Validation & Assay Performance Summary

LanthaScreen™ ERK2 A375

Cat. no. K1798

Modification Detected: Phosphorylation of Tyr185/Tyr187

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 Ras, Raf, MEK, and Erk1/2. Activated MEK phosphorylates Erk2 (Mapk1) proteins at a specific Thr-Tyr motif (Thr/Tyr 185/187). A number of constitutive active kinase mutants within the MAP kinase pathway have been implicated in oncogenesis. One of these mutants is BRAF(V600E), which is the most predominant oncogenic BRAF mutant. The human melanoma cell line A375 endogenously expresses BRAF(V600E), which leads to the constitutive activation of the MAP kinase pathway and phosphorylation of Erk2 in the absence of ligands. LanthaScreen™ Erk2 A375 constitutively expresses GFP-Erk2 under control of a CMV promotor. 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-pTpY-185/187-Erk2 antibody, in a time-resolved FRET (TR-FRET) readout. This cell line can be used to evaluate compound activity against BRAF(V600E).

GFP-Erk2 lentivirus was transduced into A375 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 (pTpY 185/187) antibody.

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

Z'-Factor (EC_{100})	= 0.72
Relative Response Ratio	= n.a.
EC_{50} Raf Inhibitor I	= 153.1 nM
Recommended cell no. cells/well	= 15000
Recommended [DMSO]	= 0.5%
Recommeded Treatment Time	= 30 - 60 min
Recom. Lysis/Equil. Time	= 90 min
Max. [Stimulation]	= n.a.

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 DMSO concentration**

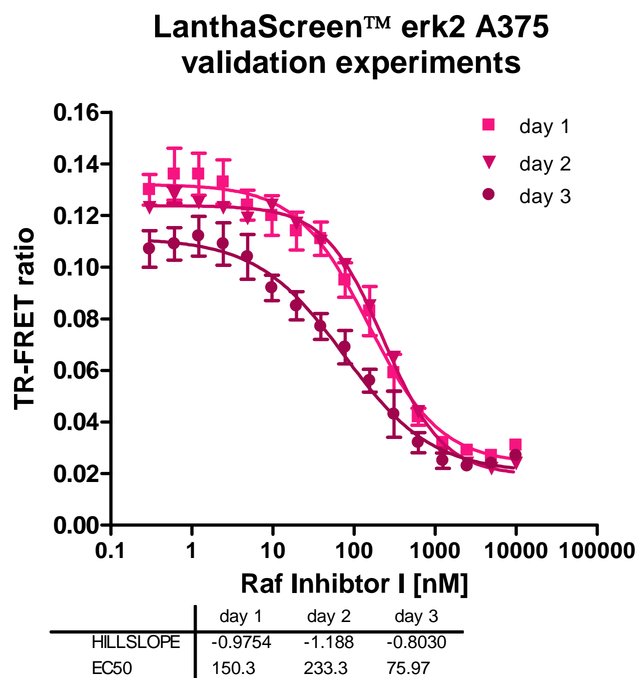
5. **Assay performance with variable lysis/equilibration time**

6. **Assay performance with alternate intihibitors**

7. **Assay performance using addition only protocol**

Validation Experiments (3 Separate Days)

Figure 1 — Inhibition of constitutive GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 A375 cells under optimized conditions



	day 1	day 2	day 3
HILLSLOPE	-0.9754	-1.188	-0.8030
EC50	150.3	233.3	75.97

LanthaScreen™ Erk2 A375 (15000 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 the cells were treated with the indicated concentrations of Raf Inhibitor I (GW5074) for 30 minutes. The medium was aspirated followed by addition of 20 µl lysis buffer, which included 2 nM Tb-anti-phospho-Erk2 (pTpY 185/187) antibody, and incubated for 90 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.

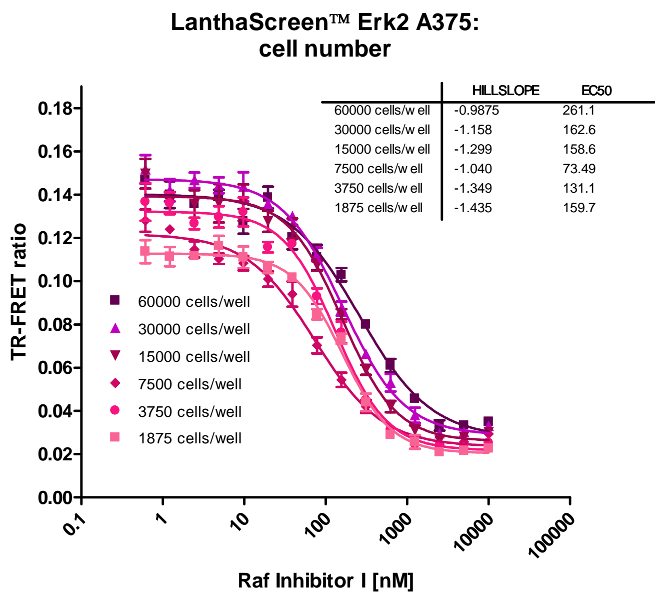
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM w/ L-GLUTAMAX™	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

