

# Cellomics<sup>®</sup> STAT3 Activation Kit

## High-Content Screening Reagents

1802.2

| Number            | Description  |
|-------------------|--|
| <b>K01-0008-1</b> | <b>STAT3 Activation Kit</b> , sufficient materials for 5 × 96 wells  |
| <b>R01-0505-1</b> | <b>STAT3 Activation Kit</b> , sufficient materials for 50 × 96 wells |

| Kit Contents:                                | K0100081 | R0105051 |
|--|----------|----------|
| STAT3 primary antibody, rabbit               | 154 µl   | 1.66 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.

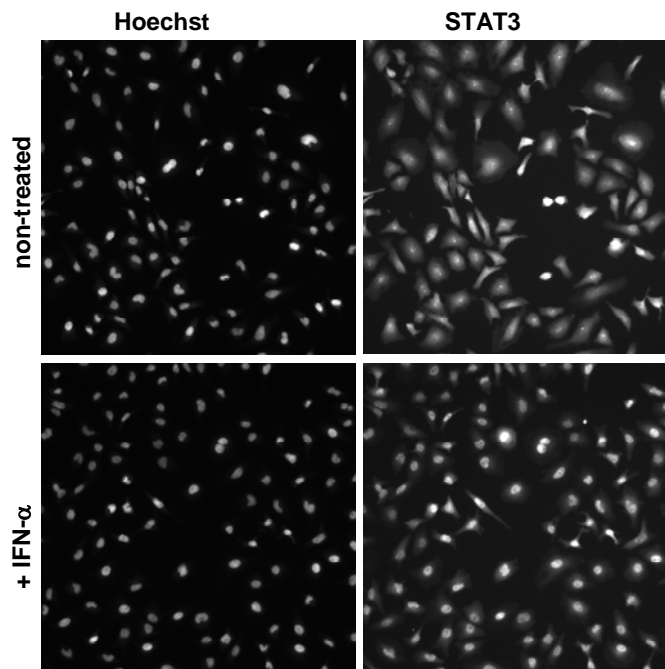
**Warning:** Please completely read these instructions and the accompanying material safety data sheets before using this product. The Cellomics Reagents are not for diagnostic use in humans or animals.

## Introduction

The STAT3 Activation Kit provides the reagents and protocol necessary to quantify STAT3 activation by directly measuring its translocation from the cytoplasm to the nucleus. The protocol is performed on live cells growing on standard high-density microplates, and detection is accomplished via immunofluorescence. The kit includes a primary STAT3 antibody and a DyLight 488-Conjugated Secondary Antibody. The nuclear region is identified by Hoechst Dye, which is also included.

Cytokines are important for the 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.<sup>1</sup> 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 afforded by this HCS reagent kit, where activation of STAT3 is quantified by its translocation to the nucleus.

The optimized protocol included in the STAT3 Activation HCS Reagent Kit product is for a fixed end-point assay. Inhibitors of STAT3 translocation are screened by stimulating cells with a control inducer such as interferon- $\alpha$  (IFN- $\alpha$ ) after exposure of live cells to test compounds. Replacing IFN $\alpha$  in the assay with test compounds identifies agonists of STAT3 translocation. Translocation is directly quantified as the difference in cytoplasmic to nuclear intensity of the labeled transcription factor (Figure 1). The STAT3 Activation HCS Reagent Kit, in combination with the Cellomics ArrayScan<sup>®</sup> HCS Reader and the Cytoplasm to Nucleus Translocation Application software, enables automated plate handling, focusing, cell image acquisition, analysis and quantification of STAT3 activation. For a more detailed description of the image processing algorithm, see the Cytoplasm to Nucleus Translocation Application Guide that accompanies the Cytoplasm to Nucleus Translocation Application software.



**Figure 1. HeLa cells stained before and after STAT3 activation by IFN- $\alpha$ . Top panels: STAT3 localization in non-treated cells. Bottom panels: STAT3 localization in stimulated cells (IFN- $\alpha$  – 2500 U/ml for 20 minutes).**

## Additional Materials Required

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

- IFN- $\alpha$  or other STAT3 activator (Sigma, Product No. I-4276)
- Paraformaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Black, clear-bottom microplates (Packard ViewPlate<sup>®</sup>, Product No. 6005182)

## Cell Preparation Information

- Protocol optimized for HeLa cells (ATCC, Product No. CCL-2)
- Perform cell culture 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 STAT3 activation, harvest cells with trypsin-versene mixture, dilute into EMEM Complete Medium and determine cell density.
- Dilute cells to  $5 \times 10^4$  cells/ml in EMEM Complete Medium and add 100  $\mu$ l 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<sub>2</sub>.

## STAT3 Activation Kit Protocol

### A. Solution Preparation (per 96-well plate)

|                                      |   |
|--------------------------------------|---|
| 1X Wash Buffer                       | Add 20 ml of 10X Wash Buffer to 180 ml of ultrapure water for a final volume of 200 ml. Store buffer at 4°C for up to 7 days.             |
| 1X Permeabilization Buffer           | Add 4 ml of 10X Permeabilization buffer to 36 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 of 10X Wash Buffer II to 180 ml of ultrapure water for a final volume of 200 ml. Store buffer at 4°C for up to 7 days.          |
| Fixation Solution                    | Add 6 ml of 16% paraformaldehyde to 18 ml of 1X Wash Buffer. Warm to 37°C before use. Prepare solution just before each assay.            |
| Primary Antibody Solution            | Add 27.5 µl of STAT3 antibody to 5.5 ml of 1X Wash Buffer.  |
| 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

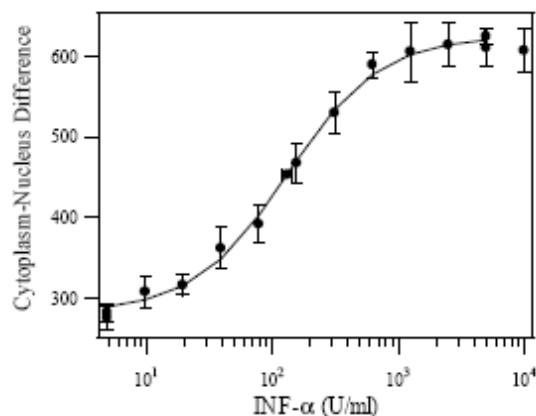
**Note:** Use 200 µl per well volume unless indicated otherwise. This protocol requires ~3 hours post-compound incubation to perform.

