Validation & Assay Performance Summary

LanthaScreen™ PRAS40 HEK293E

Cat. no. K1528

Modification Detected: Phosphorylation of T246

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 serine/threonine protein kinase Akt (also known as Protein Kinase B; PKB) was initially identified as one of the downstream targets of phosphatidylinositol-3 kinase (PI3K). The PI3K/Akt pathway mediates signals for cell survival, cell growth, cell-cycle progression, apoptosis inhibition, transcription, translation, and glucose uptake. Activation of Akt results in the phosphorylation of a wide range of protein substrates, including the adaptor protein PRAS40 (proline-rich Akt substrate 40 kDa). Many stimuli (e.g., insulin and insulin-like growth factor; IGF-1) induce Akt phosphorylation at Thr-246 of PRAS40. The result of this modification is not completely understood, but it is known that phosphorylation at Thr246 is required for its metabolic and cell survival / anti-apoptotic signaling roles. Interestingly, PRAS40 has been show to bind to and inhibit mTOR signaling and is itself a substrate of the mTOR kinase (at residue Ser183). Stimulation of cells with insulin results in Akt phoshporylation of PRAS40, interaction with 14-3-3 scaffolding proteins, dissociation from the mTORC1 complex (with Raptor), relieving the inhibition of downstream signaling (i.e., phosphorylation of 4E-BP1 or p70S6K).

LanthaScreen™ PRAS40 HEK293E is a human cell line which constitutively expresses GFP-PRAS40 fusion proteins. This kinase target was introduced using lentivirus and the cells are a clonal population isolated by FACS, using GFP fluorescence as a sorting marker and Blasticidin to maintain the cells under selection. The PI3K/Akt pathway is known to be active in this insulin-responsive HEK293E cell line (much more so than HEK293T, which shows constitutive activation of the PI3K/Akt signaling pathway); therefore, the GFP-PRAS40 fusion protein can serve as an Akt substrate and a robust readout for PI3K/Akt signaling. Using this cell line, a homogenous immunoassay has been developed in which the phosphorylation state of GFP-PRAS40 is detected in cell lysates using a terbium-labeled anti-phospho-Thr246-PRAS40 antibody, in a time-resolved FRET (TR-FRET) readout. This cell line has been validated for EC_{50} values and Z' factors under optimized conditions using insulin as ligand for Akt-mediated GFP-PRAS40 phosphorylation. This assay has also been tested for assay performance under variable experimental conditions, including cell plating density, agonist stimulation time, DMSO tolerance and assay development time. Additional information using alternate stimuli and small-molecule inhibitor of the PI3K/Akt pathway is also included.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format (addition only protocol) using the LanthaScreen™ Tb-anti-PRAS40 [pT246] Antibody (Invitrogen Cat # PV5102 or PV5103).

1. **Primary agonist dose-response under optimized conditions (ave. of ≥3 expts)**
   Z'-Factor (EC_{100}) = 0.73
   Relative Response Ratio = 5.87
   EC_{50} insulin (pM) = 2.2

   Recommended cell #. = 20,000 cells/well
   Recommended [DMSO] = 0.1%
   Recommended Stim. Time = 30 min
   Recom. Assay Incubation = 60 min
   Max. [Stimulation] = 100 nM

2. **Alternate Stimuli**
   IGF-1 (EC_{50}) = 7.4 pg/mL

3. **Small-molecule inhibitor Testing**
   LY294002 (IC_{50}) = 2.53 µM
   PI-103 (IC_{50}) = 301 nM
   PI-3Kα inhibitor IV = 1.02 µM
   Wortmannin (IC_{50}) = 142 nM

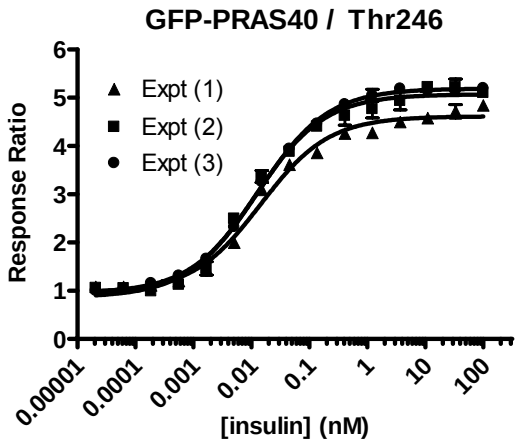
4. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

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**
9. **Assay performance with alternate agonists**
10. **Assay performance with small-molecule inhibitors**

Primary Agonist Dose-Response and Determination of Maximum Assay Window

Figure 1 — Insulin-induced phosphorylation of LanthaScreen™ PRAS40 HEK293E cells under optimized conditions



LanthaScreen™ PRAS40 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. 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-PRAS40 [pT246] 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 BMG PHERAstar plate reader set to TR-FRET mode. The 520/490 nm emission ratios are plotted for each experiment, with n=16 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 10% and 90% confluency. Do not allow cells to become overconfluent.

