Validation & Assay Performance Summary

LanthaScreen™ PRAS40 HEK293E

Cat. no.: K1528

Modification Detected: Phosphorylation of S183

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/mTOR 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. PRAS40 is also part of the mTOR complex 1 (mTORC1) and has been shown to inhibit mTOR signaling downstream. Interestingly, growth factor stimulation also results in phosphorylation of PRAS40 at Ser183 by mTORC1. The consequences of these two modifications is not completely understood, but it is known that phosphorylation at Ser183 is rapamycin-sensitive while Thr246 is not.

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/mTOR pathway is known to be active in this insulin-responsive HEK293E cell line (much more so than HEK293T, which shows constitutive activation of the signaling pathway). Using this cell line, two homogenous immunoassays have been developed in which the phosphorylation state of GFP-PRAS40 can be monitored in cell lysates using terbium-labeled anti-phospho-PRAS40 antibodies (specific for Ser183 or Thr246) 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 an agonist. The assay described here for Ser183 phosphorylation has 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 inhibitors of the PI3K/Akt/mTOR pathway is also included.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using the LanthaScreen™ Tb-anti-PRAS40 [pS183] Antibody.

1. **Primary agonist dose-response under optimized conditions (ave. of ≥3 expts)**
   Z'-Factor (EC_{100}) = 0.69
   Relative Response Ratio = 3.3
   EC_{50} insulin (nM) = 0.050

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

2. **Alternate Stimuli**
   IGF-1 (EC_{50}) = 0.102 ng/mL
   PMA = 31.1 ng/mL

3. **Stealth™ RNAi Testing**
   N/A

4. **Small-molecule inhibitor Testing**
   LY294002 (IC_{50}) = 1.3 µM
   PI-103 (IC_{50}) = 106 nM
   rapamycin = 0.5 nM

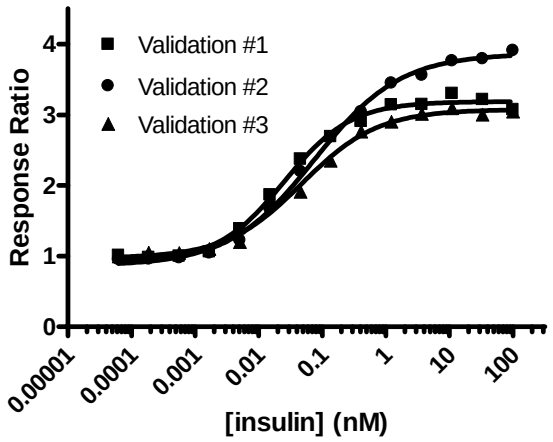
5. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

6. **Assay performance with variable cell number**
7. **Assay performance with variable agonist stimulation time**
8. **Assay performance with variable DMSO concentration**
9. **Assay performance with variable antibody equilibration time**
10. **Assay performance with alternate agonists**
11. **Assay performance with small-molecule inhibitors**
12. **Western blot validation**
13. **Instrument comparison**
14. **Alternate assay format (indirect Tb-Ab detection)**

Primary Agonist Dose-Response and Determination of Maximum Assay Window

Figure 1 —Phosphorylation of LanthaScreen™ PRAS40 HEK293E cells at Ser183 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. The assay media was then aspirated and cells were subsequently lysed by addition of 20 µL lysis buffer, which included 5 nM of Tb-anti-PRAS40 [pS183] 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 fluorescence plate reader (BMG LABTECH) using the LanthaScreen filter module. To obtain response ratios (RR), all TR-FRET emission ratios (520/490 nm) were normalized against unstimulated values (average of zero agonist or zero compound) for that experiment. The response ratios are plotted for each experiment with n≥6 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

