Validation & Assay Performance Summary

LanthaScreen™ PDCD4 HEK293E

Cat. no. K1593

Modification Detected: Phosphorylation of S457

LanthaScreen™ Cellular Assay Validation Packet

This cell-based assay has been thoroughly tested and validated by Invitrogen and is suitable for immediate use in high-throughput screening (HTS) applications. 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 has been underscored recently by the identification of two distinct cellular complexes: mTORC1 (includes raptor and is rapamycin sensitive) and mTORC2 (includes rictor and is insensitive to rapamycin). Activation of the PI3K/AKT pathway through insulin (or other mitogens) can lead to the phosphorylation of programmed cell death protein 4 (PDCD4) by mTORC1 (indirectly through the ribosomal protein p70 S6 kinase).

LanthaScreen™ PDCD4 HEK293E is a human cell line which constitutively expresses GFP-PDCD4 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 Ser457 on GFP-PDCD4 is detected in cell lysates using a terbium-labeled phosphospecific antibody. This cell line has been validated with different stimuli and inhibitors (shows correct EC_{50} and IC_{50} values) and RNAi duplexes. This assay has also 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 high signal-to-background.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using the LanthaScreen™ Tb-anti-PDCD4 [pS457] Antibody (Cat# PV5103 or PV5105).

1. **Primary agonist dose-response under optimized conditions (ave. of ≥3 expts)**
   Z'-Factor (EC_{100}) = 0.69
   Relative Response Ratio = 2.77
   EC_{50} insulin (pM) = 35.5

   Recommended cell #. = 30,000 cells/well
   Recommended [DMSO] = up to 0.1%
   Recommended Stim. Time = 30 min
   Recom. Assay Incubation = 60 min
   Max. [Stimulation] = 100 nM

2. **Alternate agonist dose-response**
   IGF-1 (EC_{50}) = 107 pg/mL
   PMA = 1314 ng/mL
   TNFα = no response

3. **Small-molecule Inhibitor Testing**
   LY294002 (IC_{50}) = 1.7 μM
   PI-103 (IC_{50}) = 21 nM
   Rapamycin = 0.25 nM

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

Assay Testing Summary

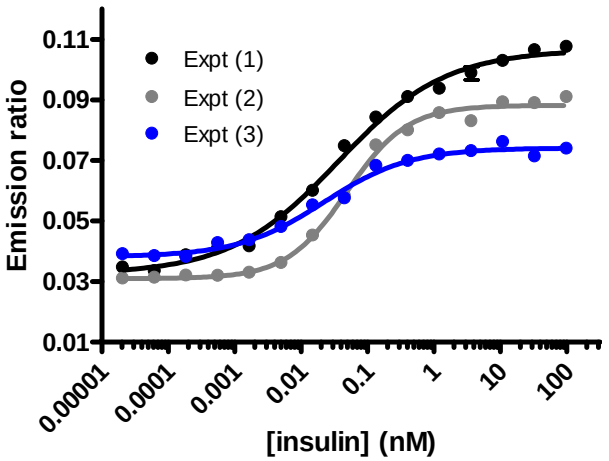
5. **Assay performance with variable cell number**
6. **Assay performance with variable agonist stimulation time**
7. **Assay performance with variable DMSO concentration**
8. **Assay performance with variable antibody equilibration time**

Additional Information

9. **Western Blot validation**
10. **Instrument comparison**

Primary Agonist Dose-Response and Determination of Maximum Assay Window

Figure 1 — Insulin-induced phosphorylation of GFP-PDCD4 HEK293E cells under optimized conditions

