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1.0 OVERVIEW OF CELLULAR LANTHASCREEN™ TECHNOLOGY

Time-resolved FRET (TR-FRET) has been recognized as a method to overcome interfering signals in screening applications. Similar to standard FRET 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 of the acceptor fluorophore. Invitrogen's LanthaScreen TR-FRET technology uses long life time terbium chelates (Tb) as donor species which are unique in their extended excited state lifetime. The excited state lifetime of terbium is in the range of milliseconds as opposed to nanoseconds for the majority of fluorophores. This unique feature allows the measurement of FRET between terbium and a suitable acceptor after a time delay, typically 50 to 100 microseconds after excitation by a flash lamp excitation source. This delay overcomes interference caused by 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 specific molecular steps within endogenous signal transduction cascades. 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™ STAT3 GripTite™ cell line allows the accurate monitoring of the JAK-mediated phosphorylation of STAT3 (at Tyr-705), in response to cytokines such as IL-6. Following JAK/STAT pathway activation, the change in phosphorylation state of the GFP-STAT3 fusion proteins is detected in cell lysates using LanthaScreen™ Tb-pSTAT3 [pTyr705] antibody. This assay has been designed for use in high-throughput screening applications.

2.0 MATERIALS SUPPLIED

Cell Line Name:	LanthaScreen™ STAT3 GripTite™
Description	LanthaScreen™ STAT3 GripTite™ cells contain a stably integrated expression vector encoding a GFP-STAT3 fusion under control of a CMV promoter. The GFP-STAT3 DNA expression construct was transfected into HEK-293 MSR (GripTite™) cells using Lipofectamine™ 2000, followed by selection with Blasticidin. This cell line is a clonal population isolated by flow cytometry using GFP fluorescence as sorting marker.
Catalog Number:	K1488
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-STAT3
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin (5 µg/ml)
Vector Used:	Vivid Colors™ pcDNA™6.2/N-EmGFP-STAT3 Vector
***Mycoplasma* Testing:**	Negative
BioSafety Level:	2

3.0 MATERIALS REQUIRED, BUT NOT SUPPLIED

Media/Reagents	Recommended Source	Part #
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
DMEM with GlutaMAX™	Invitrogen	10569-010
DMSO	Fluka	41647
Fetal bovine serum (FBS), dialyzed, tissue-culture grade (**DO NOT SUBSTITUTE!**)	Invitrogen	26400-044
OPTI-MEM® I (with HEPES/L-Gln, without Phenol Red)	Invitrogen	11058-021
Fetal Bovine Serum (Charcoal Stripped)	Invitrogen	12676-029
Nonessential amino acids (NEAA)	Invitrogen	11140-050
Sodium Pyruvate	Invitrogen	11360-070
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136
HEPES (1 M, pH 7.3)	Invitrogen	15630-080
Trypsin/EDTA	Invitrogen	25300-062
Recombinant Human Interleukin-6 (IL-6)	Invitrogen	PHC0065
Blasticidin (antibiotic)	Invitrogen	R210-01
LanthaScreen™ Tb-anti-pSTAT3 [pTyr705] Antibody	Invitrogen	PV4846
Lysis buffer	Not a product (see section 5.2.5)	
Protease Inhibitor mix	SIGMA Aldrich	P8340
Phosphatase Inhibitor mix	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**)	Chroma Technology Corp.
Optional: Microplate centrifuge	Various

4.0 CELL CULTURE CONDITIONS

4.1 Media Required

Component	Thawing Medium	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	500 ml bottle	500 ml bottle	—	—
OPTIMEM (without Phenol Red)	—	—	500 ml bottle	—
Dialyzed FBS	50 ml	50 ml	—	—
Charcoal stripped FBS	—	—	5 ml	—
NEAA	5 ml	5 ml	5 ml	—
HEPES (pH 7.3)	12.5 ml	12.5 ml	—	—
Penicillin/Streptomycin	5 ml	5 ml	5 ml	—
Sodium Pyruvate	—	—	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 Growth Conditions

For detailed cell growth and maintenance directions, see **Section 7.0**.

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

1. Thaw cells in Thaw 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 reach confluence.
2. Freeze cells at 2×10^6 cells/ml in Recovery™ Cell Culture Freezing Medium.

5.0 ASSAY PROCEDURE

The following instructions outline the recommended procedure for monitoring the IL-6 induced phosphorylation of GFP-STAT3 using LanthaScreen™ Tb-anti pSTAT3 [pTyr705] 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. 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 other solvents and/or solvent concentrations are used, change the following assay conditions and optimize appropriately.