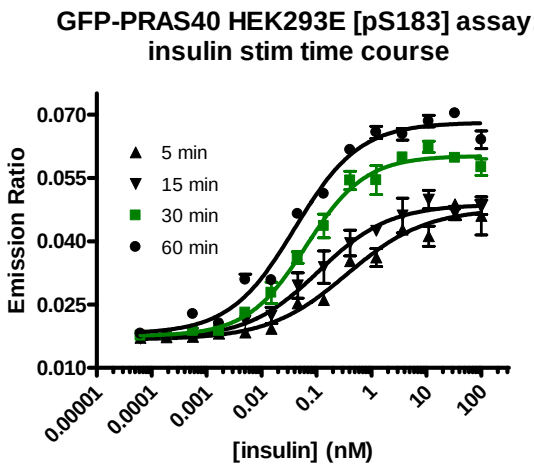
Component	Growth Medium	Assay Medium	Freezing Medium
D-MEM (w/ GlutaMAX™)	90%	—	—
D-MEM (low glucose)	—	99%	—
Dialyzed FBS **Do Not Substitute!**	10%	—	—
Bovine Serum Albumin (BSA), ultrapure	—	0.1%	—
Non-Essential Amino Acids	0.1 mM	—	—
HEPES (pH 7.3)	25 mM	—	—
Penicillin (antibiotic)	100 U/mL	—	—
Streptomycin (antibiotic)	100 µg/mL	—	—
Blasticidin (antibiotic)	5 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance w/ Variable Cell Number

NO data generated, but experiment has been performed using PRAS40 [pT246] assay. Recommended number is 20,000–30,000 cells/well.

Assay Performance w/ Variable Stimulation Times

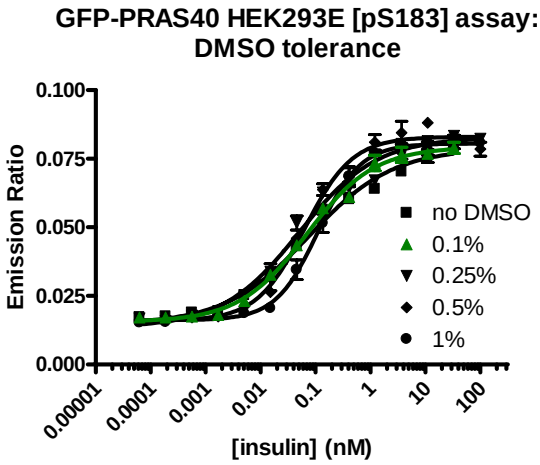
Figure 2 — Time course of insulin treatment



LanthaScreen™ PRAS40 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 (5, 15, 30, and 60 min). Cells were next lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-PRAS40 [pS183] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar fluorescence plate reader (BMG LABTECH) using the LanthaScreen filter module. Emission ratios (520/490 nm) are plotted for each experiment, with n=4 replicates per data point. The green curve indicates the recommended time for agonist stimulation.

Assay Performance w/ Variable DMSO Concentrations

Figure 3 – Insulin dose-response curves in the presence of different final concentrations of DMSO

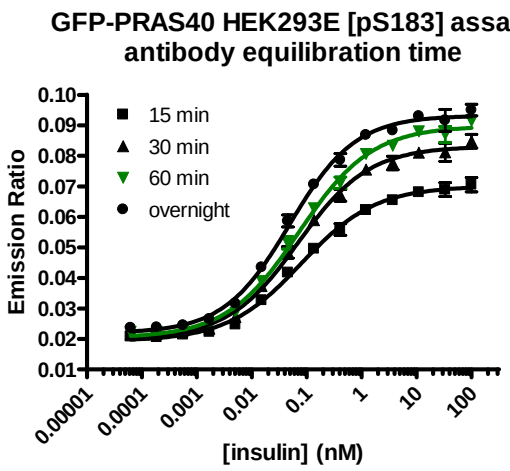


LanthaScreen™ PRAS40 HEK293E cells (20,000 cells/well) were plated the day prior to the assay in a 384-well format and serum starved overnight in assay medium. Cells were then treated with the indicated concentrations of insulin in the presence of varying concentration of DMSO (0, 0.1%, 0.25%, 0.5%, and 1%). Cells were next lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-PRAS40 [pS183] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar fluorescence plate reader (BMG LABTECH) using the LanthaScreen filter module. Emission ratios (520/490 nm) are plotted for each experiment, with n=4 replicates per data point. The green curve indicates the recommended percentage of DMSO.

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 relatively unchanged, however.

