Validation & Assay Performance Summary

LanthaScreen™ AKT HEK293E

Cat. no. K1615

Modification Detected: Phosphorylation of S473

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.

Pathway / Assay Description

The PI3K/AKT pathway mediates signals for cell growth, cell survival, transcription, translation, and glucose uptake. Because of the complexity of this signaling cascade, especially as applied to the regulation of the mammalian target of rapamycin (mTOR), cell-based methods are critical for proper identification of small-molecule mediators of this pathway. The significance of mTOR as a kinase has been underscored recently by the identification of two distinct multimeric complexes inside the cell: mTORC1 (includes raptor and is rapamycin sensitive) and mTORC2 (includes rictor and is insensitive to acute rapamycin exposure). mTORC2 has been shown to phosphorylate AKT at residue Ser473 for complete activation of this prosurvival kinase.

LanthaScreen™ AKT HEK293E is a human cell line which constitutively expresses GFP-AKT fusion proteins. This kinase target was introduced using lipid transfection and these cells are a clonal population isolated by FACS, using GFP fluorescence as a sorting marker and Blasticidin to maintain cells under selection. Using this cell line, a homogenous immunoassay was developed with a time-resolved FRET (TR-FRET) readout in which the insulin-induced phosphorylation of Ser473 on GFP-AKT is detected in cell lysates using a terbium-labeled phosphor-specific antibody. This cell line has been validated with different stimuli/inhibitors and shows correct EC_{50} / IC_{50} values. Moreover, this assay has been optimized for performance under variable experimental conditions (including cell plating density, agonist stimulation time, DMSO tolerance and assay development time) and displays excellent statistical data (Z' > 0.6) and good signal-to-background.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using the LanthaScreen™ Tb-anti-AKT [pS473] Antibody (Invitrogen #PV5123 or #PV5124).

1. **Primary agonist dose-response under optimized conditions (ave. of ≥3 expts)**
   Z'-Factor (EC_{100}) = 0.68
   Relative Response Ratio = 2.32
   EC_{50} IGF-1 (ng/mL) = 0.422

   Recommended cell #. = 20,000 cells/well
   Recommended [DMSO] = up to 0.1%
   Recommended Stim. Time = 30 min
   Recom. Assay Incubation = 120 min
   Max. [Stimulation] = 100 ng/mL

2. **Alternate agonist dose-response**
   insulin (EC_{50}) = 264 pM
   PMA = no response
   PDGF-BB = no response

3. **Small-molecule Inhibitor Testing**
   IGF-1R inhibitor II (IC_{50}) = 2.4 μM
   PI3-Ka inihibitor IV (IC_{50}) = 16.7 μM
   PI-103 (IC_{50}) = 78.2 nM
   Wortmannin (IC_{50}) = 2.8 nM
   Rapamycin = no response

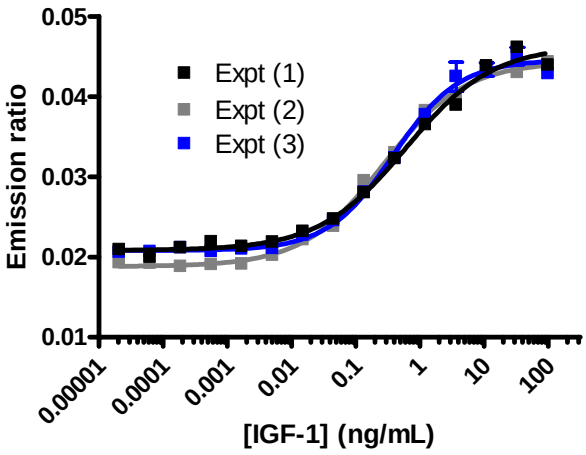
4. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

5. **Assay performance with variable cell number**
6. **Assay performance with variable agonist stimulation time**
7. **Assay performance with variable DMSO concentration**
8. **Assay performance with variable antibody equilibration time**
9. **Western blot validation**
10. **Instrument comparison**

Primary Agonist Dose-Response

Figure 1 — IGF-1-induced phosphorylation of Ser473 on GFP-AKT in HEK293E cells under optimized conditions

