Validation & Assay Performance Summary

LanthaScreen™ IGF-1R GripTite™ Cells

Cat. no. K1834

Modification Detected: Phosphorylation of Multiple Tyr Residues on IGF-1R

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 IGF-1R/PI3K/AKT pathway mediates signals for cell growth, cell survival, transcription, translation, and glucose uptake. IGF-1R is a receptor tyrosine kinase (RTK) that resides at the top of the pathway. Upon growth factor binding extracellularly, this RTK undergoes auto-phosphorylation at multiple intracellular tyrosine residues (resulting in pathway activation).

The LanthaScreen™ IGF-1R GripTite™ (HEK 293 MSR) cellular assay utilizes a human cell line that constitutively expresses IGF-1R fusion proteins with yellow fluorescent protein (YFP). This kinase target was introduced using lipid transfection and these cells are a clonal population isolated by FACS, using YFP 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 multiple tyrosine residues on IGF-1R is detected in cell lysates using a generic terbium-labeled phosphospecific antibody (Tb-anti-pY20). 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-pY20 antibody (Invitrogen #PV3552 or #PV3553).

1. **Primary agonist dose-response under optimized conditions (ave. of 3 expts)**
   Z'-Factor (EC_{100}) = 0.73
   Relative Response Ratio = 5.46
   EC_{50} IGF-1 (ng/mL) = 30.9

   Recommended cell #. = 20,000 cells/well
   Recommended [DMSO] = up to 0.1%
   Recommended Stim. Time = 30 min
   Recom. Assay Incubation = 120 min
   Max. [Stimulation] = 1000 ng/mL

2. **Small-molecule Inhibitor Testing**
   IGF-1R inhibitor II (IC_{50}) = 2.4 μM

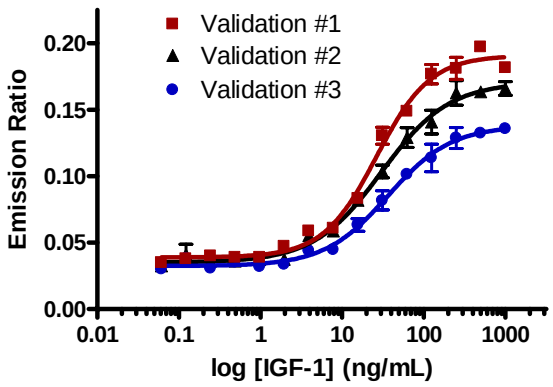
3. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

4. **Assay performance with variable cell number**
5. **Assay performance with variable agonist stimulation time**
6. **Assay performance with variable DMSO concentration**
7. **Assay performance with variable antibody equilibration time**
8. **Assay performance with alternate assay format**
9. **Assay performance with variable final concentrations of Tb-Ab**

Primary Agonist Dose-Response and Determination of Maximum Assay Window

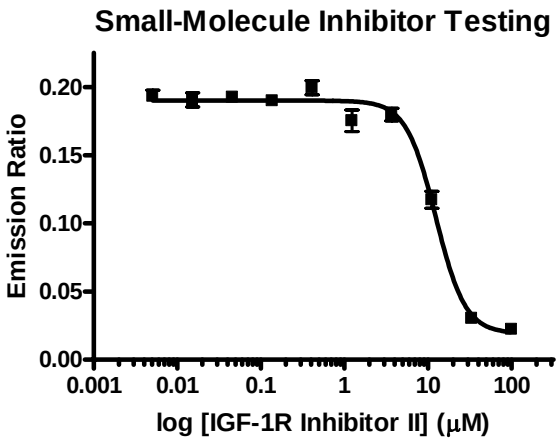
Figure 1 — LanthaScreen™ Cellular Assay to detect growth factor-induced phosphorylation of IGF-1R.



LanthaScreen™ IGF-1R GripTite™ 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) in assay medium. 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 (2-fold dose-response) for 30 min. Next, the assay medium was removed via aspiration and cells were subsequently lysed by addition of 20 μL lysis buffer that included 2 nM of Tb-anti-pY20 antibody and 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Following incubation of the assay plate 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. The 520/490 nm emission ratios (without background subtraction) are plotted for each experiment, with n≥6 replicates per data point.

Small-Molecule Inhibitor Testing

Figure 2 — Inhibition of IGF-1R auto-phosphorylation



LanthaScreen™ IGF-1R GripTite™ 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) in assay medium. On the day of the assay, cells were first treated with 4 μL of 10X the indicated concentrations of small-molecule inhibitor (IGF-1R inhibitor II – CalBiochem #407248) for 60 min. Next, cells were treated with 4 μL of a stimulating concentration of IGF-1 corresponding to the EC_{80} for 30 min (in a total reaction volume of 40 μL). The assay medium was subsequently removed via aspiration and the cells were lysed by addition of 20 μL lysis buffer that included 2 nM of Tb-anti-pY20 antibody and 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Following incubation of the assay plate 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. The 520/490 nm emission ratios are plotted for this experiment, with n=4 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 HEK293 (GripTite™) cells once mature (>80% confluency) prior to performing the assay.

