LanthaScreen™ c-Fos HeLa Cell-based Assay Protocol

invitrogen™

Catalog no. K1579 **Shipping: Dry Ice** **Storage: Liquid Nitrogen**

Protocol part no. K1579.pps Rev. date: 14 May 2008

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1. Overview of LanthaScreen™ Cellular Assay 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 lifetime terbium chelate (Tb) fluorophore as a donor species. The majority of fluorophores possess excited-state lifetimes on the order of nanoseconds. In contrast, terbium fluorophores display extended excited state lifetimes on the range of milliseconds. This unique feature allows the measurement of FRET between interacting donor and acceptor molecules after an extended 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.

2. Materials Supplied

Cell Line Name:	LanthaScreen™ c-Fos HeLa
Description:	LanthaScreen™ c-Fos HeLa cells contain a stably integrated expression vector encoding GFP-ERK2 fusion under control of a CMV promoter. The GFP-c-Fos DNA expression construct was introduced into HeLa cells using lipid based 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:	K1579
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 MAP Kinase (MAPK) signaling pathway.
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin (5 µg/ml)
Vector Used:	pcDNA™6.2/EmGFP-c-Fos 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 w/ GlutaMAX™	Invitrogen	10569-10
DMSO	Fluka	41647
Fetal bovine serum (FBS), dialyzed, tissue-culture grade (**DO NOT SUBSTITUTE!**)	Invitrogen	26400-044
Opti-MEM® I	Invitrogen	11058-021
Fetal Bovine Serum (Charcoal/Dextran-Treated)	Invitrogen	12676-029
Nonessential amino acids (NEAA)	Invitrogen	11140-050
HEPES (1 M)	Invitrogen	15630-80
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
Phosphate-buffered saline without calcium and magnesium [PBS(-)]	Invitrogen	14190-136
Trypsin/EDTA	Invitrogen	25300-062
Blasticidin (antibiotic)	Invitrogen	R210-01
LanthaScreen™ Tb-anti-c-Fos [pThr 232] Antibody	Invitrogen	PV5207 or PV5208
Tumor Necrosis Factor Alpha (TNFα)	Invitrogen	PHC3011
Lysis buffer	(see **Section 5.2.3**)	
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**)	

4. Cell Culture Conditions

4.1 Media Required

Component	Growth Medium	Assay Medium	Freezing Medium	Thaw Medium
DMEM w\ GlutaMAX™	500 ml (90%)	—	—	500 ml (90%)
Opti-MEM® I	—	500 ml (99.5%)	—	—
Dialyzed FBS	50 ml (10%)	—	—	50 ml (10%)
Charcoal / Dextran stripped FBS	—	2.5 ml (0.5%)	—	—
NEAA	5 ml (0.1 mM)	5 ml (0.1 mM)	—	5 ml (0.1 mM)
HEPES	5 ml (0.01 M)	—	—	5 ml (0.01 M)
Penicillin (antibiotic)	5 ml (100 U/ml)	5 ml (100 U/ml)	—	5 ml (100 U/ml)
Streptomycin (antibiotic)	5 ml (100 µg/ml)	5 ml (100 µg/ml)	—	5 ml (100 µg/ml)
Sodium Pyruvate	5 ml (1 mM)	5 ml (1 mM)	—	5 ml (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 Detailed Cell Handling Procedures

4.2.1 Thawing Method

1. Place 14 ml of Thaw 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 to a sterile 15-ml conical tube.
6. Add 10 ml of Thaw Medium (without Blasticidin) dropwise 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 T75 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 10% 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 (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 (7 ml for a T75 flask, 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 prewarmed Growth Medium.

4.2.3 Freezing Method

1. Harvest and count the cells, then spin cells down and resuspend in 4°C Recovery™ Cell Culture Freezing Medium at a density of 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.

4.2.4 Special Considerations for Working with this Cell Line

1. This cell line is a clonal population isolated by Fluorescence Activated Cell Sorting (FACs). Assay performance can be expected to depend upon use of the specified media as responsive cells have been chosen based on these formulations.
2. For additional information about the HeLa cellular background please contact Technical Support at 1-760-603-7200, select option 3 and enter extension 40266.
3. This cell line is tested to be compatible with Corning tissue culture-treated plates (3570) and may not be suitable for use with other plates.
4. This cell line is Blasticidin resistant.

5. Assay Procedure

The following instructions outline the recommended procedure for monitoring TNFα-induced phosphorylation of GFP-c-Fos using TR-FRET as the readout.