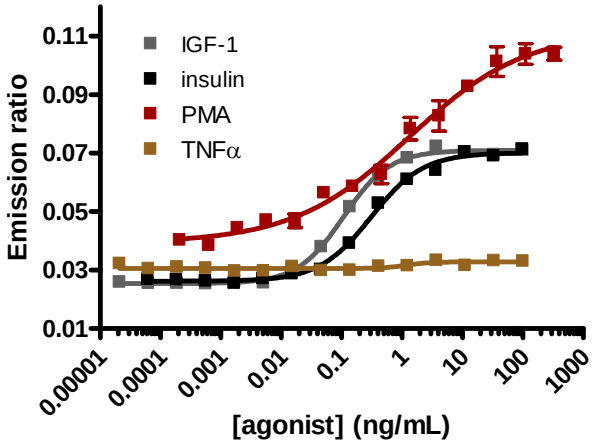


	(1)	(2)	(3)
EC50 (pM)	36	48.7	21.8
Z' factor	0.74	0.66	0.66
RR	3.39	2.91	2.01

LanthaScreen™ PDCD4 HEK293E cells (30,000 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. On the day of the assay, cells were first treated with 4 μL of 1% DMSO followed by 4 μL of 10X concentration of insulin (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-PDCD4[pS457] antibody and both protease / phosphatase inhibitor cocktails, and then incubated for 60 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



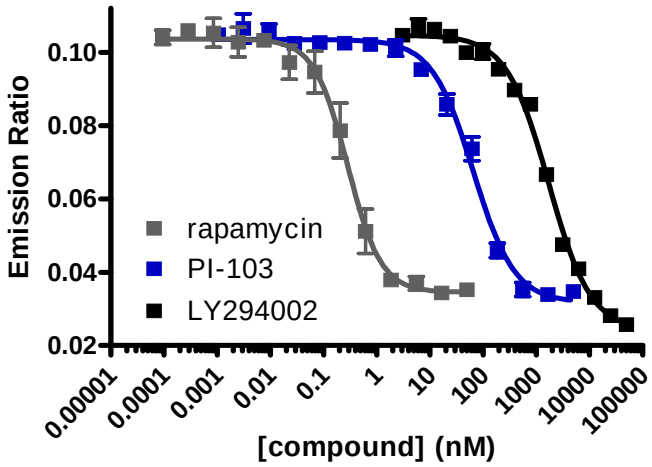
	IGF-1	insulin	PMA	TNFalpha
EC50 (ng/mL)	0.107	0.308	1.314	--
Z' factor	0.87	0.90	0.83	-0.26
RR	2.69	2.60	3.40	1.08

LanthaScreen™ PDCD4 HEK293E cells (30,000 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 agonist – either insulin (Invitrogen #12585-014), IGF-1 (recombinant human insulin-like growth factor; BioSource #PGH0074), TNF-α (recombinant human tumor necrosis factor alpha; BioSource #PHC3016), or PMA (Phorbol 12-Myristate 13-Acetate; Sigma #P1585) for 30 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-PDCD4 [pS457] antibody. The assay was allowed to equilibrate at room temperature for 60 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.

Note: The concentration of insulin is represented as ng/mL (instead of nM) on this graph.

Small-Molecule Inhibitor Testing

Figure 3 — Inhibition of PI3K/AKT/mTORC1 signaling



LanthaScreen™ PDCD4 HEK293E cells (30,000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight in assay medium. Cells were first treated with the indicated concentrations of small-molecule inhibitor (LY294002; BioSource#PHZ1144; PI-103; CalBiochem #528100; rapamycin – BioSource #PHZ1233) for 60 min and then treated with stimulating concentration of insulin 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-PDCD4 [pS457] 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 ≥ 3 replicates per 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 20% and 90% confluency. Do not allow cells to become overconfluent. Harvest cells at 80–90% confluency prior to performing the assay.

