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1. Overview of Cellular LanthaScreen™ Technology

Time-resolved FRET (TR-FRET) has been recognized as a method to overcome interfering signals from compounds in high-throughput screening 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. Invitrogen's LanthaScreen™ TR-FRET technology uses a long life time terbium chelate (Tb) as the donor species which is unique in its extended excited state lifetime. This time period is in the range of milliseconds as opposed to nanoseconds (1000-fold longer) for the majority of fluorophores. This unique feature allows the measurement of FRET between terbium and the acceptor molecule after a time delay, typically 50–100 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 specific target proteins as fusions with green fluorescent protein (GFP, a suitable TR-FRET acceptor for the excited-state Tb fluorophore) in living cells, modification-specific antibodies labeled with Tb can be used to detect stimulus-induced post-translational modifications in a lysed-cell assay format.

The LanthaScreen™ PDCD4 HEK293E cell line allows the accurate monitoring of the mTORC1-mediated phosphorylation of PDCD4 (at Ser457), in response to insulin or insulin-like growth factor (IGF-1). Following activation of the PI3K/AKT pathway with growth factor stimulation, the change in phosphorylation state of the GFP-PDCD4 fusion proteins are detected in cell lysates using LanthaScreen™ Tb-anti-PDCD4 [pSer457] antibody. This assay has been designed in a "addition only" or "mix and read" format and is compatible for use in high-throughput screening applications.

2. Materials Supplied

Cell Line Name:	LanthaScreen™ PDCD4 HEK293E
Description:	LanthaScreen™ PDCD4 HEK293E cells contain a stably integrated expression vector encoding a GFP-PDCD4 fusion under control of a CMV promoter. The GFP-PDCD4 DNA expression construct was introduced into HEK293E cells using lipid transduction, followed by selection with Blasticidin. This cell line is a clonal population isolated by flow cytometry using GFP fluorescence as sorting marker.
Product Number:	K1593
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 phosphorylation of GFP-PDCD4; read-out of mTORC1 signaling
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin (5 µg/mL)
Vector Used:	----
***Mycoplasma* Testing:**	yes
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-PDCD4 [pSer457] Antibody	Invitrogen	PV5104
Lysis buffer	(see section 5.2.6)	
Protease Inhibitor cocktail	Sigma Aldrich	P8340
Phosphatase Inhibitor cocktail	Sigma Aldrich	P2850
Trypan blue	Invitrogen	15250-061

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**)	Various
Optional: Microplate centrifuge	Various

4. Cell Culture Conditions

4.1 Media Required

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

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

4.2 Growth Conditions

For detailed cell growth and maintenance directions, see **Section 7.**

Note: We recommend passing cells for three passages after thawing before using them in this assay.

1. 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 over-confluent.
2. Freeze cells at 1–2 × 10^6 cells/mL in Freezing Medium.

5. Assay Procedure

The following instructions outline the recommended procedure for monitoring the insulin-induced phosphorylation of GFP-PDCD4 in HEK293E cells using LanthaScreen™ Tb-anti-PDCD4 [pSer457] antibody.

5.1 Quick Reference Guide

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

Plate layouts and experimental outlines will vary; in screening mode, we recommend using at least three wells for each condition: unstimulated, stimulated, and cell-free.

Note: Some solvents may affect assay performance. Assess the effect of a test compound solvent before screening

	Unstimulated Wells	Stimulated Wells	Cell-free wells
Step 1: Plate cells	32 µL cells suspended in Assay Medium (30,000 cells/well)	32 µL cells suspended in Assay Medium (30,000 cells/well)	32 µL Assay Medium (no cells)
Step 2: Incubate cells	Incubate at 37°C/5% CO_2 for 16–20 hours		
Step 3: Add DMSO	4 µL per well of 1% DMSO in Assay Medium		
Step 4: Stimulate cells	4 µL Assay Medium	4 µL 10x insulin in Assay Medium (30 minutes)	4 µL Assay Medium
Step 5: add Lysis Buffer (incl. Tb-Ab)	30 µL per well		
Step 6: Cell Lysis/Assay Equilibration	60 minutes at room temperature (protect plate from light)		
Step 7: Read Plate/ Analyze data	See **Section 6**		

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 80–95% confluency before performing the assay.

5.2.2 Plate Cells

1. Harvest cells from culture in Growth Medium and resuspend in Assay Medium at a density of 9.4×10^5 cells/mL.
2. Plate the cells into a white tissue-culture treated 384-well plate. Add 32 µL of Assay Medium to each cell-free control well. Add 32 µL of cell suspension to each unstimulated and stimulated well (final density = 30,000 cells/well).
3. After plating, centrifuge the plate briefly at room temperature (30 seconds at 14 × *g*).
4. Incubate the plate in a 37°C/5% CO_2 incubator for 16–20 hours.

5.2.4 Stimulate Cells

1. Prepare 10X insulin at EC_{80} in Assay Medium. We recommend preparing a dose response curve for insulin to determine the EC_{80} for your Stimulation Solution.
2. Add 4 µL of Assay Medium to each unstimulated, stimulated, and cell-free well.
3. Add 4 µL of 10X insulin in Assay Medium to each stimulated well and 4 µL of Assay Medium to each unstimulated and cell-free well.
4. Incubate the assay plate in a humidified 37°C/5% CO_2 incubator for 60 minutes.

