INSTRUCTIONS

Cellomics® STAT2 Activation Kit

High-Content Screening Reagents

1800.1

Number	Description
K01-0005-1	**STAT2 Activation Kit,** sufficient materials for 5 × 96 wells
R01-0504-1	**STAT2 Activation Kit,** sufficient materials for 50 × 96 wells

Kit Contents:	K0100051	R0105041
STAT2 primary antibody, rabbit	154 µl	1.67 ml
DyLight™ 488 Conjugated Goat Anti-Rabbit IgG	75 µl	1 ml
Hoechst Dye	30 µl	165 µl
Wash Buffer (10X)	100 ml	--
Wash Buffer II (10X)	100 ml	--
Permeabilization Buffer (10X)	100 ml	--
Thin Plate Seal Assembly	7/pack	--

Storage: Upon receipt store all kit components at 4°C. Keep vial containing DyLight 488 conjugated goat anti-rabbit IgG protected from light. Allow buffers to warm to room temperature before use. See the **Solution Preparation** section for storage and stability of prepared solutions.

Warning: Please completely read these instructions and the accompanying material safety data sheets before using this product. The Cellomics Reagents are not for diagnostic use in humans or animals.

Introduction

The Thermo Scientific Cellomics STAT2 Activation Kit enables direct quantitation of STAT2 activation by its translocation from the cytoplasm to the nucleus using a fixed end-point assay. The protocol is performed on live cells growing on standard high-density microplates. The kit is supplied with a primary STAT2 antibody and a DyLight 488-conjugated Secondary Antibody. The nuclear region is identified by Hoechst Dye, which is also included.

Cytokines are important for proliferation and differentiation of hematopoietic cells. Many cells respond to cytokine induction via the janus kinases-signal transducers and activators of transcription (JAK/STAT) pathway, resulting in the transcription of selected genes.[1] The complement of induced genes and associated response varies according to the cell type and stimulus, which is reflected by tyrosine phosphorylation by specific JAKs. Consequently, it is crucial that screens for potential drugs that affect this collection of pathways consider specificity among the six known STAT targets. This specificity is addressed by this kit because activation of STAT2 is quantified by its translocation to the nucleus.

The STAT2 Activation Kit enables screening of STAT2 inhibitors by stimulating cells with a control inducer such as interferon–α (IFN-α) after exposing live cells to test compounds. Replacing IFN-α in the assay with test compounds identifies agonists of STAT2 translocation. Translocation is directly quantified as the difference in cytoplasmic to nuclear intensity of the labeled transcription factor (Figure 1). This kit, in combination with the Thermo Scientific ArrayScan HCS Reader and the Cytoplasm to Nucleus Translocation BioApplication software, enables automated plate handling, focusing, cell image acquisition, analysis and quantification of STAT2 activation.

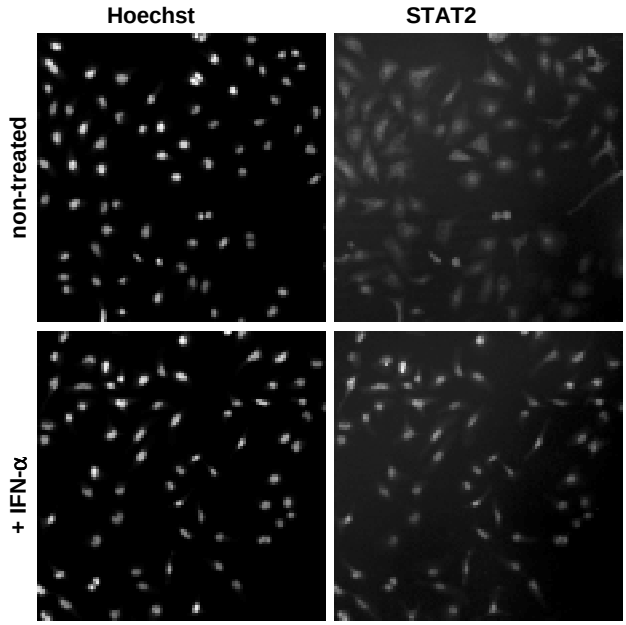

Figure 1. Stained HeLa cells before and after activation of STAT2. Top panel: STAT2 localization in unstimulated cells. **Bottom panel:** STAT2 localization in cells stimulated with 500 U/ml of IFN-α for 20 minutes.

Additional Materials Required

Note: For the screening size kit, Wash Buffer, Permeabilization Buffer, and Wash Buffer II are available separately. Contact customer service for more information.