Note:

- We recommend using white, tissue-culture-treated, 384-well assay plates with low fluorescence background, and have optimized this assay for use with Corning Assay Plates (Part# 3570).
- 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 0.1%. 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 Wells	Stimulated Wells (Positive control Agonist)	Cell-free Wells	Test Compound Wells
Step 1 Plate cells	32 µl cells in Assay Medium (25,000 cells/well)	32 µl cells in Assay Medium (25,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium (25,000 cells/well)
Step 2 Incubate 16-20 hours	Incubate cells for 16–20 hours in a humidified 37°C/5% CO_2 incubator			
Step 3 Prepare complete 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 TNFα 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 minutes. The optimal stimulation time may vary depending on the agonist.			
Step 6 Aspirate Media	Aspirate Media from each well using a multichannel aspirator			
Step 7 Lyse Cells	Add 20 µl/well of complete lysis buffer including terbium-labeled antibody			
Step 8 Equilibrate Reaction	Incubate 1 hour at room temperature			
Step 9 Read Plate	See **Section 5.3**			

Antagonist Assay Quick Reference Guide

	Unstimulated Wells	Stimulated Wells	Positive Control Compound Wells	Cell-free Wells	Test Compound Wells
Step 1 Plate cells	32 µl cells in Assay Medium (25,000 cells/well)	32 µl cells in Assay Medium (25,000 cells/well)	32 µl cells in Assay Medium (25,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium (25,000 cells/well)
Step 2 Incubate 16-20 hours	Incubate cells for 16-20 hours in a humidified 37°C/5% CO_2 incubator				
Step 3 Prepare complete 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 minutes before proceeding				
Step 5 Add Agonist	4 µl Assay Medium	4 µl 10X TNFα in Assay Medium	4 µl 10X TNFα in Assay Medium	4 µl 10X TNFα in Assay Medium	4 µl 10X TNFα in Assay Medium
Step 6 Incubate cells	Incubate in a humidified 37°C/5% CO_2 incubator for 30 minutes.				
Step 7 Aspirate Media	Aspirate Media from each well using a multichannel aspirator				
Step 8 Lyse Cells	Add 20 µl/well of complete lysis buffer including terbium-labeled antibody				
Step 9 Equilibrate Reaction	Incubate 1 hour at room temperature				
Step 10 Read Plate	See **Section 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 60–90% 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 7.81×10^5 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 humidified 37°C/5% CO2 incubator for 16–20 hours.

5.2.3 Prepare Lysis buffer

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-c-Fos [pThr232] antibody) should be prepared on the day of the experiment before stimulation of cells.
2. Determine the volume of complete lysis buffer needed for the assay (assuming 20 µl/well using the standard protocol and 30 µl/well for the optional addition-only protocol; see **Section 7**). 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-c-Fos [pThr232] antibody to the lysis buffer to a final concentration of 2 nM. Mix gently by inversion several times.
4. Place 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 TNFα that gives the maximum stimulation (top of the dose response curve). We recommend running a dose response curve to determine the optimal concentration (EC_{100}) for your TNFα solution. See **Section 6** for a representative curve. From this example data, we determined the EC_{100} to be 5 ng/ml or 286 pM.

1. Prepare a stock solution of 0.5% DMSO in Assay Medium.
2. Prepare a 5X stock of Test Compounds in Assay Medium with 0.5% DMSO (or if test compound is dissolved in DMSO, make sure the DMSO concentration for the 5X solution is 0.5%).
3. Reconstitute TNFα (Tumor Necrosis Factor alpha) at 100 µg/ml according to the manufacturer's protocol. Avoid repeated freeze thaws or long-term storage above -20ºC.
4. Prepare a 5X stock of TNFα in Assay Medium containing 0.5% DMSO. We recommend running a dose response curve to determine the EC_{100} for your TNFα solution. See **Section 6** for a representative curve.
5. Add 8 µl of the stock solution of 0.5% DMSO in Assay Medium to the Unstimulated Control and Cell-free Control wells.
6. Add 8 µl of the 5X stock solution of TNFα to the Stimulated Control wells.
7. Add 8 µl of the 5X stock of Test Compounds to the Test Compound wells.
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.**

