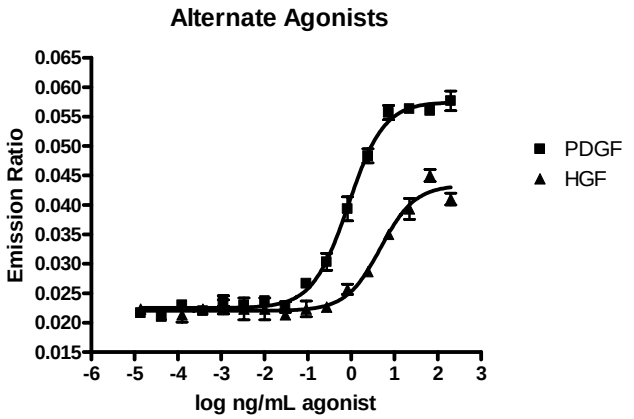
Figure 5 — EGF induced GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 U2OS cells using variable cell lysis / equilibration times



LanthaScreen™-Erk2 U2OS (12000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were pretreated with 4 µL of 1% DMSO before treatment with the indicated concentration of EGF (4 µL addition) for 6 minutes +/- 1 minute. Media were aspirated and lysed by addition of 20 µl assay buffer, which included Tb-anti-phospho-Erk2 (pTpY 185/187) antibody, and incubated for the indicated time 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, representing the average of at least three replicates at each data point.

Alternate Agonist Profiles for LanthaScreen™ ERK2 U2OS

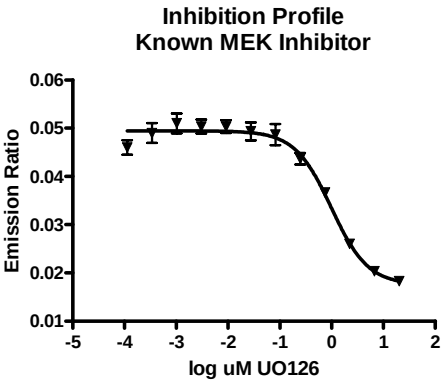
Figure 6 –GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 U2OS cells using alternate agonists



LanthaScreen™-Erk2 U2OS (12000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were treated with the indicated concentration of PDGF or HGF for 6 minutes +/- 1 minute. Media were aspirated and lysed by addition of 20 µl assay buffer, which included Tb-anti-phospho-Erk2 (pTpY 185/187) 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, representing the average of at least three replicates at each data point.

Small Molecule Inhibitor for LanthaScreen™ ERK2 U2OS

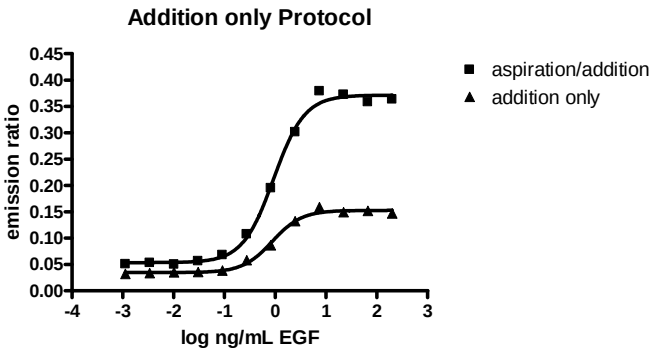
Figure 7 — Inhibition of EGF induced GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 U2OS cells



LanthaScreen™-Erk2 U2OS (12000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were pretreated with 4 µL of UO126 inhibitor before treatment with EGF (4 µL addition to 20 ng/mL final) for 6 minutes. Media were aspirated and lysed by addition of 20 µl assay buffer, which included Tb-anti-phospho-Erk2 (pTpY 185/187) 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, representing the average of at least three replicates at each data point.

Optional Addition Only Lysis Protocol for LanthaScreen™ ERK2 U2OS

Figure 8 — Inhibition of EGF induced GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 U2OS cells



LanthaScreen™-Erk2 U2OS (12000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were pretreated with 4 µL of 1% DMSO before treatment with the indicated concentration of EGF (4 µL addition) for 6 minutes +/- 1 minute. For the addition only protocol, 30 uL of lysis buffer was directly added to stimulated cells, which included 2nM Tb-anti-phospho-Erk2 (pTpY 185/187) antibody. Cells were then incubated for 2h at room temperature. For the two-step protocol, media were aspirated and lysed by addition of 20 µl assay buffer, which included 2nM Tb-anti-phospho-Erk2 (pTpY 185/187) antibody, and incubated for 2h 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, representing the average of at least three replicates at each data point.