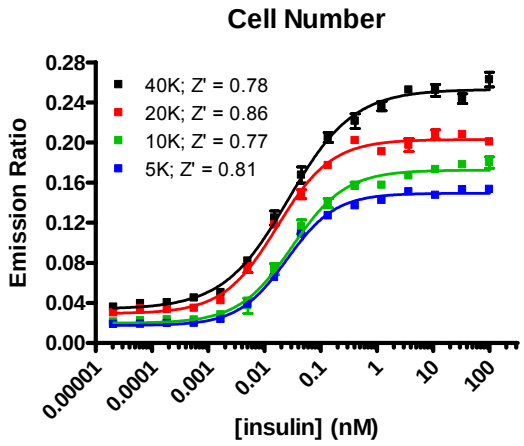
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
D-MEM (w/ GlutaMAX™)	90%	—	—
D-MEM (low glucose)	—	99%	—
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	—	—
Sodium Pyruvate	—	—	—
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

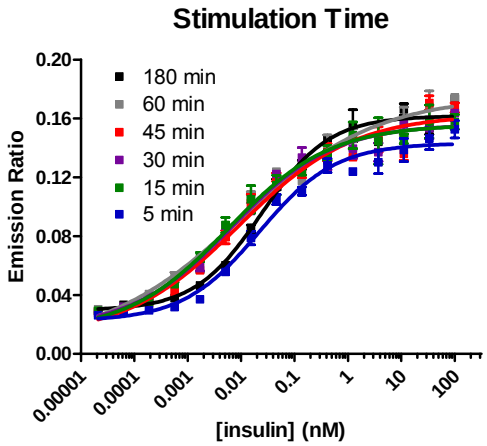
Figure 2 — Insulin dose-response curves when plating different numbers of cells/well



LanthaScreen™ PRAS40 HEK293E cells were plated at variable densities (40000, 20000, 10000, or 5000 cells/well) 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 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-PRAS40 [pT246] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a BMG PHERAstar plate reader 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 Stimulation Times

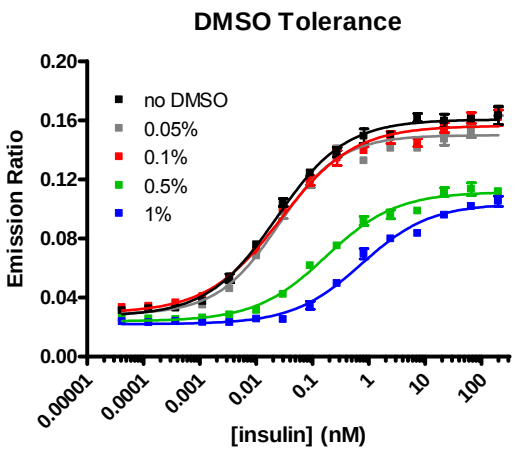
Figure 3 — Time course of insulin treatment



LanthaScreen™ PRAS40 HEK293E cells (20000 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 (5, 15, 30, 45, 60, and 180 min). Cells were next lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-PRAS40 [pT246] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a BMG PHERAstar plate reader set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with n=4 replicates per data point.

Assay Performance w/ Variable DMSO Concentrations

Figure 4 – Insulin dose-response curves in the presence of different final concentrations of DMSO

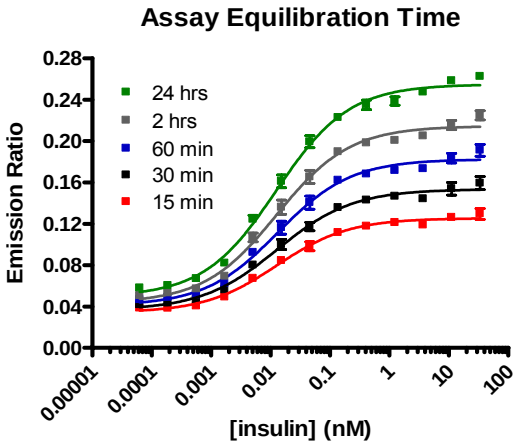


LanthaScreen™ PRAS40 HEK293E cells (20000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight in assay medium. Cells were then treated with the indicated concentrations of insulin in the presence of varying concentration of DMSO (0, 0.05%, 0.1%, 0.5%, and 1%). Next, cells were lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-PRAS40 [pT246] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a BMG PHERAstar plate reader set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with n=4 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 w/ Variable Assay Equilibration Times

