LanthaScreen™ AKT HEK293E Cell-based Assay Protocol

invitrogen™

Catalog no. K1615 | **Shipping: Dry Ice** | **Storage: Liquid Nitrogen**

Protocol part no. K1615.pps | Rev. date: 14 May 2008

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1. Overview of LanthaScreen™ GFP Cellular Assay Technology

Time-resolved FRET (TR-FRET) has been recognized as a method to overcome interfering signals from compounds in high-throughput screening (HTS) applications. Similar to standard FRET-based assays, TR-FRET relies on the proximity dependent energy transfer between an excited donor fluorophore and a suitable acceptor fluorophore, which can be detected by an increased emission from the acceptor molecule. LanthaScreen™ TR-FRET technology from Invitrogen is unique in that it uses a long lifetime terbium chelate (Tb) as the donor species. The extended excited state for Tb is 1000-fold longer than the majority of fluorophores (milliseconds as opposed to nanoseconds). This unique feature allows the measurement of FRET between Tb and the acceptor molecule after a time delay (typically 100 microseconds) after excitation by a flash lamp excitation source. This delay overcomes interference caused by such things as autofluorescent compounds and precipitate-induced light scatter. A complete guide to commonly asked questions and answers regarding LanthaScreen™ technology can be found at www.invitrogen.com/lanthascreen.

The LanthaScreen™ GFP Cellular Assays represent a unique and sensitive way to utilize TR-FRET technology for the interrogation of target-specific phosphorylation events within endogenous signal transduction pathways. By expressing the kinase target protein in living cells as a fusion with green fluorescent protein (GFP), which is a suitable TR-FRET acceptor for the donor molecule Tb, modification-specific antibodies labeled with Tb can be used to detect stimulus-induced post-translational modifications (e.g., phosphorylation or ubiquitination) in a lysed-cell assay format.

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2. Materials Supplied

Cell Line Name: LanthaScreen™ AKT HEK293E

Description: LanthaScreen™ AKT HEK293E cells contain a stably integrated expression vector encoding GFP-AKT fusion under control of a CMV promoter. This DNA construct was introduced into HEK 293E cells using lipid transfection followed by selection with Blasticidin. This cell line is a clonal population isolated by flow cytometry using GFP fluorescence as sorting marker.

Product Number: K1615

Shipping Condition: Dry Ice

Storage Condition: Liquid nitrogen. **Immediately upon receipt, cells must be stored in liquid nitrogen or thawed for immediate use. Cells stored at –80°C can quickly lose viability.**

Quantity: ~2,000,000 (2×10^6 cells/ml)

Application: Detection of agonists/antagonists of the PI3K/AKT signaling pathway (mTORC2 readout)

Growth Properties: Adherent

Cell Phenotype: Epithelial

Selection Marker: Blasticidin (5 µg/ml)

Vector Used: Vivid Colors™ pcDNA™6.2/EmGFP-Bsd/V5-DEST

***Mycoplasma* Testing:** Negative

BioSafety Level: 2

3. Materials Required, but Not Supplied

Media/Reagents	Recommended Source	Part #
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
Dulbecco's Modified Eagle Medium (D-MEM) with GlutaMAX™-1 and sodium pyruvate	Invitrogen	10569-010
DMSO	Fluka	41647
Fetal bovine serum (FBS), dialyzed, tissue-culture grade (**DO NOT SUBSTITUTE!!**)	Invitrogen	26400-044
D-MEM, low glucose, w/o L-glutamine or phenol red	Invitrogen	11054-020
Bovine Serum Albumin (BSA), 10% Ultrapure	Invitrogen	P2046
Non-Essential Amino Acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
HEPES (pH = 7.3)	Invitrogen	15630-080
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136
Trypsin/EDTA (0.05%)	Invitrogen	25300-062
Blasticidin (antibiotic)	Invitrogen	R210-01
LanthaScreen™ Tb-anti-AKT [pSer473] Antibody	Invitrogen	PV5123 or PV5124
Insulin-like Growth Factor 1 (IGF-1)	Invitrogen	PHG9071
Lysis buffer	(see **Section 5.2.3**)	
Protease Inhibitor cocktail	Sigma Aldrich	P8340
Phosphatase Inhibitor cocktail	Sigma Aldrich	P2850