- IFN-α (Sigma, Product No. I-4276) or other STAT2 activator
- Paraformaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Black, 96-well clear-bottom microplates (Packard ViewPlate®, Product No. 6005182)

Cell Preparation Information

- The protocol is optimized for HeLa cells (ATCC, Product No. CCL-2).
- Culture cells in Minimum Essential Medium-Eagle (EMEM) containing the following supplements (=EMEM Complete Medium): 10% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin solution.
- Split cells when they reach 70-80% confluency (2-3 times per week) at a dilution of 1:2 to 1:6.
- For STAT2 activation, harvest cells with trypsin-versene mixture, dilute into EMEM Complete Medium, and determine cell density.
- Dilute cells to 5×10^4 cells/ml in EMEM Complete Medium and add 100 µl of the cell suspension per well of a 96-well microplate (= 5,000 cells/well). Incubate cells for 18-24 hours at 37°C in 5% CO_2.

STAT2 Activation Kit Protocol

A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml ultrapure water for a final volume of 200 ml. Store buffer at 4°C for up to 7 days.
1X Permeabilization Buffer	Add 2 ml of 10X Permeabilization buffer to 18 ml of ultrapure water for a final volume of 20 ml. Store buffer at 4°C for up to 7 days.
1X Wash Buffer II	Add 20 ml 10X Wash Buffer II to 180 ml ultrapure water for a final volume of 200 ml. Store buffer at 4°C for up to 7 days.
Fixation Solution	Add 6 ml 16% paraformaldehyde to 18 ml 1X Wash Buffer. Warm to 37°C before use. Prepare solution just before each assay.
Primary Antibody Solution	Add 27.5 µl of STAT2 antibody to 5.5 ml 1X Wash Buffer. Prepare solution just before each assay.
Secondary Antibody Staining Solution	Add 3 µl of Hoechst Dye and 12 µl of the DyLight 488 Goat Anti-Rabbit to 6 ml of 1X Wash Buffer. Prepare solution just before each assay.

B. Procedure

Note: Use 200 µl per well unless indicated otherwise. This protocol requires ~3 hours post-compound incubation to perform.

1. Dilute IFN-α to 2,500 U/ml in culture medium. Add 25 µl/well and mix thoroughly.
2. Incubate 20 minutes at 37°C, 5% CO_2. For an agonist screen, the compound replaces stimulator. For an antagonist screen, add compound before the stimulator.
3. Aspirate culture medium and add 200 µl of 1X Wash Buffer pre-warmed to 37°C.

   Note: Perform all wash steps carefully to maintain cell integrity and attachment. For best results, use low-velocity fluid dispensing.
4. Aspirate Wash Buffer and add 200 µl of pre-warmed Fixation Solution to each well. Incubate plate in a fume hood at room temperature for 10 minutes. Pre-warming fixative is critical to maintaining cell integrity.
5. Aspirate Fixation Solution and wash plate once with 200 µl/well of 1X Wash Buffer.
6. Aspirate Wash Buffer, add 200 µl of 1X Permeabilization Buffer and incubate for 15 minutes.
7. Aspirate Permeabilization Buffer and wash plate once with 200 µl/well of 1X Wash Buffer.
8. Aspirate Wash Buffer, add 50 µl/well of Primary Antibody Solution and incubate for 1 hour.
9. Aspirate Primary Antibody Solution, add 200 µl/well of 1X Wash Buffer II and incubate for 5-10 minutes.
10. Aspirate Wash Buffer II and wash twice with 200 µl/well of 1X Wash Buffer.
11. Aspirate Wash Buffer, add 50 µl/well of Staining Solution per well and incubate for 1 hour protected from light.
12. Aspirate Staining Solution, add 200 µl/well of 1X Wash Buffer II and incubate for 5-10 minutes.
13. Aspirate Wash Buffer II and wash twice with 200 µl/well of 1X Wash Buffer. Leave Wash Buffer from the last wash in wells.
14. Seal plate and evaluate on ArrayScan HCS Reader.
15. Store sealed plates in the dark at 4°C.

Additional Information

A. Dose-response Curve and Time-course Experiment

Dose-response curves were produced by stimulating HeLa cells with IFN-α for 20 minutes stained as described in the protocol (Figure 2). For the time-course experiments, HeLa cells were incubated at 37°C with IFN-α at maximal dose (500 U/ml) (Figure 3).

