LanthaScreen™ ATF2 (19-106) A549 Cell-based Assay

Catalog no. K1556 | **Shipping Condition: Dry Ice** | **Storage: Liquid Nitrogen**

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1. OVERVIEW OF 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 as donor species which are unique in their extended excited stage lifetime. The excited stage 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 signaling cascades. By expressing modification targets as fusion with green fluorescent protein (GFP, a suitable TR-FRET acceptor for terbium), modification specific antibodies labeled with the TR-FRET donor terbium can be used to quantitatively detect stimulus induced changes in posttranslational modification following cell lysis . The LanthaScreen™ IkB HEK293 cell line allows the accurate monitoring of the IKK mediated phosphorylation of IkBα, a critical step in the NFkB signaling pathway, in response to different stimuli. The change in phosphorylation status of the GFP-IkB fusion protein can be analyzed in cell lysates in a homogenous TR-FRET assay using a terbium-labeled anti-pS32-IkB antibody as detection reagent. This assay can be utilized for the development of cell-based assays for the screening of drug candidates that modulate IKK activity.

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2. MATERIALS SUPPLIED

Cell Line Name: LanthaScreen™ ATF2 (19-106) A549

Description: LanthaScreen™ ATF2 (19-106) A549 cells contain a stably integrated expression vector encoding for a GFP-ATF2 (19-106) fusion protein under control of the CMV promoter. Treatment of LanthaScreen™ ATF2 (19-106) A549 cells with agonist of the jnk pathway, such as TNF or EGF will lead to the transient phosphorylation of the GFP-ATF2 (19-106) fusion protein. The phosphorylated GFP-ATF2 fusion protein can be quantitated in cell lysates using a terbium labeled anti-pT71-ATF2 antibody.

Product Number: K1556

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-ATF2

Growth Properties: Adherent

Cell Phenotype: Epithelial

Selection Marker: Blasticidin (5 µg/ml)

Vector Used: Vivid Colors™ pcDNA6.2/N-EmGFP-ATF2-19-106 Vector

***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
DMEM with GlutaMAX™	Invitrogen	10569-010
DMSO	Fluka	41647
Fetal bovine serum (FBS), dialyzed, tissue-culture grade (**DO NOT SUBSTITUTE!**)	Invitrogen	26400-044
OPTIMEM with GlutaMAX™	Invitrogen	51985-034
Fetal bovine serum (charcoal stripped)	Invitrogen	12676-029
Nonessential amino acids (NEAA)	Invitrogen	11140-050
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
0.05% Trypsin/EDTA	Invitrogen	25300-054
Blasticidin (antibiotic)	Invitrogen	R210-01
LanthaScreen Tb-anti-pATF2 (pThr71) Antibody	Invitrogen	PV4452
Lysis buffer B (for cell lysis after medium removal)	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	3704

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

3.1 Optional Equipment and Materials

- Epifluorescence- or fluorescence-equipped microscope with appropriate filters
- Microplate centrifuge

4. CELL CULTURE CONDITIONS

4.1 Media Required

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	—	—
OPTIMEM with GlutaMAx™/HEPES	—	99%	—
Dialyzed FBS	10%	—	—
Charcoal / Dextran stripped FBS	—	1%	—
NEAA	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	—	—
Penicillin (antibiotic)	100 U/ml	100 U/ml	—
Streptomycin (antibiotic)	100 µg/ml	100 µg/ml	—
Sodium Pyruvate	—	1 mM	—
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.**.

Note: We recommend passing cells for three passages after thawing before using them in GFP-ATF2 phosphorylation 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 10% and 90% confluency. Do not allow cells to reach confluence.
2. Freeze cells at 1-2 × 10^6 cells/ml in Freezing Medium.

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5. ASSAY PROCEDURE

The following instructions outline the recommended procedure for monitoring the TNF induced phosphorylation of GFP-ATF2 (19-106) in a TR-FRET based assay using a Tb-labeled anti pT71-ATF2 antibody. This protocol has been extensively used with the LanthaScreen™ ATF2 (19-106) cell line.

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 (10000 cells/well)	32 µl cells suspended in Assay Medium (10000 cells/well)	32 µl Assay Medium (no cells)
Step 2 Incubate cells	Incubate at 37°C/5% CO_2 for 18 hours		
Step 3 Add Medium	Prepare a stock of Assay Medium. Add 4 µl per well		
Step 4 Add agonist	4 µl Assay Medium	4 µl 10x TNF in assay medium	4 µl Assay Medium
Step 5 Incubate cells	Incubate the plate at 37°C/5% CO_2 for 30 min.		
Step 6 Prepare Lysis Buffer (during cell incubation)	Prepare lysis buffer (see **Section 5.2.5**): • add 1:100 Protease inhibitor (SIGMA P8340) • add 1:100 Phosphatase inhibitor (SIGMA P2850) • add Tb-anti-pT71 ATF2 Ab (final concentration 2 nM)		
Step 7 Remove medium	Use a multichannel aspirator to remove the medium		
Step 8 add Lysis Buffer (incl. antibody)	20 µl per well		
Step 9 incubation	60 minutes at RT in the dark (cover plate)		
Step 10 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 3.1×10^5 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 18 hours.