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 test compound in Assay Medium with 1% DMSO. (Or if the test compound is dissolved in DMSO, prepare a 10X stock of test compound in Assay Medium and make sure the DMSO concentration for the 10X solution is 1.0%).
3. Reconstitute TNFα (Tumor Necrosis Factor alpha) at 100 µg/ml according to the manufacturer's protocol. Avoid repeated freeze/thaw cycles or long-term storage above –20ºC.
4. Prepare a 10X stock of TNFα in Assay Medium at an EC_{80} concentration. We recommend running a dose response curve to determine the EC_{80} for your TNFα solution. See **Section 6** for representative data. From this example data, we determined the EC_{80} to be 1.6 ng/ml or 91.4 pM.
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 the 10X stock of test compound to each Test Compound well.
7. Add 4 µl of the stock solution of 1.0% 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-minute incubation is sufficient.
10. Add 4 µl of the 10X EC_{80} stock solution of TNFα 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.

5.2.6 Cell Lysis

Note: See **Section 7** for an optional protocol without an aspiration step.

1. Remove assay plate from the humidified 37°C/5% CO_2 incubator
2. Carefully aspirate media from each well using a multichannel aspirator. In order to minimize cell loss, avoid direct contact with the adhered cells on the bottom of the well when aspirating. Cell loss is avoided by contacting the side of the well with the tip of the aspirator.
3. Immediately add 20 µl of complete lysis buffer to each well and cover plate.
4. Incubate covered plate at room temperature for 1 hour.

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

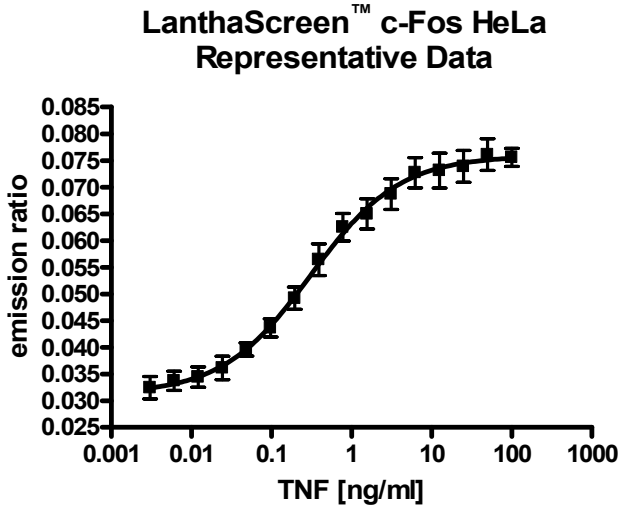
5.3.2 Reading an Assay Plate

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 (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.

6. Representative Data



EC_{50}	0.4 ng/ml (22.9 pM)
EC_{80}	~ 1.6 ng/ml (91.4 pM)
EC_{100}	~ 5 ng/ml (286 pM)
Z′ at EC_{100}	0.71

Figure 1. Dose response of LanthaScreen™ c-Fos Hela cells to TNFα. LanthaScreen™ c-Fos HeLa cells were treated with agonist TNFα in a 384-well format. Cells were plated overnight and then incubated for 30 minutes with indicated concentrations of TNFα in the presence of 0.1% DMSO. After incubation, the cells were lysed in the presence of LanthaScreen™ Tb-anti-c-Fos [pThr232] Antibody and the reaction was equilibrated for 1 hour. TR-FRET values were obtained using a BMG PHERAstar fluorescence plate reader and the 520/490 ratios were plotted against the concentration of the agonist.

7. Optional Addition-Only Cell Lysis Protocol

This section provides directions for performing an "addition-only" protocol for cell lysis.

Note: This protocol may not be suitable for use with this cell line. Experimental results using this addition-only protocol with this cell line have indicated that assay response ratios may be less than two-fold, and therefore not suitable for screening applications.

Note: This protocol requires the use of a larger volume of lysis buffer (and therefore a larger quantity of antibody per well). Typically, the addition-only format results in slightly lower response ratios than the standard protocol (**Section 5.2.6**). However, the data quality (Z′) is similar in either format. One format may be preferred over the other, depending on the needs of the user.

1. Remove assay plate from the humidified 37°C/5% CO_2 incubator
2. Immediately add 30 µl of complete lysis buffer including 5 nM LanthaScreen™ Tb-anti-c-Fos [pThr232] antibody to the stimulated cells in assay medium.
3. Incubate covered plate at room temperature for 1 hour.
4. Proceed to **Section 5.3** for detection.

8. References

Robers MB., *et al,* **High-Throughput Cellular Assays for Regulated Posttranslational Modifications,** (2008) *Anal Biochem*. 2008 Jan 15;372(2):189-97.

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