LanthaScreen™ STAT5 (JAK2 V617F) U2OS Cell-based Assay Protocol

invitrogen™

Catalog no. K1588 **Shipping: Dry Ice** **Storage: Liquid Nitrogen**

Protocol part no. K1588.pps Rev. date: 14 May 2008

Table of Contents

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™ STAT5 (JAK2 V617F) U2OS
Description:	LanthaScreen™ STAT5 U2OS (JAK2 V617F) cells contain a stably integrated expression vectors encoding GFP-STAT5 and GST-JAK2 V617F fusion proteins under control of CMV promoters. The GFP-STAT5 DNA expression construct was introduced into U2OS cells using lentiviral transduction, followed by selection with Blasticidin. The GST-JAK2 V617F expression construct (JAK2 JH1-JH2 domains including a V617F substitution) was then introduced using lipid transfection, followed by selection with Geneticin. This cell line is a clonal population isolated by flow cytometry using GFP fluorescence as sorting marker.
Product Number:	K1588
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 antagonists of JAK2 V617F
Growth Properties:	Adherent
Cell Phenotype:	Epithelial
Selection Markers:	Blasticidin (5 µg/ml) and Geneticin (500 µg/ml)
Vector Used:	pLenti-*bsd* /EmGFP-STAT5 and pEXP27-JAK2 V617F
***Mycoplasma* Testing:**	Negative
BioSafety Level:	1

3. Materials Required, but Not Supplied

Media/Reagents	**Recommended Source**	**Part #**
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
McCoy's 5A with L-Glutamine	Invitrogen	16600-082
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
Sodium Pyruvate	Invitrogen	11360-070
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
Geneticin® (antibiotic)	Invitrogen	10131-027
LanthaScreen™ Tb-anti-mouse antibody	Invitrogen	PV3764 or PV3767
Anti-STAT5 A/B [pTyr 694/699] Antibody	Invitrogen	PV5262 or PV5263
Lysis buffer	(see **Section 5.2.3**)	
Protease Inhibitor mix	SIGMA Aldrich	P8340
Phosphatase Inhibitor mix	SIGMA Aldrich	P2850
JAK Inhibitor I (Pyridone 6)	Calbiochem	420099
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
McCoy's5A w/ L-glutamine	500 ml (90%)	—	—	500 ml (90%)
Opti-MEM® I	—	500 ml (99%)	—	—
Dialyzed FBS	50 ml (10%)	—	—	50 ml (10%)
Charcoal / Dextran stripped FBS	—	5 ml (1.0%)	—	—
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	—	—	—
Geneticin (antibiotic)	500 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/Geneticin) 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/Geneticin) 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/Geneticin).

   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/Geneticin) and place flask in a humidified 37°C/5% CO_2 incubator.
10. Switch to passaging cells in Growth Medium with Blasticidin and Geneticin once cells appear to be growing at consistent rates for the given cellular background.

4.2.2 Propagation Method

1. Cells should be passaged or fed at least two times a week. Cells should be maintained between 20% and 90% confluency. Do not allow cells to reach confluence. Cells which have grown to confluence many not show expected antagonist response in the assay.
2. To passage cells, 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. 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 U2OS 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 and Geneticin resistant.

5. Assay Procedure

The following instructions outline the recommended procedure for monitoring the constitutive JAK2 V617F-mediated phosphorylation of GFP-STAT5 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.
- This assay measures the activity of constitutively-active JAK2 V617F and is therefore useful for the identification of JAK2 V617F inhibitors. The assay is not designed for the identification of agonists of JAK2 V617F activity.

5.1 Quick Reference Guide

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

Antagonist Assay Quick Reference Guide

	Untreated Wells	Positive Control Antagonist	Cell-free Wells	Test Compound Wells
Step 1 Plate cells	32 µl cells in Assay Medium (12,000 cells/well)	32 µl cells in Assay Medium (12,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium (12,000 cells/well)
Step 2 Incubate cells	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 JAK Inhibitor I in Assay Medium with 0.5% DMSO	8 µl Assay Medium with 0.5% DMSO	8 µl 5X Test Compound in 0.5% DMSO
Step 5 Incubate cells	Incubate in a humidified 37°C/5% CO_2 incubator for 2 hours. The optimal stimulation time may vary depending on the antagonist.			
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 detection antibodies			
Step 8 Equilibrate Reaction	Incubate 2 hours at room temperature			
Step 9 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 80–95% 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 3.75×10^5 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-mouse and Anti-STAT5 A/B [pTyr 694/699] antibodies) 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-mouse antibody to 10 nM final concentration and add Anti-STAT5 A/B [pTyr 694/699] antibody to 5 nM final concentration in the supplemented lysis buffer. Mix gently by inversion several times.
4. Place complete lysis buffer on ice until use.