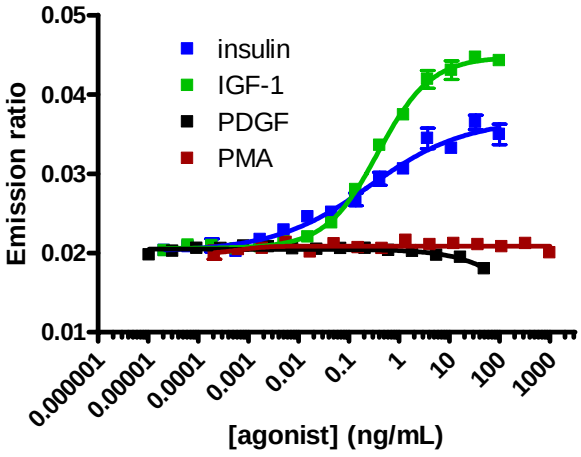


	(1)	(2)	(3)
EC50 (ng/mL)	0.606	0.273	0.386
Z' factor	0.66	0.67	0.71
RR	2.44	2.30	2.22

LanthaScreen™ AKT HEK293E cells (20,000 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 to serum starve overnight (16–20 h). On the day of the assay, cells were first treated with 4 μL of 1% DMSO followed by 4 μL of 10X concentration of IGF-1 (dose response) for 30 min. Cells were subsequently lysed by addition of 30 μL lysis buffer (to 70 μL total volume; "addition-only" protocol), which included 5 nM of Tb-anti-AKT [pS473] antibody and both protease / phosphatase inhibitor cocktails, and then incubated for 120 min at room temperature. Fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar plate reader (BMG LABTECH) set to TR-FRET mode. The 520/490 nm emission ratios are plotted for each experiment, with n≥8 replicates per data point.

Alternate Agonist Dose-Response

Figure 2 – Treatment of cells with alternate ligands

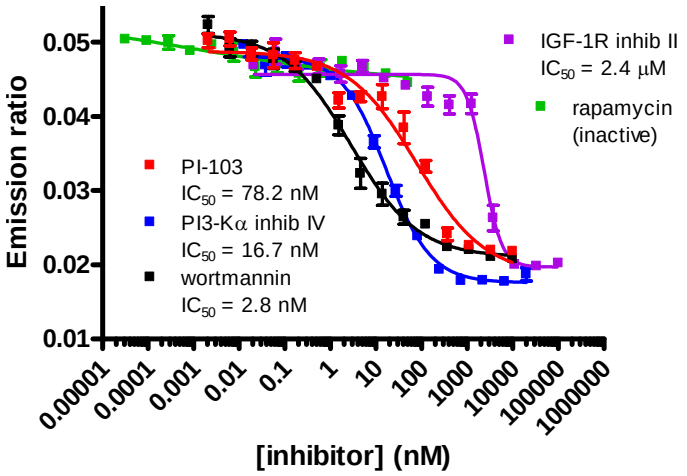


	insulin	IGF-1	PDGF	PMA
EC50 (ng/mL)	1.553	0.377	--	--
Z' factor	0.67	0.81	--	--
RR	1.80	2.18	0.88	1.05

LanthaScreen™ AKT HEK293E cells (20,000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight (16–20 h) in assay medium. Cells were then treated with a 3-fold serial dilution of agonist – either insulin (Invitrogen #12585-014) or IGF-1 (recombinant human insulin-like growth factor; BioSource #PGH0074), for 30 min or PDGF-BB (recombinant human platelet-derived growth factor BB homodimer; BioSource #PHG0046) or PMA (Phorbol 12-Myristate 13-Acetate; Sigma #P1585) for 15 min in a total reaction volume of 40 µL. Cells were next lysed by the addition of 30 µL of lysis buffer that included 5 nM of Tb-anti-AKT [pS473] antibody. The assay was allowed to equilibrate at room temperature for 120 min and then fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar plate reader (BMG LABTECH) set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with n=4 replicates per data point.