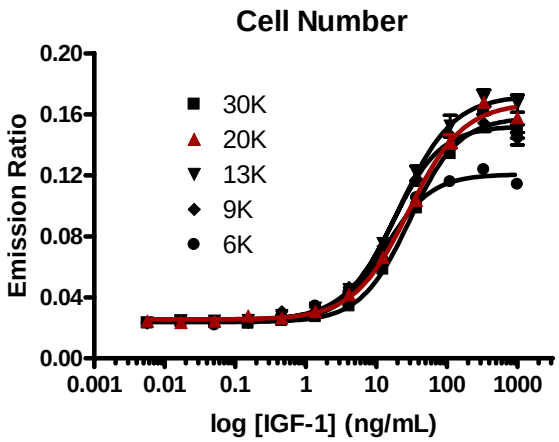
Note: We recommend passing cells for three passages after thawing before using them in the LanthaScreen™ assay. For more detailed cell growth and maintenance directions, please refer to the protocol.

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

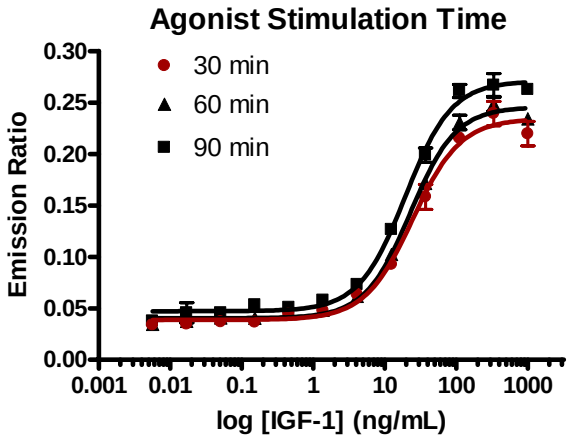
Figure 3 — IGF-1 dose-response curves when plating different numbers of cells per well



LanthaScreen™ IGF-1R GripTite™ cells were plated at variable densities (30000, 20000, 13000, 9000 or 6000 cells/well in 32 µL of assay medium, 384-well format) and serum starved 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 (3-fold dose-response) for 30 min. Next, the assay medium was removed via aspiration and cells were subsequently lysed by addition of 20 µL lysis buffer that included 2 nM of Tb-anti-pY20 antibody and 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Following incubation of the assay plate 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. The 520/490 nm emission ratios (without background subtraction) are plotted for each experiment, with n=4 replicates per data point.

Assay Performance with Variable Stimulation Times

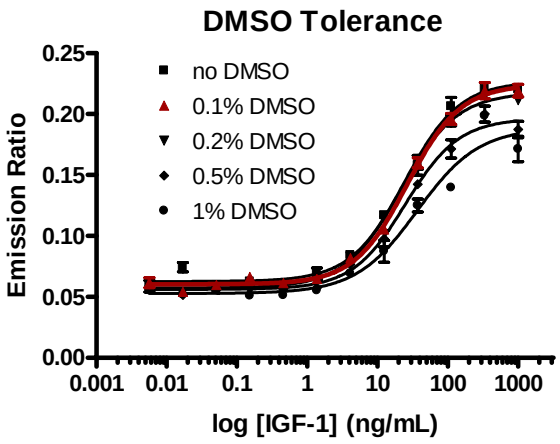
Figure 4 — Time course of IGF-1 stimulation



LanthaScreen™ IGF-1R GripTite™ cells were plated at a density of 20,000 cells/well (in 32 µL of assay medium, 384-well format) and serum starved overnight (16–20 h). The next day, cells were first treated with 4 µL of 1% DMSO followed by 4 µL of 10X concentration of IGF-1 (3-fold dose-response) for either 30, 60, or 90 minute stimulation times. Next, the assay medium was removed via aspiration and cells were subsequently lysed by addition of 20 µL lysis buffer that included 2 nM of Tb-anti-pY20 antibody and 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Following incubation of the assay plate 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. The 520/490 nm emission ratios (without background subtraction) are plotted for each experiment, with n=3 replicates per data point.

Assay Performance with Variable DMSO Concentrations

Figure 5 – IGF-1 dose-response curves in the presence of different final concentrations of DMSO

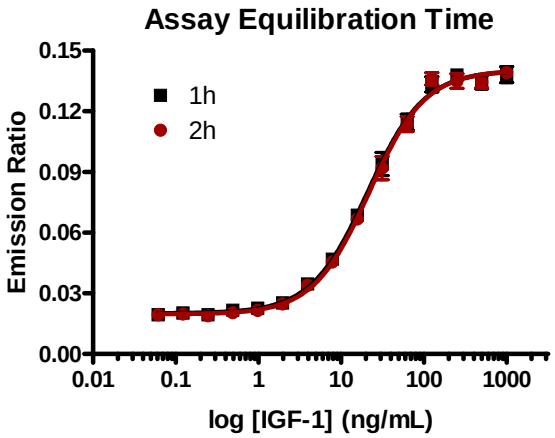


LanthaScreen™ IGF-1R GripTite™ cells were plated at a density of 20,000 cells/well (in 32 µL of assay medium, 384-well format) and serum starved overnight (16–20 h). The next day, cells were first treated 4 µL of various final concentrations of DMSO (0, 0.1%, 0.2%, 0.5%, and 1%), followed by 4 µL of 10X concentrations of IGF-1 (3-fold dose-response) to stimulate the cells for 30 minutes. Next, the assay medium was removed via aspiration and cells were subsequently lysed by addition of 20 µL lysis buffer that included 2 nM of Tb-anti-pY20 antibody and 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Following incubation of the assay plate 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. The 520/490 nm emission ratios (without background subtraction) 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%.