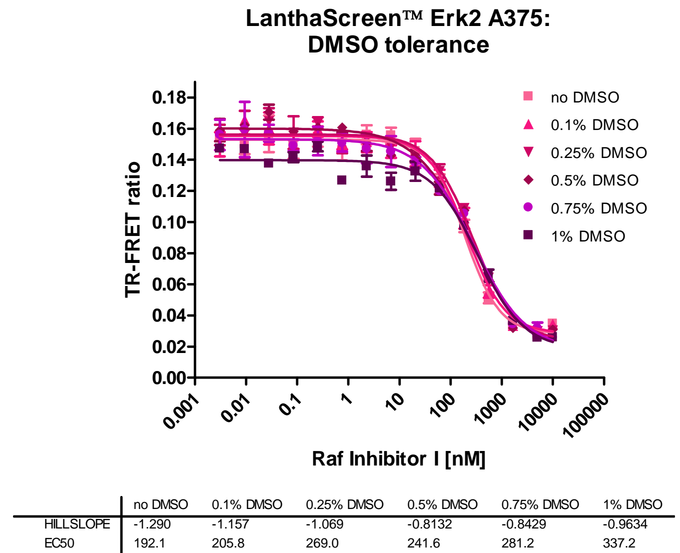
Figure 2 — Inhibition of constitutive GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 A375 cells with variable cell number / well



LanthaScreen™-Erk2 A375 (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 the cells treated with the indicated concentration of Raf Inhibitor I (GW5074) for 30 minutes. The medium was aspirated followed by addition of 20 µl lysis buffer, which included 2 nM Tb-anti-phospho-Erk2 (pTpY 185/187) antibody, and incubated for 90 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 – Inhibition of constitutive GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 A375 cells with variable DMSO concentrations

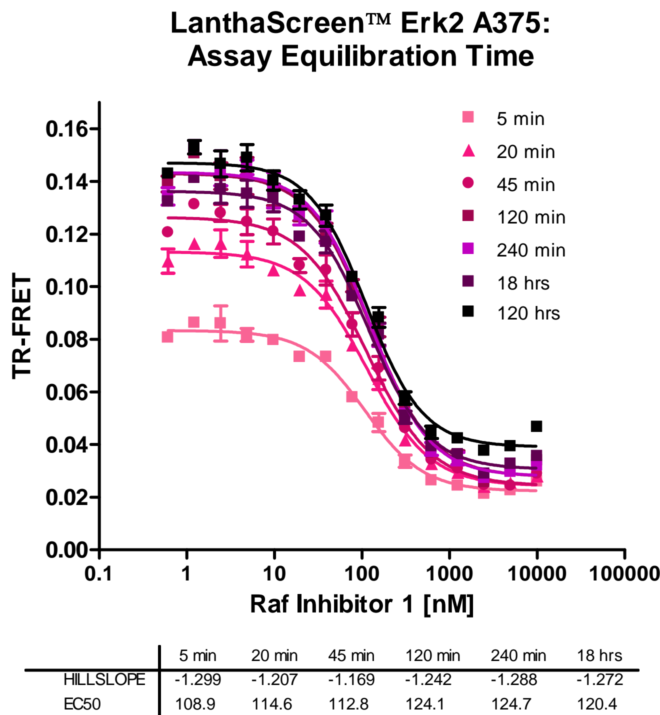


	no DMSO	0.1% DMSO	0.25% DMSO	0.5% DMSO	0.75% DMSO	1% DMSO
HILLSLOPE	-1.290	-1.157	-1.069	-0.8132	-0.8429	-0.9634
EC50	192.1	205.8	269.0	241.6	281.2	337.2

LanthaScreen™-Erk2 A375 (15000 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 the cells treated with the indicated concentration of Raf Inhibitor I (GW5074) for 30 minutes. The medium was aspirated followed by addition of 20 µl lysis buffer, which included 2 nM Tb-anti-phospho-Erk2 (pTpY 185/187) antibody, and incubated for 90 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 — Inhibition of constitutive GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 A375 cells using variable cell lysis / equilibration times