Small-Molecule Inhibitor Testing

Figure 3 — Inhibition of PI3K/AKT/mTORC2 signaling



LanthaScreen™ AKT HEK293E cells (20,000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight (16–20 h) in assay medium. Cells were first treated with the indicated concentrations of small-molecule inhibitor (wortmannin – BioSource#PHZ1301; PI3-Kα inhibitor IV – CalBiochem #528111; PI-103 – CalBiochem #528100; rapamycin – BioSource #PHZ1233; IGF-1R inhibitor II – CalBiochem #407248) for 60 min and then treated with stimulating concentration of IGF-1 corresponding to the EC_{80} for 30 min in a total reaction volume of 40 µL. Next, cells were lysed by the addition of 30 µL of lysis buffer that included 5 nM of Tb-anti-AKT [pS473] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar plate reader (BMG LABTECH) set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with $n \geq 3$ replicates per data point.

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blasticidin (preferably into a smaller flask – e.g., T25) 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 20% and 90% confluency. Do not allow cells to become overconfluent. Harvest cells at 70–90% confluency prior to performing the assay.

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.

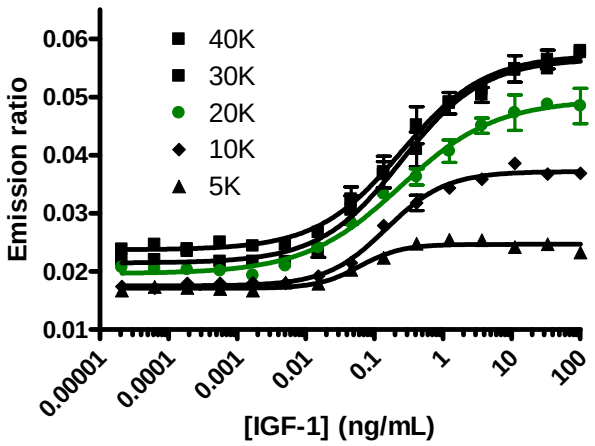
Note: These cells are less adherent than the parental HEK293E cell line and can therefore be more easily dislodged from the tissue culture flask.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
D-MEM (w/ GlutaMAX™)	90%	—	—
D-MEM (low glucose)	—	99.9%	—
Dialyzed FBS **Do Not Substitute!**	10%	—	—
Bovine Serum Albumin (BSA), ultrapure	—	0.1%	—
Non-Essential Amino Acids	0.1 mM	—	—
HEPES (pH 7.3)	25 mM	—	—
Penicillin (antibiotic)	100 U/mL	—	—
Streptomycin (antibiotic)	100 µg/mL	—	—
Blasticidin (antibiotic)	5 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

Figure 4 — IGF-1 dose-response curves when plating different numbers of cells per well

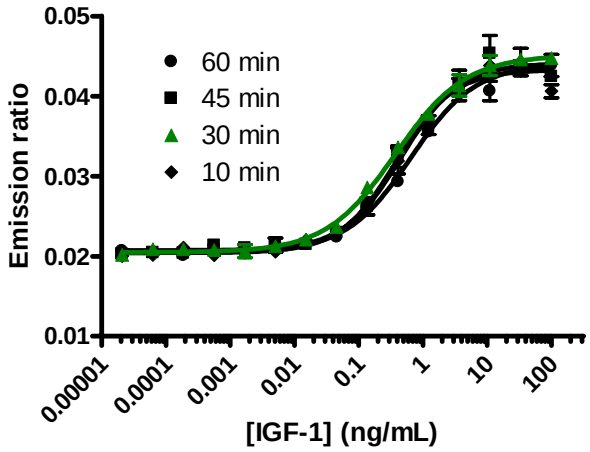


	5K	10K	20K	30K	40K
EC50 (ng/mL)	0.061	0.149	0.237	0.247	0.242
Z' factor	0.42	0.66	0.88	0.86	0.87
RR	1.35	2.13	2.71	2.52	2.30

LanthaScreen™ AKT HEK293E cells were plated at variable densities (40,000; 30,000; 20,000; 10,000; or 5,000 cells/well) the day prior to the assay in a 384-well format and serum starved overnight (16–20 h) in assay medium. Cells were then treated with a 3-fold serial dilution of IGF-1 for 30 minutes in a final reaction volume of 40 µL. Next, cells were lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-AKT [pS473] antibody and protease/phosphatase inhibitor cocktails. After incubation at room temperature for 120 min, fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar plate reader (BMG LABTECH) set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with n=3 replicates per data point.