Consumables	Recommended Source	Part #
White tissue culture treated, 384-well assay plates	Corning Life Sciences	3570

Equipment	Recommended Source
Fluorescence plate reader with top-read and TR-FRET capability	Various
Filters, if required for plate reader (see **Section 5.3**)	

4. Cell Culture Conditions

4.1 Media Required

Component	Growth Medium	Assay Medium	Freezing Medium	Thaw Medium
Dulbecco's Modified Eagle Medium (D-MEM) with GlutaMAX™-1 and sodium pyruvate	90% (500 ml)	—	—	90% (500 ml)
D-MEM, low glucose, w/o L-glutamine or phenol red	—	99.9% (500 ml)	—	—
Dialyzed FBS	10% (50 ml)	—	—	10% (50 ml)
Bovine Serum Albumin (BSA)	—	0.1% (0.5 ml)	—	—
NEAA	0.1 mM (5 ml)	—	—	0.1 mM (5 ml)
Penicillin (antibiotic)	100 U/ml (5 ml)	—	—	100 U/ml (5 ml)
Streptomycin (antibiotic)	100 µg/ml (5 ml)	—	—	100 µg/ml (5 ml)
Blasticidin (antibiotic)	5 µg/ml	—	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%	—

Note: Unless otherwise stated, have all media and solutions at least at room temperature (we recommend 37°C for optimal performance) before adding them to the cells.

4.2 Detailed Cell Handling Procedures

4.2.1 Thawing Method

1. Place 9 ml of Thaw Medium (without Blasticidin) into a T25 flask.
2. Place the flask in a humidified 37°C/5% CO_2 incubator for 15 minutes to allow medium to equilibrate to the proper pH and temperature.
3. Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1–2 minutes. Do not submerge vial in water.
4. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet.
5. Transfer the vial contents to a sterile 15-ml conical tube.
6. Add 10 ml of Thaw Medium (without Blasticidin) drop-wise into the cell suspension.
7. Centrifuge cells at 200 × *g* for 5 minutes.
8. Aspirate supernatant and resuspend the cell pellet in 1 ml of fresh Thaw Medium (without Blasticidin).

   Note: This step is important to fully remove the DMSO present from the Recovery™ Cell Culture Freezing Medium.
9. Transfer contents to the T25 tissue culture flask containing pre-equilibrated Thaw Medium (without Blasticidin) and place flask in a humidified 37°C/5% CO_2 incubator.
10. Switch to passaging cells in Growth Medium with Blasticidin, once cells appear to be growing at consistent rates for the given cellular background.

4.2.2 Propagation Method

Cells should be passaged or fed at least two times a week. Cells should be maintained between 20% and 90% confluency. Do not allow cells to reach confluence. Cells which have grown to confluence many not show expected agonist response in the assay. To passage cells:

1. Aspirate medium, rinse once with PBS, add Trypsin/EDTA (1 ml for a T25 flask, 3 ml for a T75 flask, 5 ml for a T175 flask, and 8 ml for T225 flask) and swirl to coat the cells evenly. Cells usually detach after ~2 minutes exposure to Trypsin/EDTA.
2. Add Growth Medium (3 ml for a T25 flask, 7 ml for a T75 flask, and 10 ml for T175 and T225 flasks) to inactivate Trypsin and mix. Verify under a microscope that cells have detached and clumps have completely dispersed.
3. Transfer required amount to a new flask containing pre-warmed Growth Medium.

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4.2.3 Freezing Method

1. Harvest and count the cells, then spin cells down and resuspend in 4°C Recovery™ Cell Culture
2. Freezing Medium at a density of 2×10^6 cells/ml.
3. Dispense 1-ml aliquots into cryogenic vials.
4. Place in an insulated container for slow cooling and store overnight at –80°C.
5. Transfer to liquid nitrogen the next day for storage.