5.2.3 Prepare Stock Solutions

1. Prepare Assay Medium.
2. Prepare 10X TNF-α, at EC_{80}, in Assay Medium. We recommend preparing a dose response curve for TNF-α 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 TNF-α 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

Note: This protocol is designed for the lysis of cells **after removal of medium**. It is recommended to control for cell loss (*e.g.,* due to compound toxicity), which can lead to false positive results. An easy way to determine cell loss is to measure total GFP signal before addition of the Tb-labeled antibody. Any substantial decrease in total GFP signal might indicate loss of cells during the medium removal (detached cells due to toxicity).

1. Prepare lysis buffer: 20 mM Tris, pH 7.4, 5 mM EDTA, 5 mM NaF, 150 mM NaCl, % NP-40)). The lysis buffer can be prepared in large batches and stored at -20°C. The lysis buffer can be subjected to repeated freeze/thaw cycles. The phosphatase / protease inhibitor cocktails as well as the terbium labeled (Tb) anti pT71-ATF2 antibody should be added immediately before the experiment (or during stimulation) to the exact amount of lysis buffer needed for the experiment (20 µl/well = approx. 8 ml/384-well plate). Remaining buffer should be discarded.
2. Addition of 1/100 volume of each protease inhibitor and phosphatase inhibitor mix to the lysis buffer. Mix well by pipetting up and down several times.
3. Add Tb-anti pT71-ATF2 antibody to a final concentration of 2 nM to the lysis buffer. The lysis buffer should be used within 90 minutes after addition of the antibody.
4. Remove assay plate from the humidified 37°C/5% CO_2 incubator.
5. Remove medium using a multi channel aspirator. Excessive contact with the cell layer should be avoided.
6. Add 20 ml of complete lysis buffer to each well.
7. Incubate in the dark 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. 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	337 nm
Emission filter	490 nm	520 nm

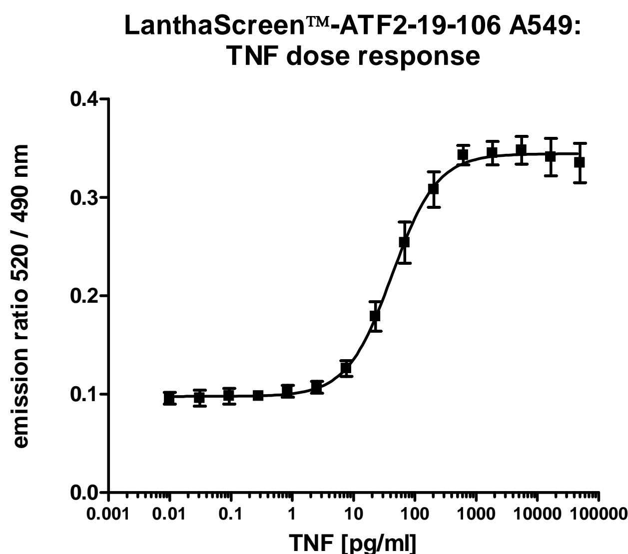
4. Calculate the acceptor/donor Emission Ratio for each well, by dividing the acceptor emission values by the donor emission values.

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 emission from the Cell-Free wells at 520 nm (Average acceptor background).
3. Subtract the Average acceptor background (data collected at 520 nm) from all of the acceptor emission data.
4. Calculate the acceptor/donor Emission Ratio for each well, by dividing the background-subtracted acceptor emission values by the donor emission values.

6.2 Representative Data



EC_{50}	42.4 pg/ml
EC_{80}	200 pg/ml
EC_{100}	600 ng/ml
Z′ at EC_{100}	0.712

Figure 1. Dose response of LanthaScreen™ ATF2 (19-106) A549 cells to TNF-α. LanthaScreen™ ATF2 (19-106) A549 cells were treated with TNF-α over the indicated concentration range in a 384-well format. Cells were incubated for 30 minutes with TNF-α and then lysed by addition of 20 µl lysis buffer (including 2 nM Tb-anti-pT71-ATF2 detection antibody) following medium removal. Fluorescence emission values at 490 nm and 520 nm were obtained using a standard fluorescence plate reader and the 520/490 nm ratios were plotted against the concentration of the agonist. No background subtraction was applied.

7. DETAILED CELL HANDLING PROCEDURES

7.1 Thawing Method

1. Place 14 ml of Growth 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 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 T75 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 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.

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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.

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