Assay Performance with Variable Stimulation Times

Figure 5 — Agonist stimulation time course



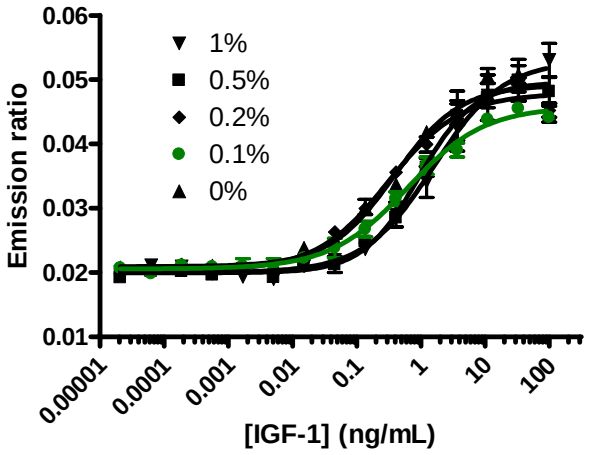
	10 min	30 min	45 min	60 min
EC50 (ng/mL)	0.390	0.377	0.460	0.651
Z' factor	0.71	0.81	0.96	0.92
RR	1.99	2.18	2.02	2.20

LanthaScreen™ AKT HEK293E cells (20,000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight in assay medium. Cells were then treated with the indicated concentrations of insulin for varying intervals of time (10, 30, 45, and 60 min). Cells were next lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-AKT [pS473] antibody. After incubation at room temperature for 120 min, fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar plate reader (BMG LABTECH) set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with n=4 replicates per data point.

Note: The response ratios (RR) are equivalent at each time point, but the EC_{50} values do shift slightly over time.

Assay Performance with Variable DMSO Concentrations

Figure 6 – IGF-1 dose-response curves in the presence of different final concentrations of DMSO



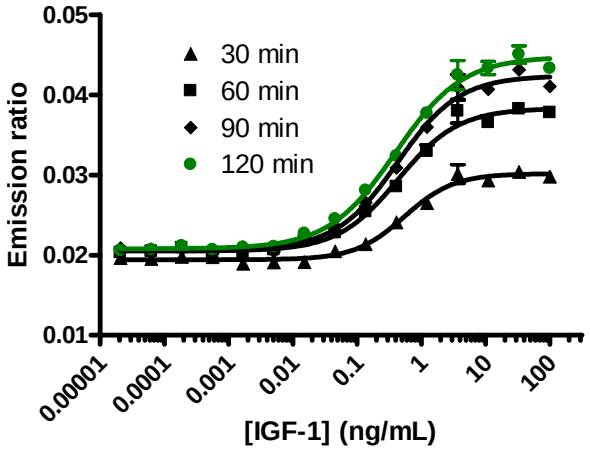
	1%	0.5%	0.2%	0.1%	0%
EC50 (ng/mL)	1.591	0.927	0.310	0.386	0.387
Z' factor	0.42	0.57	0.66	0.71	0.61
RR	2.59	2.45	2.17	2.22	2.23

LanthaScreen™ AKT HEK293E cells (20,000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight in assay medium. Cells were then treated with the indicated concentrations of IGF-1 in the presence of varying concentration of DMSO (0, 0.1%, 0.2%, 0.5%, and 1%). Next, cells were lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-AKT [pS473] antibody and protease/phosphatase inhibitor cocktails. After incubation at room temperature for 120 min, fluorescence emission values at 520 nm and 490 nm were obtained using a BMG PHERAstar plate reader (BMG LABTECH) set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with n=3 replicates per data point.

Note: We observed significant changes in the EC_{50} values and data quality for IGF-1 at DMSO concentrations at or above 0.5%. The assay window and Z' remains relatively unaffected.