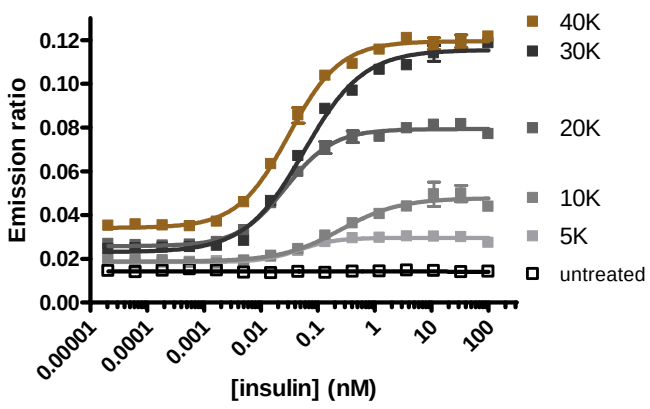
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

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 — Insulin dose-response curves when plating different numbers of cells / well

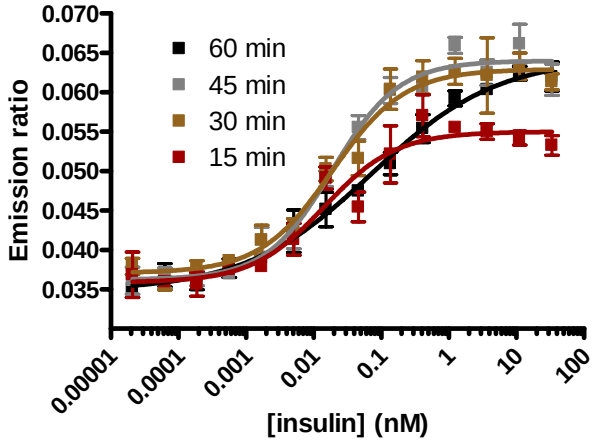


	5K	10K	20K	30K	40K
EC50 (pM)	45.9	25.1	27.3	61.2	30.7
Z' factor	0.39	0.42	0.86	0.72	0.89
RR	1.44	1.97	2.75	4.54	3.36

LanthaScreen™ PDCD4 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 insulin 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-PDCD4 [pS457] antibody and protease/phosphatase inhibitor cocktails. 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=3 replicates per data point.

Assay Performance with Variable Stimulation Times

Figure 5 — Time course of insulin treatment

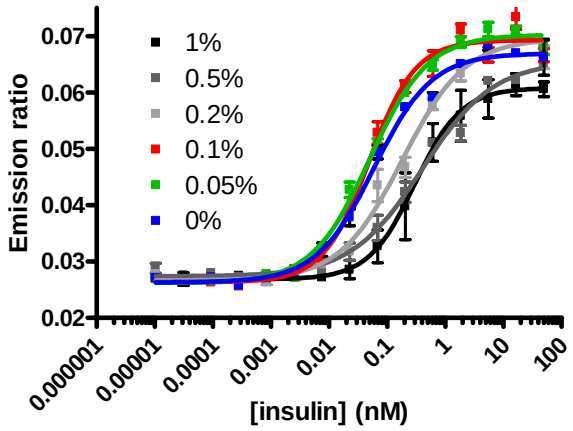


	60 min	45 min	30 min	15 min
EC50 (pM)	70.7	18.8	18.7	14.2
Z' factor	0.51	0.75	0.75	0.58
RR	1.57	1.75	1.64	1.47

LanthaScreen™ PDCD4 HEK293E cells (30,000 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 (15, 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-PDCD4 [pS457] 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=4 replicates per data point.

Assay Performance with Variable DMSO Concentrations

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



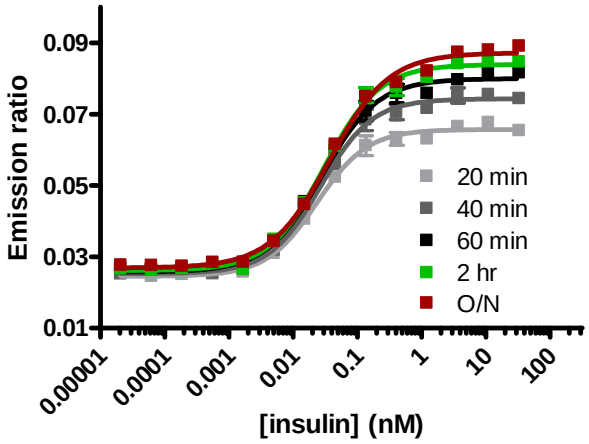
	1%	0.5%	0.2%	0.1%	0.05%	0
EC50 (pM)	301.7	403.6	184.6	49.2	50.4	59.5
Z' factor	0.74	0.91	0.90	0.90	0.80	0.89
RR	2.19	2.35	2.54	2.60	2.55	2.53

