LanthaScreen® c-jun (1-79) HeLa Cell-based Assay Protocol

Catalog no. K1425 **Shipping: 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. 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 GFP-jun-1-79 HeLa cell line allows the accurate monitoring of the JNK-mediated phosphorylation in response to different stimuli and can be utilized for the development of cell based assays for the screening of drug candidates. The change in phosphorylation status of the GFP-jun fusion protein can be analyzed in cell lysates in a homogenous time resolved FRET assay using a terbium labeled anti pS73-c-jun antibody.

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2. Materials Supplied

Cell Line Name:	LanthaScreen® c-jun (1-79) HeLa
Description:	LanthaScreen® c-jun (1-79) HeLa cells contain a stably integrated expression vector encoding for a GFP-jun-1-79 fusion protein under control of the CMV promoter. Treatment of LanthaScreen® c-jun (1-79) HeLa cells with agonist of the JNK pathway, such as TNF or EGF, will lead to the transient phosphorylation of the GFP-c-jun-1-79 fusion protein. The phosphorylated GFP-c-jun fusion protein can be quantitated in cell lysates using a terbium labeled anti pS73-c-Jun antibody.
Product Number:	K1425
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-c-jun
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin (5 µg/ml)
Vector Used:	Vivid Colors™ pcDNA6.2/N-EmGFP-jun-1-79 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-pc-Jun(pSer73) Antibody	Invitrogen	PV4452
6X LanthaScreen® Cellular Assay Lysis Buffer	Invitrogen	A12891
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**)	
Optional: Epifluorescence- or fluorescence-equipped microscope with appropriate filters	Various
Optional: Microplate centrifuge	Various

4. Cell Culture Conditions

4.1 Media Required

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	—	—
OPTIMEM w 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-c-jun 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.

5. Assay Procedure

The following instructions outline the recommended procedure for monitoring the TNF induced phosphorylation of GFP-c-jun-1-79 in a TR-FRET based assay using a Tb-labeled anti pS73 jun antibody. This protocol has been extensively used with the LanthaScreen® c-jun-(1-79) Hela 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	24 µl cells suspended in Assay Medium (10,000 cells/well)	24 µl cells suspended in Assay Medium (10,000 cells/well)	24 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 3 µl per well		
Step 4 Add agonist	3 µl Assay Medium	3 µl 10x TNF in assay medium	3 µl Assay Medium
Step 5 Incubate cells	Incubate the plate at 37°C/5% CO_2 for 30 minutes		
Step 6 Prepare Complete 6X Lysis Buffer)	To 1 mL of 6X Lysis Buffer, add 30 µL 100X protease inhibitor, 30 µL 100X phosphatase inhibitor, and LanthaScreen® Tb-anti-pc-Jun (pSer73) Antibody to 12 nM		
Step 7 Add Complete 6X Lysis Buffer	6 µL per well		
Step 8 add Lysis Buffer (incl. antibody)	20 µl per well		
Step 8 Cell Lysis/Assay Equilibration	60 minutes at room temperature (protect plate from light)		
Step 9 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 60 to 90% confluency.

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5.2.2 Plate Cells

1. Harvest cells from culture in growth medium and resuspend in assay medium at a density of 4.2×10^5 cells/ml.
2. Plate the cells into white tissue culture-treated 384-well plates. Add 24 µl per well of Assay Medium to the cell-free control wells. Add 24 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 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 3 µl Assay Medium with to the Unstimulated, Stimulated and Cell-free wells.
2. Add 3 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 Prepare Complete 6X Lysis Buffer and Lyse Cells

Note: When preparing complete 6X lysis buffer, avoid commonly used phosphatase inhibitors such as sodium orthovanadate ($NaVO_4$) and sodium pyrophosphate, as these can interfere with the integrity of Tb chelate.

1. During incubation, prepare **complete 6X lysis buffer** by adding both protease inhibitor and phosphatase inhibitor cocktails to the provided 6X lysis buffer at a 1:33 dilution of 100X stock (e.g., 30 µL of 100X stock inhibitors per 1,000 µL of 6X Lysis Buffer). Next, add LanthaScreen™ Tb-anti-pc-Jun [pSer73] antibody to a final concentration of 12 nM. Mix gently by inversion several times. Store on ice until use.
2. Remove assay plate from the humidified 37°C/5% CO_2 incubator.
3. Add 6 µL of complete lysis buffer to each well of cells in media, and cover the plate to protect from light and evaporation.
4. 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 drugdiscoverytech@lifetech.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.

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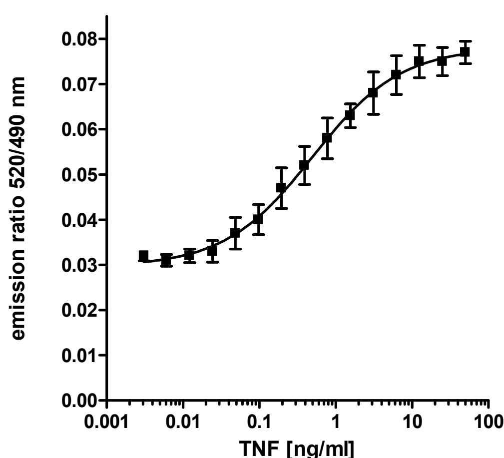
6. Data Analysis

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 (520 nm) from all 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

LanthaScreen™-c-Jun-HeLa: TNF Dose Response



EC_{50}	405 pg/ml
EC_{80}	1400 pg/ml
EC_{100}	12.5 ng/ml
Z′ at EC_{100}	0.692

Figure 1. Dose response of LanthaScreen® c-Jun (1-79) HeLa cells to TNF-alpha. LanthaScreen® c-Jun (1-79) HeLa cells were treated with TNF-alpha over the indicated concentration range in a 384-well format. Cells were incubated for 30 minutes with TNF-alpha and then lysed. Fluorescent 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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