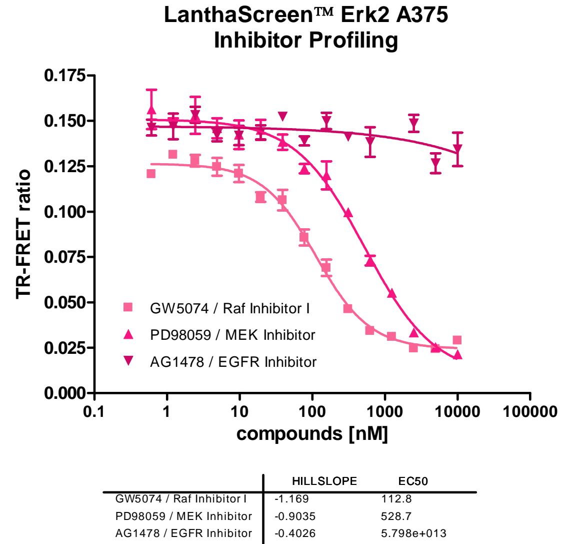


	5 min	20 min	45 min	120 min	240 min	18 hrs
HILLSLOPE	-1.299	-1.207	-1.169	-1.242	-1.288	-1.272
EC50	108.9	114.6	112.8	124.1	124.7	120.4

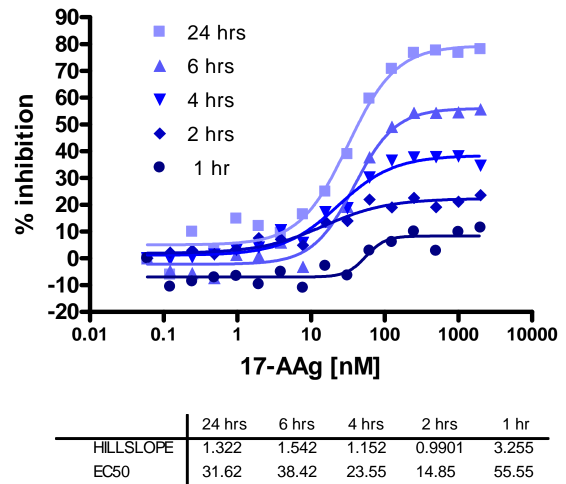
LanthaScreen™-Erk2 A375 (15000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay the cells treated with the indicated concentration of Raf Inhibitor I for 30 minutes. The medium was aspirated followed by addition of 20 µl lysis buffer, which included 2 nM 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.

Inhibitor Profiles for LanthaScreen™ Erk2 A375

Figure 6 –Inhibitor profiling of GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 A375 cells



	HILLSLOPE	EC50
GW5074 / Raf Inhibitor I	-1.169	112.8
PD98059 / MEK Inhibitor	-0.9035	528.7
AG1478 / EGFR Inhibitor	-0.4026	5.798e+013

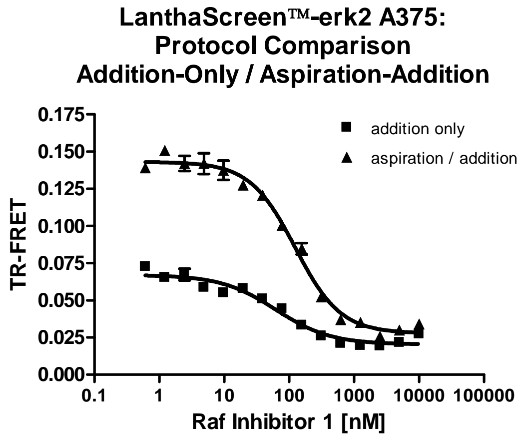


	24 hrs	6 hrs	4 hrs	2 hrs	1 hr
HILLSLOPE	1.322	1.542	1.152	0.9901	3.255
EC50	31.62	38.42	23.55	14.85	55.55

LanthaScreen™-Erk2 A375 (15000 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 different kinase inhibitors for 60 minutes or 17 AAG for the indicated periods of time. The medium was aspirated followed by addition of 20 µl lysis buffer, which included 2 nM Tb-anti-phospho-Erk2 (pTpY 185/187) antibody, and incubated for 90 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.

Alternate Addition Only Protocol for LanthaScreen™ Erk2 A375

Figure 7 — Inhibition of constitutive GFP-Erk2 phosphorylation in LanthaScreen™-Erk2 A375 cells Addition only protocol



	addition only	aspiration / addition
HILLSLOPE	-1.025	-1.242
EC50	62.31	124.1

LanthaScreen™-Erk2 A375 (15000 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 the cells treated with the indicated concentration of Raf Inhibitor I for 30 minutes. For the standard aspiration / addition protocol the medium was aspirated followed by addition of 20 μl lysis buffer containging 2 nM Tb- anti-phospho-Erk2 (pTpY 185/187) antibody and incubation for 90 min at room temperature. For the addition only protocol the cells were lysed by addition of 30 μl lysis buffer, which included 5 nM Tb-anti-phospho-Erk2 (pTpY 185/187) antibody, and incubated for 90 min at room temperature. For addition 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.