LanthaScreen™ PDCD4 HEK293E cells (30,000 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 in the presence of varying concentration of DMSO (0, 0.05%, 0.1%, 0.5%, and 1%). Next, cells were lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-PDCD4 [pS457] antibody and protease/phosphatase inhibitor cocktails. 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=3 replicates per data point.

Note: We observed significant changes in the EC_{50} values and data quality for insulin at DMSO concentrations at or above 0.5%. The assay window and Z' remains unchanged, however.

Assay Performance with Variable Assay Equilibration Times

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

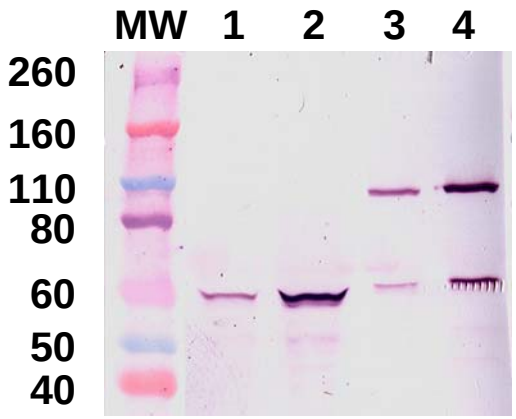


	20 min	40 min	60 min	2 h	O/N
EC50 (pM)	23.4	26.8	28.2	30.3	36.7
Z' factor	0.90	0.85	0.89	0.92	0.93
RR	2.54	2.77	2.91	2.98	3.17

LanthaScreen™ PDCD4 HEK293E cells (30,000 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-PDCD4 [pS457] antibody and protease/phosphatase inhibitor cocktails. The assay was allowed to equilibrate at room temperature for different time periods (20, 40, 60, 120 min and overnight (O/N)), 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™ PDCD4 HEK293E via Western blot

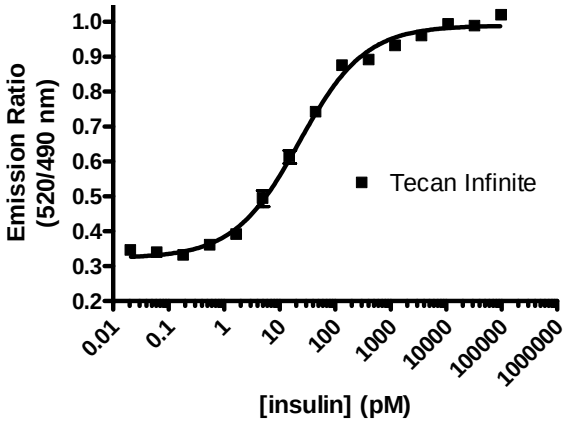


WB: anti-PDCD4 [pS457]

HEK293E parental cells (Lanes 1 and 2) and LanthaScreen™ PDCD4 HEK293E cells (Lanes 3 and 4) were grown to near confluency in 10-cm^2 dishes 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 (Lanes 1 and 3) or stimulated with insulin (100 nM) for 30 min (Lanes 2 and 4). Cells were then 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 nitrocellulose membrane was accomplished using iBlot, which was then probed with an anti-PDCD4 [pS457] antibody.

Instrument Comparison

Figure 9 – Assay plate read on different instrument.



	Tecan Infinite	BMG PHERAstar
EC50 (pM)	22.8	35.5
Z'	0.81	0.69
RR	2.95	2.77

LanthaScreen™ PDCD4 HEK293E cells (30,000 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 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-PDCD4 [pS457] antibody and protease/phosphatase inhibitor cocktails. After incubation at room temperature for 60 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.