4.2.4 Special Considerations

1. This cell line is a clonal population isolated by FACS using GFP fluorescence as a sorting marker.
2. Assay performance can be expected to depend on the specified media described within this document as responsive cells have been selected based on these formulations.
3. The LanthaScreen™ GFP cellular assay developed with this cell line has been tested with Corning 384-well white flat bottom polystyrene tissue culture-treated microplates (#3570) and may or may not be compatible with other assay plates.
4. This cell line does not adhere tightly to plastic surfaces. Therefore, it is recommended to be gentle when handling the cells as they may become dislodged from the culture flask or assay plate. This feature has no impact on the assay.
5. For additional information about the HEK293E cellular background, please contact Technical Support.
6. This cell line is resistant to Blasticidin.

5. Assay Procedure

The following instructions outline the recommended procedure for monitoring IGF-1-induced phosphorylation of GFP-AKT at Ser473 using TR-FRET as the readout.

- We recommend using Corning 384-well white tissue culture-treated assay plates with low fluorescence background.
- Some solvents may affect assay performance. Assess the effect of a test compound solvent before screening. This cell line has been qualified for DMSO tolerance up to 1%. See validation packet at www.invitrogen.com/lanthascreen for the assay performance of this cell line in the presence of various DMSO concentrations. The cell stimulation described below is carried out in the presence of 0.1% DMSO to simulate the effect that a test compound solvent might have on the assay. If you use other solvents and/or solvent concentrations, change the following assay conditions and optimize appropriately.

5.1 Quick Reference Guide

For more detailed protocol information, see **Section 5.2**.

Agonist Assay Quick Reference Guide

	Unstimulated (DMSO only)	Stimulated (primary agonist)	Cell-free Wells	Test Compound
Step 1 Plate cells in 384-well format	32 µl cells in Assay Medium (20,000 cells/well)	32 µl cells in Assay Medium (20,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium (20,000 cells/well)
Step 2 Serum starve cells (16–20 hr)	Incubate in a humidified 37°C/5% CO_2 incubator overnight (16–20 hours)			
Step 3 Prepare lysis buffer	Before Stimulation of the cells, prepare a suitable volume of complete lysis buffer by adding the necessary protease/phosphatase inhibitors and detection antibodies to the incomplete lysis buffer.			
Step 4 Add Agonist or Test Compounds	8 µl Assay Medium With 0.5% DMSO	8 µl 5X IGF-1 in Assay Medium with 0.5% DMSO	8 µl Assay Medium with 0.5% DMSO	8 µl 5X Test Compounds in 0.5% DMSO
Step 5 Incubate cells	Incubate in a humidified 37°C / 5% CO_2 incubator for 30 min. The optimal stimulation time may vary depending on the agonist.			
Step 6 Lyse Cells	Add 30 µl/well of complete lysis buffer including Tb-labeled antibody (5 nM)			
Step 7 Equilibrate Reaction	Incubate for 120 minutes at room temperature			
Step 8 Read Plate	See **Sections 5.3**			

Antagonist Assay Quick Reference Guide

	Unstimulated (DMSO only)	Stimulated (primary agonist)	Control Compound (known inhibitor)	Cell-free Wells	Test Compound
Step 1 Plate cells in 384-well format	32 µl cells in Assay Medium (20,000 cells/well)	32 µl cells in Assay Medium (20,000 cells/well)	32 µl cells in Assay Medium (20,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium (20,000 cells/well)
Step 2 Serum starve cells (16–20 hr)	Incubate in a humidified 37°C/5% CO_2 incubator overnight (16–20 hours)				
Step 3 Prepare lysis buffer	Before Stimulation of the cells, prepare a suitable volume of complete lysis buffer by adding the necessary protease/phosphatase inhibitors and detection antibodies to the incomplete lysis buffer.				
Step 4 Add Antagonist or Test Compounds	4 µl Assay Medium with 1% DMSO	4 µl Assay Medium with 1% DMSO	4 µl 10X control compound in Assay Medium with 1% DMSO	4 µl Assay Medium with 1% DMSO	4 µl 10X Test Compounds in Assay Medium with 1% DMSO
Optional Step:	Incubate plate with Antagonist for 30–60 minutes before proceeding				
Step 5 Add Agonist	4 µl Assay Medium	4 µl 10X IGF-1 in Assay Medium	4 µl 10X IGF-1 in Assay Medium	4 µl 10X IGF-1 in Assay Medium	4 µl 10X IGF-1 in Assay Medium
Step 6 Incubate cells	Incubate in a humidified 37 °C / 5% CO_2 incubator for 30 min.				
Step 7 Lyse Cells	Add 30 µl/well of complete lysis buffer including Tb-labeled antibody (5 nM)				
Step 8 Equilibrate Reaction	Incubate for 120 minutes at room temperature				
Step 9 Read Plate	See **Sections 5.3**				