1. Dilute IFN- $\alpha$  to 12,500 U/ml in culture medium. Add 25 µl/well and mix thoroughly. Incubate 20 minutes at 37°C, 5% CO<sub>2</sub>. For an agonist screen, compound would replace stimulator. For an antagonist screen, compound addition precedes stimulator addition.
2. Aspirate culture medium and add 200 µl of 1X Wash Buffer pre-warmed to 37°C. Perform all wash steps carefully to maintain cell integrity and attachment. Low velocity fluid dispensing is recommended.
3. Aspirate Wash Buffer and add 200 µl of prewarmed Fixation Solution to each well. Incubate in fume hood at room temperature for 10 minutes. Pre-warming fixative is critical to maintaining cell integrity.
4. Aspirate Fixation Solution and wash plate once with 200 µl of 1X Wash Buffer.
5. Aspirate Wash Buffer, add 200 µl of 1X Permeabilization Buffer and incubate for 15 minutes.
6. Aspirate Permeabilization Buffer and wash plate once with 200 µl of 1X Wash Buffer.
7. Aspirate Wash Buffer, add 50 µl/well of Primary Antibody Solution and incubate for 1 hour.
8. Aspirate Primary Antibody Solution, add 200 µl of 1X Wash Buffer II and incubate for 5-10 minutes.
9. Aspirate Wash Buffer II and wash twice with 200 µl of 1X Wash Buffer.
10. Aspirate Wash Buffer, add 50 µl/well of Staining Solution and incubate for 1 hour protected from light.
11. Aspirate Staining Solution, add 200 µl of 1X Wash Buffer II and incubate for 5-10 minutes.
12. Aspirate Wash Buffer II and wash twice with 200 µl of 1X Wash Buffer. Leave Wash Buffer from last wash in wells.
13. Seal plate and evaluate on ArrayScan HCS System.
14. Store sealed plates in the dark at 4°C.

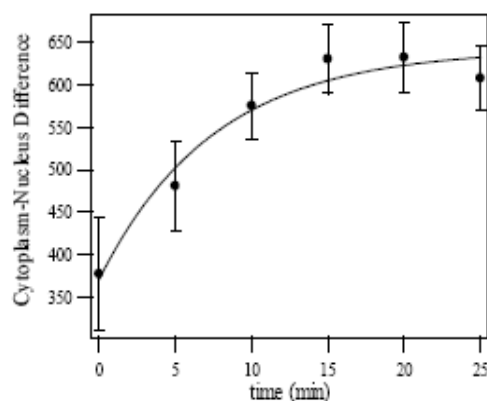
## Additional Information

### A. Dose Response Curve and Time Course

HeLa cells were stimulated with IFN- $\alpha$  for 20 minutes and labeled and fixed as described in the protocol (Figure 2A). HeLa cells were incubated at 37°C with IFN- $\alpha$  at a maximal dose (2,500 U/ml) (Figure 2B).



**Figure 2A. Dose-response curve for STAT3 activation by IFN- $\alpha$  in HeLa cells.** An EC<sub>50</sub> of 132 U/ml was determined.



**Figure 2B. Time course for STAT3 activation by IFN- $\alpha$  in HeLa cells.** Maximum stimulation was evident after 15-20 minutes where complete translocation from cytoplasm to nucleus was observed. A  $t_{1/2}$  ~7.5 minute was determined.

### B. Performance Data

#### Typical Performance Data

| Cell Type                    | HeLa                              |              |
|------------------------------|-----------------------------------|--------------|
| Stimulus                     | IFN- $\alpha$                     |              |
| Output Feature               | Mean Nucleus-Cytoplasm Difference |              |
|                              | <u>Magnification</u>              |              |
|                              | 20X                               | 10X          |
| Max/Min*                     | 7.3                               | 7.3          |
| Z'                           | 0.43                              | 0.43         |
| Well to Well (COV)           | 7.1                               | 7.1          |
| 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 minimal signal.

\*\*These items 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 a Thermo Scientific Multidrop Combi or WellMate Dispenser.
- **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<sub>2</sub> 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.

| <b>Kit Component</b>        | <b>96-Well Plates</b><br>( $\mu$ l/well) | <b>384-Well Plates</b><br>( $\mu$ l/well) | <b>24-Well Plates</b><br>( $\mu$ l/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

|                                 |  |
|---------------------------------|--|
| <b>S50-5001-1 or S50-2001-1</b> | <b>Cytoplasm to Nucleus Translocation BioApplication</b> |
| <b>S50-5019-1 or S50-2019-1</b> | <b>Molecular Translocation BioApplication</b>            |
| <b>S50-5017-1 or S50-2017-1</b> | <b>Compartmental Analysis BioApplication</b>             |

## 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](http://www.thermo.com/cellomics) or call 800-432-4091 (toll free) or 412-770-2500.

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