Assay Performance w/ Variable Assay Equilibration Times

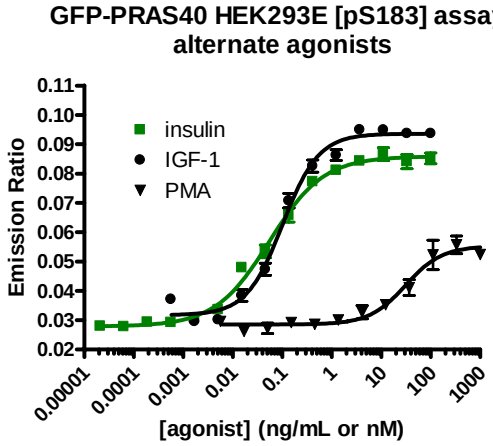
Figure 4 —Variable assay equilibration times (cell lysis and antibody incubation)



LanthaScreen™ PRAS40 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-PRAS40 [pS183] antibody. The assay was allowed to equilibrate at room temperature for different time periods (15, 30, 60 min, and overnight), and then fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar fluorescence plate reader (BMG LABTECH) using the LanthaScreen filter module. Emission ratios (520/490 nm) are plotted for each experiment, with n=4 replicates per data point. The green curve indicates the recommended time for assay equilibration.

Assay Performance with Alternate Agonists

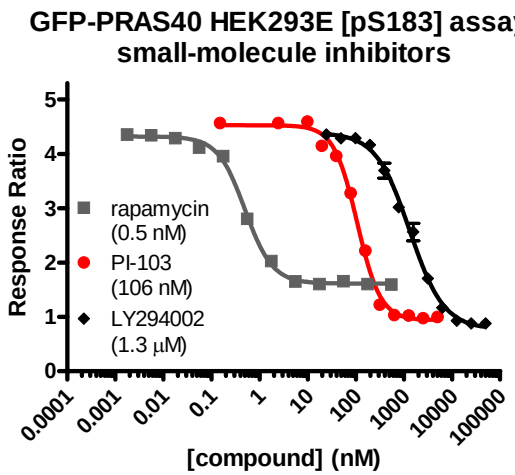
Figure 5 – Stimulation of PRAS40 [S183] phosphorylation with alternate agonists



LanthaScreen™ PRAS40 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 agonist – either IGF-1 (recombinant human insulin-like growth factor; Invitrogen #PGH0074) for 30 min, insulin (Invitrogen #12585-014) for 30 min, or PMA (Phorbol 12-Myristate 13-Acetate; Sigma #P1585) for 15 min in a total reaction volume of 40 µL (0.1% DMSO final concentration. Cells were next lysed by the addition of 30 µL of lysis buffer that includes 5 nM of Tb-anti-PRAS40 [pS183] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar fluorescence plate reader (BMG LABTECH) using the LanthaScreen filter module. Emission ratios (520/490 nm) are plotted for each experiment, with n=4 replicates per data point. The green curve indicates the recommended agonist (insulin).

Assay Performance with Small-Molecule Inhibitors

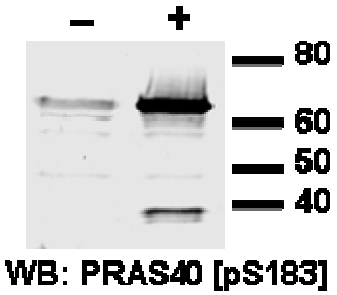
Figure 6 — Inhibition of PI3K/Akt/mTORC1 signaling in GFP-PRAS40 HEK293E using [Ser183] as the readout



LanthaScreen™ PRAS40 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 first treated with 4 µL of the indicated concentrations of small-molecule inhibitor dissolved in DMSO (LY294002-BioSource #PHZ1144; PI-103–CalBiochem #528100; rapamycin–BioSource #PHZ1233) for 60 min and then treated with 4 µL of a stimulating concentration of insulin corresponding to the EC_{80} (~10 nM) 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 includes 5 nM of Tb-anti-PRAS40 [pS183] antibody. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 490 nm were obtained using a PHERAstar fluorescence plate reader (BMG LABTECH) using the LanthaScreen filter module. Emission ratios (520/490 nm) are plotted for each experiment, with n=3 replicates per data point. Representative IC_{50} values are indicated on the graph.