Assay Performance with Variable Assay Equilibration Times

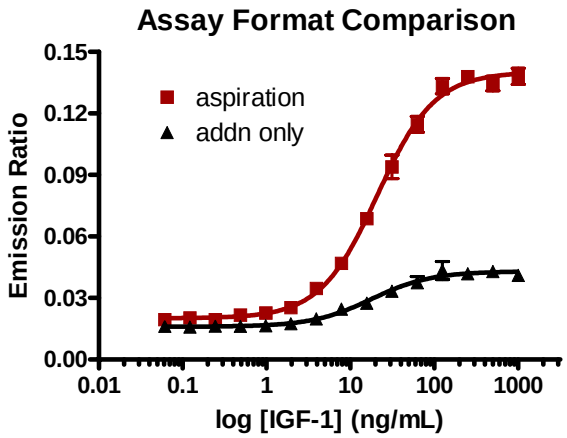
Figure 6 —Variable assay equilibration time (cell lysis and antibody incubation)



LanthaScreen™ IGF-1R GripTite™ cells were plated at a density of 20,000 cells/well (in 32 µL of assay medium, 384-well format) and serum starved overnight (16–20 h). The next day, cells were first treated with 4 µL of 1% DMSO, followed by 4 µL of 10X concentration of IGF-1 (2-fold dose-response) for 30 minutes. Next, the assay medium was removed via aspiration and cells were subsequently lysed by addition of 20 µL lysis buffer that included 2 nM of Tb-anti-pY20 antibody and 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Following incubation of the assay plate at room temperature for 60 or 120 minutes, 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 (without background subtraction) are plotted for each experiment, with n=3 replicates per data point. Although the data is not shown here, similar assay windows were observed as soon as 10 minutes and even after 24 hours.

Assay Performance w/ Alternate Assay Format

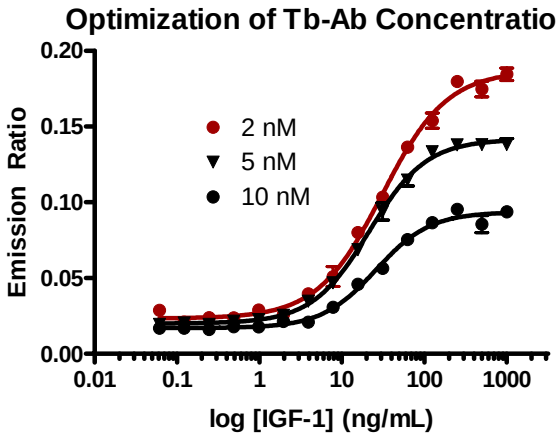
Figure 7 – IGF-1 dose-response curves in the presence of different final concentrations of DMSO



LanthaScreen™ IGF-1R GripTite™ cells were plated at a density of 20,000 cells/well (in 32 µL of assay medium, 384-well format) and serum starved overnight (16–20 h). The next day, cells were first treated 4 µL of 1% DMSO (i.e., 0.1% final concentration), followed by 4 µL of 10X concentrations of IGF-1 (2-fold dose-response) to stimulate the cells for 30 minutes. The following cell lysis step was performed in one of two ways: (1) the assay medium was either removed via aspiration and cells were subsequently lysed by addition of 20 µL lysis buffer that included 2 nM of Tb-anti-pY20 antibody and 1X protease inhibitor and 1X phosphatase inhibitor cocktails, or (2) cells were lysed by the addition of 30 µL of lysis buffer (same contents as described above) to the existing 40 µL of cells and media already in the well ("addition only" protocol). Following incubation of the assay plate 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. The 520/490 nm emission ratios (without background subtraction) are plotted for each experiment, with n=3 replicates per data point.

Assay Performance w/ Variable Concentrations of Tb-Ab

Figure 8 – IGF-1 dose-response curves in the presence of different final concentrations of DMSO



LanthaScreen™ IGF-1R GripTite™ cells were plated at a density of 20,000 cells/well (in 32 µL of assay medium, 384-well format) and serum starved overnight (16–20 h). The next day, cells were first treated with the indicated concentrations of IGF-1 (2-fold dose-response) for 30 minutes in the presence of 0.1% DMSO. Next, the assay medium was removed via aspiration and cells were subsequently lysed by addition of 20 µL lysis buffer that included either 2 nM, 5 nM, or 10 nM of Tb-anti-pY20 antibody and 1X protease inhibitor and 1X phosphatase inhibitor cocktails. Following incubation of the assay plate 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. The 520/490 nm emission ratios (without background subtraction) are plotted for each experiment, with n=4 replicates per data point.