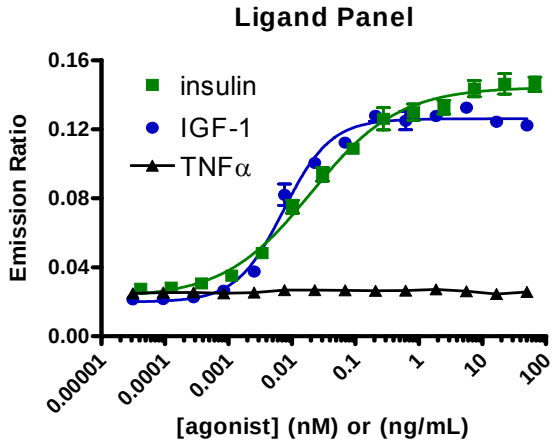
Figure 5 —Variable assay equilibration times (cell lysis and antibody incubation)



LanthaScreen™ PRAS40 HEK293E cells (20000 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-PRAS40 [pT246] antibody. The assay was allowed to equilibrate at room temperature for different time periods (15, 30, 60, 120 min and 24 hr), and then fluorescence emission values at 520 nm and 490 nm were obtained using a BMG PHERAstar plate reader 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 Alternate Agonists

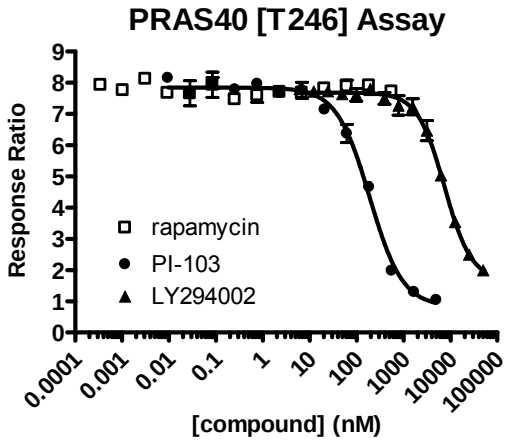
Figure 6 – Treatment of LanthaScreen™ PRAS40 HEK293E cells with alternate agonists



LanthaScreen™ PRAS40 HEK293E cells (20000 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 IGF-1 (recombinant human insulin-like growth factor; BioSource #PGH0074) or TNF-α (recombinant human tumor necrosis factor alpha; BioSource #PHC3016) for 30 min in a total reaction volume of 40 µL. Cells were subsequently lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-PRAS40 [pT246] 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 BMG PHERAstar plate reader 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 Small-Molecule Inhibitors

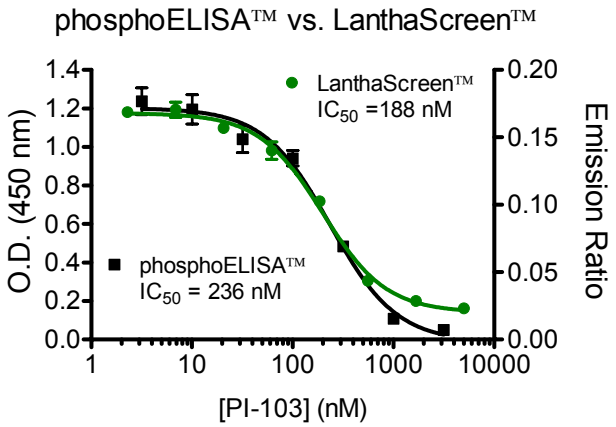
Figure 7 — Inhibition of PI3K/Akt signaling using LanthaScreen™ PRAS40 HEK293E as the readout



LanthaScreen™ PRAS40 HEK293E cells (20000 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; wortmannin – BioSource #PHZ1301; PI-3Kα inhibitor IV – CalBiochem #528111; PI-103 – CalBiochem #528100) for 60 min and then treated with an EC_{80} stimulating concentration of insulin 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-PRAS40 [pT246] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a BMG PHERAstar plate reader set to TR-FRET mode. Emission ratios (520/490 nm) are plotted for each experiment, with n ≥ 3 replicates per data point.

Phospho-ELISA Validation

Figure 8 – Comparison of data generated with phosphor-ELISA and LanthaScreen™ Cellular Assay



HEK293E parental cells were treated with the indicated concentrations of PI-103 and analyzed using the PRAS40 [T246] phosphoELISA (■) kit from Invitrogen. These data were compared to the LanthaScreen™ PRAS40 [T246] cellular assay (●) results with PI-103 inhibition. Both curves overlay well and the observed IC_{50} values are essentially the same (~200 nM).