5.2.5 Lysis Buffer Preparation and Cell Lysis

1. Prepare lysis buffer: 20 mM Tris-HCl, 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 and protease inhibitor cocktails as well as the LanthaScreen™ Tb-anti-pPDCD4 [Thr246] antibody) should be prepared on the day of the experiment.
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 ($NaVO_4$) and sodium pyrophosphate can interfere with the integrity of Tb chelate and should be avoided.
3. Add LanthaScreen™ Tb-anti-pPDCD4 [pSer457] antibody to the lysis buffer to a final concentration of 5 nM. Mix gently by inversion several times.
4. Remove assay plate from the humidified 37°C/5% CO_2 incubator.
5. Add 30 µL of complete lysis buffer to each well of cells in media, and cover the plate to protect from light and evaporation.
6. Incubate the covered plate at room temperature for 60 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 BMG PHERAstar plate reader using the LanthaScreen™ filter block available from BMG. The assay can be performed on a variety of plate readers, including those from Tecan. We do not recommend the use of monochromator-based instruments for LanthaScreen™ GFP Cellular Assays, 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 the 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	337 nm
Emission filter:	490 nm	520 nm

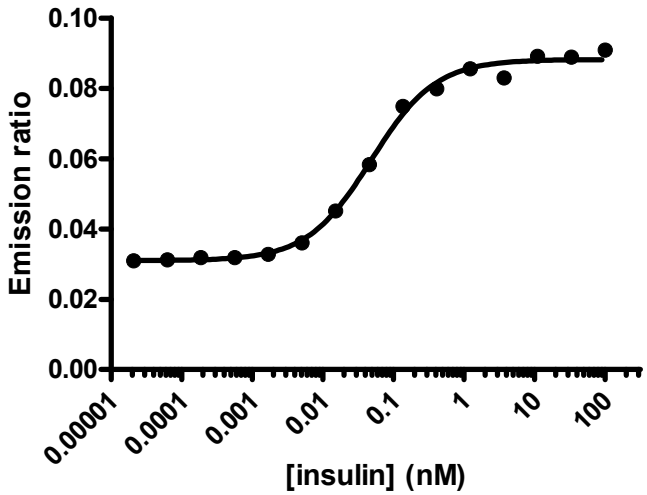
4. Calculate the acceptor/donor Emission Ratio (TR-FRET Ratio) for each well, by dividing the acceptor emission values by the donor emission values.

6. Data Analysis

6.1 Background Subtraction (optional)

1. Use the assay plate layout to identify the location of the cell-free wells. These control wells are used for background subtraction.
2. Determine the average 520/490 nm emission ratio from the cell-free wells (average antibody background).
3. Subtract the average antibody background (520/490 nm ratio) from all of the emission ratio data.

6.2 Representative Data



EC_{50} insulin	49 pM
EC_{80} insulin	207 pM
Max Stim Used	100 nM
Z′ (at max stim)	0.66
Response Ratio	3.0

Figure 1. Insulin dose-response curve. Insulin dose-response curve. LanthaScreen™ PDCD4 HEK293E cells (30,000 cells/well in 32 µL of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were pretreated with 4 µL of 1% DMSO before treatment with the indicated concentration of insulin (4 µL addition) for 30 minutes. Cells were lysed by addition of 30 µL lysis buffer (to 70 µL total volume), which included both 5 nM of LanthaScreen™ Tb-anti-PDCD4 [pSer457] antibody and protease/phosphatase inhibitors. The plate was 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 8 replicates at each data point.

7. Detailed Cell Handling Procedures

7.1 Thawing Method

1. Place 10 mL of Growth 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 drop-wise into 10 mL of Growth Medium without Blasticidin in a sterile 15-mL conical tube.
6. Centrifuge cells at 200 × *g* for 5 minutes.
7. Aspirate supernatant and resuspend the cell pellet in 1 mL of fresh Growth Medium without Blasticidin.
8. Transfer contents to the T25 tissue culture flask containing pre-equilibrated Growth Medium without Blasticidin and place flask in a humidified 37°C/5% CO_2 incubator.
9. At first passage, switch to Growth Medium with Blasticidin.

7.2 Propagation Method

1. Cells should be passaged or fed at least twice a week. Cells should be maintained between 20% and 90% confluence. Do not allow cells to become over-confluent.
2. To passage cells, aspirate medium, rinse once in 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 2–5 minutes upon exposure to Trypsin/EDTA. Add an equal volume of Growth Medium to inactivate Trypsin.
3. Verify under a microscope that cells have detached and clumps have completely dispersed.
4. Spin down cells at 200 × *g* and resuspend in Growth Medium.

7.3 Freezing Method

1. Harvest the cells as described in **Section 7.2**. After detachment, count the cells, then spin cells down at 200 × *g* and resuspend at a density of 1–2 × 10^6 cells/mL in cold (4°C) Recovery™ Cell Culture Freezing Medium.
2. Dispense 1-mL aliquots into cryogenic vials.
3. Place in an insulated container for slow cooling and store overnight at –80°C.
4. Transfer to liquid nitrogen the next day for storage.

8. References

1. Dorrello, N. V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E., and M. Pagano. (2006) *Science* **314**, 467–471.

9. Purchaser Notification

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