5.2 Detailed Assay Protocol

Plate layouts and experimental outlines will vary; in screening mode, we recommend using at least three wells for each control: Unstimulated Control, Stimulated Control, and Cell-free Control.

Note: Some solvents may affect assay performance. Assess the effects of solvent before screening.

5.2.1 Precautions

- Work on a dust-free, clean surface.
- If pipetting manually, you may need to centrifuge the plate briefly at room temperature (30 seconds at 14 × *g*) after additions to ensure all assay components are on the bottom of the wells.
- Cells should be grown to reach 80–90% confluency.
- Complete lysis buffer may need to be prepared prior to stimulation of cells, in order to avoid exceeding the stimulation time for this assay.

5.2.2 Plate Cells

1. Harvest cells from culture in growth medium and resuspend in assay medium at a density of 6.25×10^5 cells/ml.
2. Plate the cells into white tissue culture treated 384-well plates. Add 32 µl per well of Assay Medium to the cell-free control wells. Add 32 µl per well of the cell suspension to Unstimulated and Stimulated wells.
3. After plating, incubate the plates in a 37°C/5% CO_2 incubator for 16–20 hours.

5.2.3 Lysis buffer preparation

Note: Complete lysis buffer (including Tb-labeled antibody) should be made immediately before use (the next day after plating the cells and just prior to stimulating the cells).

1. Prepare lysis buffer: 20 mM Tris, pH 7.4, 5 mM EDTA, 5 mM NaF, 150 mM NaCl, 1% NP-40 (or equivalent). This incomplete lysis buffer can be prepared in large batches and stored at –20°C. The complete lysis buffer (consisting of phosphatase / protease inhibitor cocktails as well as the LanthaScreen™ Tb-anti-AKT [pSer473] antibody) should be prepared on the day of the experiment and stored on ice until use.

2. Determine the volume of complete lysis buffer needed for the assay (assuming 30 µl/well). Add 1/100 volume of each protease inhibitor and phosphatase inhibitor cocktail to the incomplete lysis buffer. Mix well by inversion several times.

Note: Commonly used phosphatase inhibitors such as sodium orthovanadate (VO_4) and sodium pyrophosphate can interfere with the integrity of Tb-chelate and should be avoided.

3. Add LanthaScreen™ Tb-anti-AKT [pSer473] antibody to the lysis buffer to a final concentration of 5 nM. Mix gently by inversion several times.
4. Store complete lysis buffer on ice until use.

5.2.4 Agonist Assay Plate Setup

Note: This subsection provides directions for performing an Agonist assay. See **Section 5.2.5** for directions for performing an Antagonist assay.

Note: The positive agonist controls are run at the concentration of IGF-1 that gives the maximum stimulation (top of the dose response curve). We recommend running a dose response curve to determine the optimal concentration (EC100) for your agonist solution. See **Section 6** for a representative curve. From this example data we determined the EC_{100} to be 100 ng/ml or 13.2 nM.