Western Blot Validation

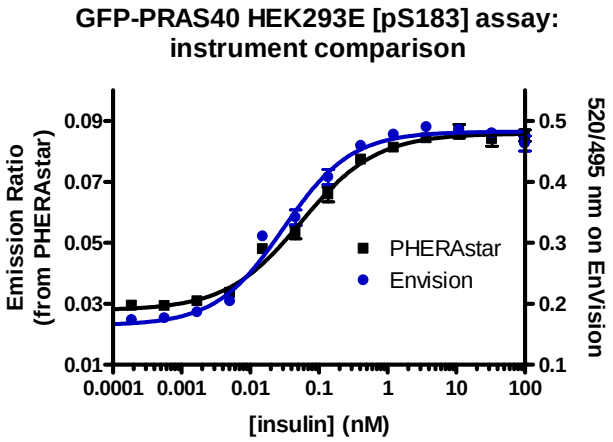
Figure 7 – Validation of LanthaScreen™ Tb-anti-PRAS40 [pS183] antibody via Western blot



LanthaScreen™ PRAS40 HEK293E cells were grown to near confluency in 10-cm^2 dishes in complete Growth Media. 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 (-) or stimulated with insulin (100 nM) for 30 min (+). Following removal of the media, cells were lysed in cold lysis buffer (1% NP-40) including 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 unlabeled rabbit anti-human PRAS40 [Ser183] polyclonal antibody. Detection of bands on the membrane was done so with the Western Breeze immunodetection kit (alkaline phosphatase). The band for GFP-PRAS40 (~70 kDa) phosphorylated at Ser183 is prominent in the insulin-treated sample. Additionally, endogenous PRAS40 is also detected ~40 kDa.

Instrument Comparison

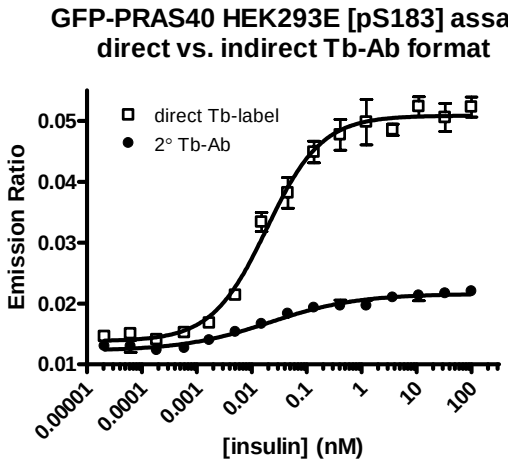
Figure 8 – Assay plate read on different instrument



LanthaScreen™ PRAS40 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 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-PRAS40 [pS183] antibody and protease/phosphatase inhibitor cocktails. After incubation at room temperature for 60 min, fluorescence emission values at 520 nm and 495 nm were obtained using an Envision plate reader (Perkin Elmer) with the appropriate filter sets (blue curve). Emission ratios are plotted for each experiment, with n=8 replicates per data point. Data acquired on the PHERAstar (BMG LABTECH; black curve) is taken from the validation summary at the beginning of this document.

Assay Performance with Alternate Assay Format

Figure 9 – Comparison of LanthaScreen™ cellular assay using directly-labeled Tb-Ab or a primary Ab / 2° Tb-Ab approach



LanthaScreen™ PRAS40 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 30 min in the presence of 0.1% DMSO. Cells were subsequently lysed by the addition of 30 µL of lysis buffer containing 1X protease and phosphatase inhibitor cocktails and 5 nM of rabbit anti-human PRAS40 [pS183] antibody. That antibody was either directly labeled with Tb (□) or the lysis buffer also contained 10 nM Tb-labeled anti-rabbit secondary antibody (● - indirect approach). After incubation at room temperature for 60 min, the fluorescence emission values at 520 nm and 490 nm were acquired on the PHERAstar (BMG LABTECH). Emission ratios are plotted for each experiment, with n=4 replicates per data point.