Assay Performance with Variable Assay Equilibration Times

Figure 7 —Variable assay equilibration times (cell lysis and antibody incubation)

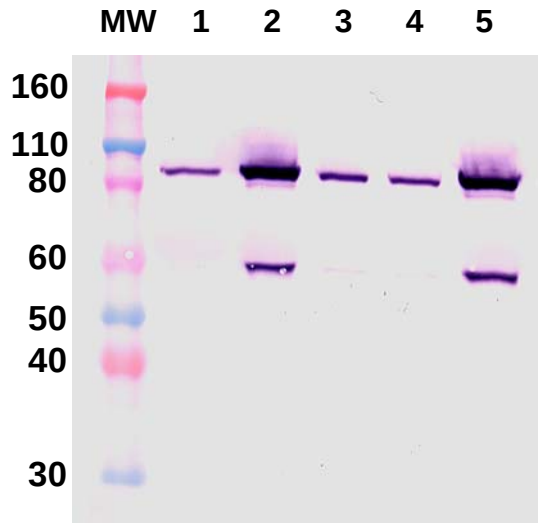


	30 min	60 min	90 min	120 min
EC50 (ng/mL)	0.544	0.432	0.427	0.396
Z' factor	0.35	0.47	0.62	0.71
RR	1.51	1.89	2.06	2.22

LanthaScreen™ AKT HEK293E cells (20,000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight in assay medium. Cells were then treated with a 3-fold serial dilution of insulin for 30 min and subsequently were lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-AKT [pS473] antibody and protease/phosphatase inhibitor cocktails. The assay was allowed to equilibrate at room temperature for different time periods (30, 60, 90, and 120 min), and then fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar plate reader (BMG LABTECH) set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with n=3 replicates per data point.

Western Blot Validation

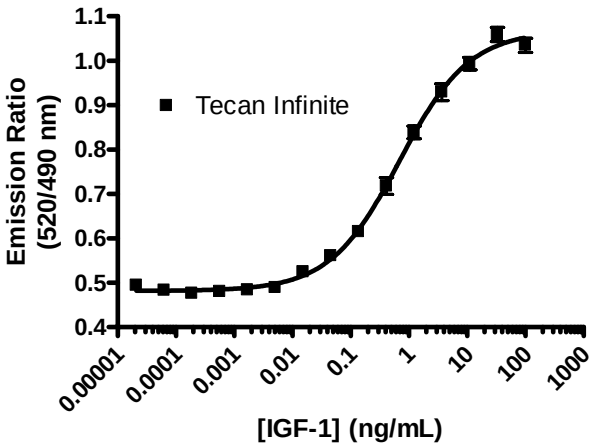
Figure 8 – Validation of LanthaScreen™ AKT HEK293E via Western blot



LanthaScreen™ AKT HEK293E cells were grown to near confluency in 6-well plates in complete Growth Media. All cells were gently rinsed with PBS and then serum starved overnight in Assay Media (16–20 h). The next day, cell were either left untreated (Lane 1); stimulated with insulin (100 nM) for 30 min (Lane 5); or treated with rapamycin (50 ng/mL) (Lane 2), wortmannin (10 uM) (Lane 3), or LY294002 (20 uM) (Lane 4) for 60 min followed by insulin stimulation for 30 min. All inhibitors were from BioSource™. Media was removed from the cells and were then subsequently lysed in cold lysis buffer that includes protease and phosphatase inhibitor cocktails. Lysates were normalized for total protein concentration using Bradford Assay and samples were resolved using SDS-PAGE (4-20% gradient gel). Transfer to a PVDF membrane was accomplished using iBlot, which was then probed with an anti-AKT/PKB [pS473] antibody from BioSource™ (Cat. no. 44-621G).

Instrument Comparison

Figure 9 – Assay plate read on different instrument.



	Tecan Infinite	BMG PHERAstar
EC50 (ng/mL)	0.692	0.422
Z' factor	0.71	0.68
RR	2.10	2.32

LanthaScreen™ AKT HEK293E cells (20,000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight in assay medium. Cells were then treated with the indicated concentrations of IGF-1 for 30 min in the presence of 0.1% DMSO. Cells were subsequently lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-AKT [pS473] antibody and protease/phosphatase inhibitor cocktails. After incubation at room temperature for 120 min, fluorescence emission values at 520 nm and 490 nm were obtained using an Infinite F500 plate reader (Tecan) with the appropriate filter sets. Emission ratios (520/490 nm) are plotted for each experiment, with n=8 replicates per data point. Data presented for the BMG PHERAstar is taken from the validation summary at the beginning of this document.