1. Prepare a stock solution of 0.5% DMSO in Assay Medium.
2. Prepare a 5X stock of each test compound in Assay Medium with 0.5% DMSO (or if the test compound is dissolved in DMSO, make sure the DMSO concentration for the 5X solution is 0.5%).
3. Reconstitute insulin-like growth factor 1 (IGF-1) to 0.1 mg/ml according to the manufacturer's recommendation and store in aliquots at –20°C for long-term storage (up to several months). Aliquots of IGF-1 are freeze/thaw sensitive, but once thawed can be stored at 4°C for up to 1 week.
4. Prepare a 5X stock of IGF-1 in Assay Medium containing 0.5% DMSO. We recommend running a dose response curve to determine the EC_{100} for your IGF-1 solution. See **Section 6** for a representative curve. From this example, we determined the EC_{100} to be 100 ng/ml or 13.2 nM.
5. Add 8 µl of the stock solution of 0.5% DMSO in Assay Medium to each Unstimulated Control well and Cell-free Control well.
6. Add 8 µl of the 5X stock solution of IGF-1 to each Stimulated Control well.
7. Add 8 µl of each 5X stock of test compound to each Test Compound well.
8. Incubate the Agonist assay plate in a humidified 37°C/5% CO_2 incubator for 30 minutes. Then proceed to **Section 5.2.6** for instructions for cell lysis.

5.2.5 Antagonist Assay Plate Setup

Note: This subsection provides directions for performing an Antagonist assay. See **Section 5.2.4** for directions for performing an Agonist assay.

1. Prepare a stock solution of 1% DMSO in Assay Medium.
2. Prepare a 10X stock of each test compound in Assay Medium with 1% DMSO (or if the test compound is dissolved in DMSO, prepare a 10X stock of Test Compounds in Assay Medium and make sure the DMSO concentration for the 10X solution is 1.0%).
3. Reconstitute insulin-like growth factor 1 (IGF-1) to 0.1 mg/ml according to the manufacturer's recommendation and store in aliquots at –20°C for long-term storage (up to several months). Aliquots of IGF-1 are freeze/thaw sensitive, but once thawed can be stored at 4°C for up to 1 week.
4. Prepare a 10X stock of IGF-1 in Assay Medium at an EC_{80} concentration. We recommend running a dose response curve to determine the EC_{80} for your IGF-1 solution. See **Section 6** for a representative curve. From this example, we determined the EC_{80} to be 1.69 ng/ml or 0.222 nM.
5. Prepare a 10X stock of positive control inhibitor (control antagonist compound) in Assay Medium with 1% DMSO. We recommend running a dose response curve to determine the optimal inhibition concentration for the Antagonist solution.
6. Add 4 µl of each 10X stock of test compound to each Test Compound well.
7. Add 4 µl of the stock solution of 1% DMSO to each Stimulated Control well, Unstimulated Control well, and Cell-free Control well.
8. Add 4 µl of the 10X stock of positive control inhibitor to each Antagonist Control well.
9. If desired, incubate the Test Compounds with the cells in a humidified 37°C/5% CO_2 incubator before proceeding. Typically, a 30–60 minute incubation is sufficient.

10. Add 4 µl of the 10X EC_{80} stock solution of IGF-1 prepared in Step 4 to each Test Compound well, Stimulated Control well, Antagonist Control well, and Cell-free Control well.
11. Add 4 µl of Assay Medium to each Unstimulated Control well.
12. Incubate the Antagonist assay plate in a humidified 37°C/5% CO_2 incubator for 30 minutes. Then proceed to **Section 5.2.6** for instructions for cell lysis.

5.2.5 Cell Lysis

1. Remove assay plate from the humidified 37°C/5% CO_2 incubator.
2. Add 30 µl of complete lysis buffer to each well and cover plate to avoid light and prevent evaporation. Total volume in each well should be 70 µl.
3. Incubate covered plate at room temperature for 120 minutes.

5.3 Detection

All TR-FRET measurements are to be made at room temperature from the top of the wells, preferably in 384-well, low volume white assay plates with low fluorescence background.

5.3.1 Instrumentation, Filters, and Plates

The data presented in this document were generated using a PHERAstar plate reader (BMG LABTECH) using the LanthaScreen™ filter block available from BMG. The assay can be performed on a variety of plate readers including those from Tecan. If you are using a LanthaScreen™ GFP cellular assay, we do not recommend the use of monochromator-based instruments, as the sensitivity of these instruments is not sufficient to adequately detect the endogenously expressed GFP fusion proteins. Visit www.invitrogen.com/lanthascreen or contact Invitrogen Discovery Sciences technical support at 800-955-6288 (select option 3 and enter 40266), or email tech_support@invitrogen.com for more information on performing LanthaScreen™ assays on your particular instrument. Recommended filters for fluorescence plate reader:

Excitation filter:	337 nm
Donor Emission filter:	490 nm
Acceptor Emission filter:	520 nm
Integration start	100 µsec
Integration time	200 µsec

5.3.2 Reading an Assay Plate

1. Set the fluorescence plate reader to top / time-resolved read mode.
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes before making measurements.
3. Use the following filter selections:

	Scan 1	**Scan 2**
Purpose:	Measure Donor (Terbium) Signal	Measure Acceptor (TR-FRET to GFP) Signal
Excitation filter:	337 nm (30 nm bandwidth)	
Emission filter:	490 nm (10 nm bandwidth)	520 nm (25 nm bandwidth)
Dichroic Mirror	Variable, see above	
Delay Time	100 µs	
Integration Time	200 µs	

4. Calculate the acceptor/donor Emission Ratio (TR-FRET Ratio) for each well, by dividing the acceptor emission values by the donor emission values

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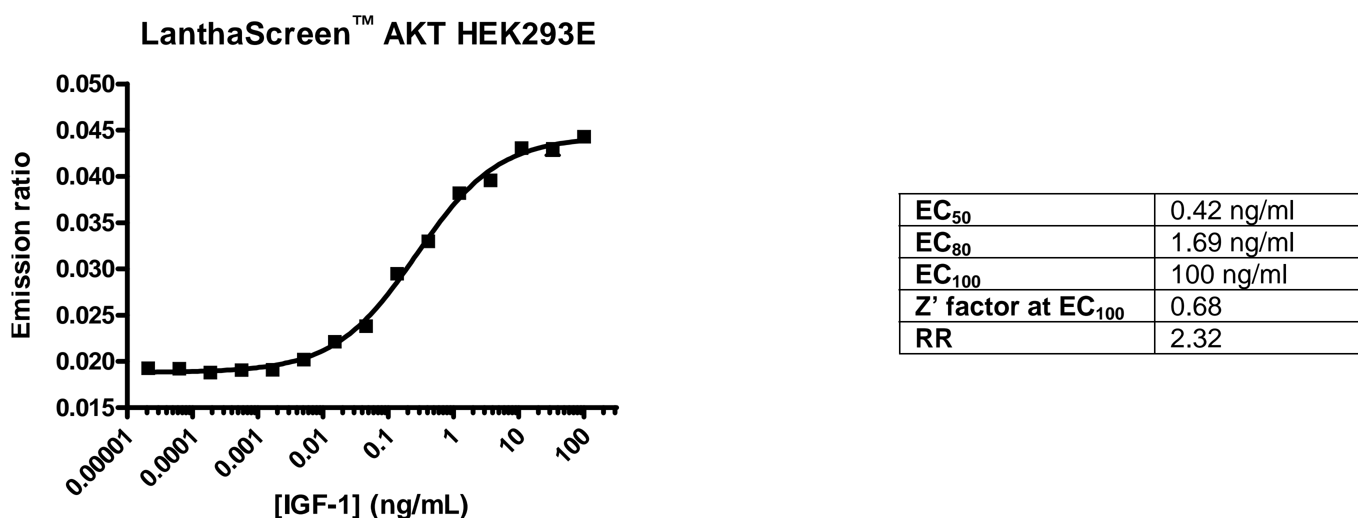
6. Representative Data



EC_{50}	0.42 ng/ml
EC_{80}	1.69 ng/ml
EC_{100}	100 ng/ml
Z' factor at EC_{100}	0.68
RR	2.32

Figure 1. Insulin-like growth factor-1 (IGF-1) dose-response with LanthaScreen™ AKT HEK293E. Cells were plated in 384-well format (20,000 cells/well in 32 µl of assay medium) on the day prior to the assay to serum starve overnight (16–20 h). On the day of the experiment, cells were assayed for response to treatment with IGF-1. 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), which included 5 nM of Tb-anti-AKT [pSer473] 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.

7. References

Robers, M.B., *et al.* "High-Throughput Cellular Assays for Regulated Posttranslational Modifications" (2008) *Anal. Biochem*. 372(2): 189–197.

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