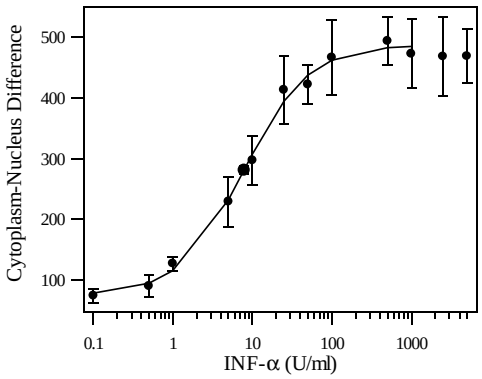

Figure 2. Dose-response curve for STAT2 activation by IFN-α in HeLa cells. EC_{50} = 11 U/ml

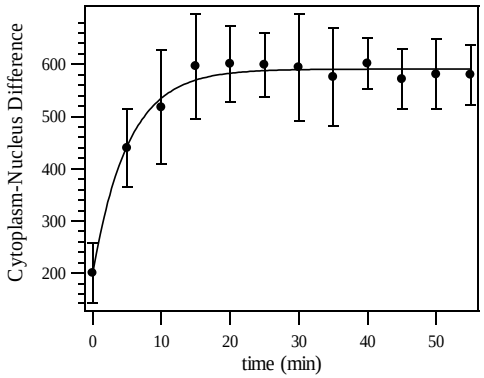

Figure 3. Time-course experiment for STAT2 activation by IFN-α in HeLa cells. Maximum stimulation was evident after 20-50 minutes where complete translocation from the cytoplasm to nucleus occurred. A $t_{1/2} \cong 4.6$ min

B. Typical Performance Data

Cell Type	HeLa	
Stimulus	IFN-α	
Output Feature	Mean Nucleus-Cytoplasm	
Magnification	**20X**	**10X**
Max/Min*	6.1	4.1
Z´ 2	0.48	0.38
Well-To-Well (CV)	10.0	10.0
Scan Time** 96-Well Plate	~ 58 min	~30 min
Scan Time** 384-Well Plate	~ 3 h, 20 min	~1 h, 45 min

* Mean maximum signal/mean minimum signal.
**Times are significantly affected by cell density. The times indicated assume recording data from at least 100 cells and usually within one optical field.

C. Microscope Information

Cells prepared and labeled according to these instructions can be used and analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization may be required when using slides, coverslips or multi-well chamber slides. Use image-processing software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are as follows:

DyLight 488 Conjugates = 494/532 nm

Hoechst Dye = 350/461 nm

D. Recommendations for Automation

- Plating Cells: To improve the uniformity and throughput of plating cells, use a liquid handling system such as Thermo Scientific Multidrop® Combi or WellMate® Dispensers.
- Dead Volumes: Every piece of automation instrumentation has a non-recoverable dead volume associated with it. Be aware of these dead volumes, priming volumes and rinsing volumes when calculating your reagent requirements.
- Nonspecific Binding: Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.

- Mixing: Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.
- Cell Washing: Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.
- Incubation: Minimize the time when plates with live cells are out of a controlled CO_2 environment. For best results, use an automated incubator to deliver plates to a pipetting deck.
- Exposure: Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.
- Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed. Some suggested starting volumes are listed in Table 1.

Table 1. Suggested volumes to use for different cell culture plates.

Kit Component	96-Well Plates (µl/well)	384-Well Plates (µl/well)	24-Well Plates (µl/well)
Fixation Solution	100	25	400
1X Wash Buffer	100	25	400
1X Wash Buffer	100	25	400
1X Permeabilization Buffer	100	25	400
Antibody Solution	50	12.5	200
Staining Solution	50	12.5	200
1X Wash Buffer (final wash)	150	37.5	200

Compatible BioApplication Software Modules

S50-5001-1 or S50-2001-1 **Cytoplasm to Nucleus Translocation BioApplication**

S50-5019-1 or S50-2019-1 **Molecular Translocation BioApplication**

S50-5017-1 or S50-2017-1 **Compartmental Analysis BioApplication**

References

1. Darnell, J. E., *et al*. (1994). *Science* **264:**1415-21.
2. Taylor, D.L., *et al*. (2007). High content screening: A powerful approach to systems cell biology and drug discovery. *Method Mol Biol* **356**. Humana Press, Totowa, N.J.
3. Zhang, J.H., *et al.* (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* **4:**67-73.

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Thermo Scientific Cellomics Reagent Kits are developed and manufactured at the same Thermo Fisher Scientific Inc. facility as Pierce Protein Research Products and are supported by Pierce Technical Support (see contact information in page footer). These kits are part of the Cellomics Total Solution Platform for HCS, which also includes Cellomics ArrayScan and other HCS Instrumentation, BioApplication Image Analysis Software and High-Content Informatics. For more information, visit *www.thermo.com/cellomics* or call 800-432-4091 (toll free) or 412-770-2500.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.