	Unstimulated Wells	Stimulated Wells	Cell-free wells
Step 1 Plate cells	32 µl cells suspended in Assay Medium (20,000 cells/well)	32 µl cells suspended in Assay Medium (20,000 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 Add Agonist	4 µL Assay Medium	4 µl 10x IL-6 in Assay Medium	4 µl Assay Medium
Step 5 Incubate Cells	Incubate at 37°C/5% CO_2 for 30 minutes		
Step 6 Prepare Lysis Buffer (during cell incubation)	Lysis Buffer (See section 5.2.5) Add 1:100 Protease inhibitor (Sigma P8340) Add 1:100 Phosphatase inhibitor (Sigma P2850) Add Tb-anti-pSTAT3 [Tyr705] antibody (final concentration 5 nM)		
Step 7 Media Removal	Aspirate media using multichannel aspirator		
Step 8 add Lysis Buffer (incl. antibody)	20 µl per well		
Step 9 Incubation	60 minutes at room temperature in the dark.		
Step 10 Read Plate/ Analyze data	See **Section 6.0**		

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 60 to 90% confluency.

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% CO2 incubator for 16-20 hours.

5.2.3 Prepare Stock Solutions

1. Prepare Assay Medium
2. Prepare 10X IL-6, at EC_{100}, in Assay Medium. We recommend preparing a dose response curve for IL-6 to determine the EC_{80} for your Stimulation Solution.

5.2.4 Stimulate Cells

1. Add 4 µl Assay Medium with to the Unstimulated, Stimulated and Cell-free wells.
2. Add 4 µl 10X IL-6 to Stimulated wells and 4 µl Assay Medium to Unstimulated and Cell-free wells.
3. Incubate the assay plate in a humidified 37°C/5% CO_2 incubator for 30 minutes.

5.2.5 Lysis buffer preparation and Cell Lysis

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-pSTAT3 [Tyr705] antibody) should be prepared on the day of the experiment.
2. Determine the volume of complete lysis buffer needed for the assay (assuming 20 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-pSTAT3 [pTyr705] 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. Carefully aspirate media from each well using a multichannel aspirator. In order to minimize cell loss, avoid direct contact with the aspirator on adhered cells when aspirating.
6. Add 20 µl of complete lysis buffer to each well and cover plate.
7. Incubate covered plate at room temperature for 60 minutes in the dark.

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. 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 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	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.

6.0 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} IL-6	2.95 ng/ml
EC_{80} IL-6	14.7 ng/ml
Max Stim Used	200 ng/ml
Z′ (max stim)	0.78

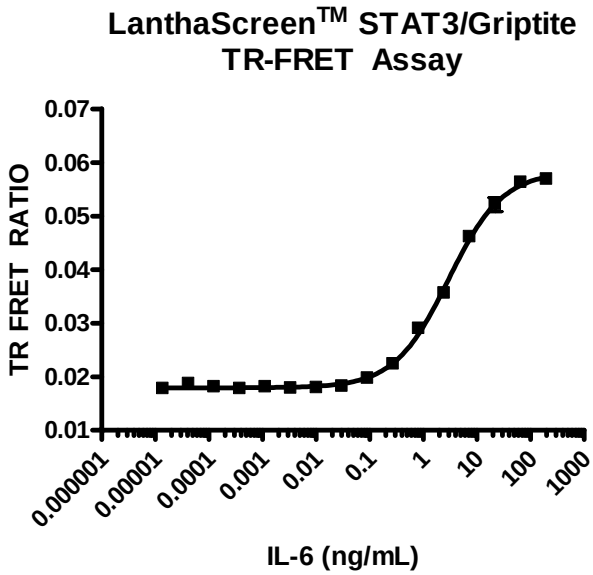

Figure 1. LanthaScreen™ STAT3 GripTite™ assay: Stimulation with IL-6. LanthaScreen™ STAT3 GripTite™ cells (20,000 cells/well in 32 µl of assay medium, 384-well format) were plated the day prior to the assay. On the day of the assay, cells were treated with IL-6 in the presence of 0.1% DMSO for 30 minutes. Cells were aspirated and lysed by addition of 20 µl lysis buffer, which included 5 nM of Tb-anti-pSTAT3 [pTyr705] antibody, and 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. No background subtraction was applied.

7.0 DETAILED CELL HANDLING PROCEDURES

7.1 Thawing Method

1. Place 14 ml of Thawing Medium (without Blasticidin) into a T75 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 Thawing 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 Thawing Medium (without Blasticidin).
8. Transfer contents to the T75 tissue culture flask containing pre-equilibrated Thawing 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 10% and 90% confluence. Do not allow cells to reach confluence.
2. To passage cells, aspirate medium, rinse once in PBS, add Trypsin/EDTA (3 ml for a T75 flask and 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 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 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 and resuspend in 4°C Cell Culture Freezing Medium to 2 × 10^6 cells/ml.
2. Dispense 1.0-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.

8.0 PURCHASER NOTIFICATION

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Use of Genetically Modified Organisms (GMO)

Information for European Customers The CellSensor™ ARE-*bla* HepG2 cell line(s) are genetically modified with the plasmids pLenti6M3 pLenti-*bsd*/ARE-*bla*. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.