5.2.4 Antagonist Assay Plate Setup

1. Prepare a stock solution of 0.5% DMSO in Assay Medium.
2. Prepare a 5X stock of each test compound in Assay Medium with 0.5% DMSO. (Or if the test compound is dissolved in DMSO, prepare a 5X stock of test compound in Assay Medium and make sure the DMSO concentration for the 10X solution is 0.5%).
3. Reconstitute JAK Inhibitor I (Pyridone 6) to 10 mM in DMSO. Store at –20ºC. Avoid repeated freeze/thaw cycles.
4. Prepare a 5X stock of positive control inhibitor (control antagonist compound) in Assay Medium with 0.5% DMSO. We recommend a starting concentration of 10 µM JAK Inhibitor I as a positive control inhibitor.
5. Add 8 µl of each 5X stock of test compound to each Test Compound well.
6. Add 8 µl of the stock solution of 0.5% DMSO to each Stimulated Control well, Unstimulated Control well, and Cell-free Control well.
7. Add 8 µl of the 5X stock of positive control inhibitor to each Antagonist Control well.
8. Add 8 µl of Assay Medium to each Unstimulated Control well and Cell-free Control well.
9. Incubate the Antagonist assay plate in a humidified 37°C/5% CO_2 incubator for 2 hours.

5.2.5 Cell Lysis

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 2 hours.

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

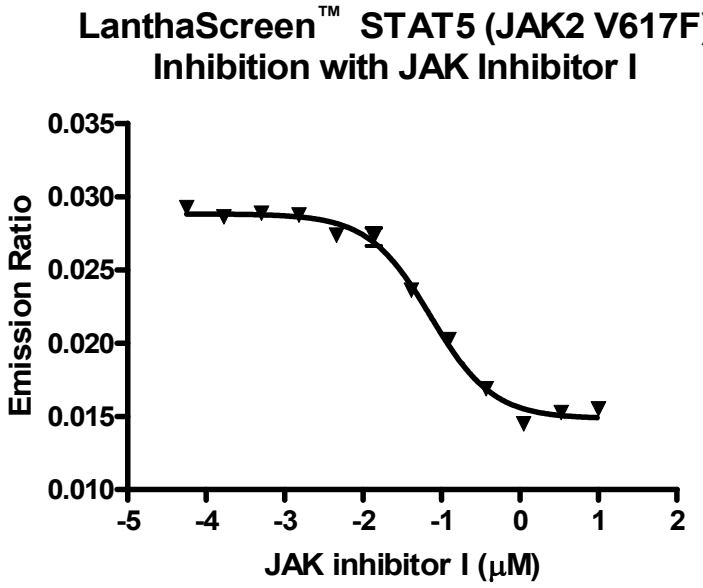
5.3.2 *Reading an Assay Plate*

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



IC_{50}	0.072 µM (22.3 pg/µl)
Z′ at IC_{100}	0.66

Figure 1. Dose response of LanthaScreen™ U2OS STAT5 (JAK2 V617F) cells to JAK Inhibitor I (Pyridone 6) LanthaScreen™ U2OS STAT5 (JAK2 V617F) cells were treated with antagonist JAK Inhibitor I over the indicated concentration range in a 384-well format. Cells were plated for 16–20 hours and then incubated for 2 hours with indicated concentrations of JAK Inhibitor I in the presence of 0.1% DMSO. The cells were then lysed in the presence of LanthaScreen™ Tb-anti-Mouse and Anti-STAT5 A/B [pTyr694/699] antibodies for 2 hours. 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. References

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

8. Purchaser Notification

Limited Use Label License No. 5: Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Invitrogen Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500. Email: outlicensing@invitrogen.com.

Limited Use Label License No. 51: Blasticidin and the Blasticidin Selection Marker

Blasticidin and the blasticidin resistance gene (*bsd*) are the subject of U.S. Patent No. 5,527,701 sold under patent license for research purposes only. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

Use of Genetically Modified Organisms (GMO)

Information for European Customers: The LanthaScreen™ STAT5 (JAK2 V617F) U2OS cell line(s) are genetically modified with the plasmid pLenti-*bsd*/EmGFP-STAT5. 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.