INSTRUCTIONS

Thermo SCIENTIFIC

Cellomics® NFAT-1 Activation Kit

High-Content Screening Reagents

1812.1

Number	Description
K01-0011-1	**NFAT-1 Activation Kit,** sufficient materials for 5 × 96 wells
R01-0515-1	**NFAT-1 Activation Kit,** sufficient materials for 50 × 96 wells

Kit Contents:	K0100111	R0105151
NFAT-1 Primary Antibody (mouse)	65 µl	735 µl
DyLight™ 488 Conjugated Goat Anti-Mouse IgG	72 µl	1 ml
Hoechst Dye	30 µl	165 µl
Wash Buffer (10X)	100 ml	--
Blocking Buffer (10X)	85 ml	2 x 85 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 the DyLight™ 488 conjugated-Goat Anti-Mouse 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 Cellomics NFAT-1 Activation Kit provides the reagents necessary to quantify NFAT-1 activation by directly measuring its translocation from the cytoplasm to the nucleus. The assay is performed on live cells growing on standard high-density microplates. The kit is supplied with an anti-NFAT-1 primary antibody and a DyLight 488-conjugated Secondary Antibody. The nuclear region is identified by Hoechst Dye, also included in the kit.

Transcription factors belonging to the NFAT (nuclear factor of activated T cells) family regulate cytokine expression in T cells and are intimately involved in regulation of early events of the immune response. NFAT resides in the cytoplasm and translocates to the nucleus upon dephosphorylation via binding to calcineurin (calcium/calmodulin-dependent phosphatase). Upon translocation, NFAT forms complexes with AP-1 proteins to regulate gene expression.

The NFAT-1 Activation Kit protocol was optimized for a fixed end-point assay. Inhibitors of NFAT-1 translocation are screened by stimulating cells with a control inducer, such as thapsigargin, after exposing live cells to the test compounds. Replacing thapsigargin in the assay with test compounds identifies agonists of NFAT-1 translocation. Translocation is directly quantified as the difference in cytoplasmic to nuclear intensity of the labeled transcription factor (Figure 1). The NFAT-1 Activation Kit, in combination with the Thermo Scientific ArrayScan® HCS System and the Cytoplasm to Nucleus Translocation Application software affords automated plate handling, focusing, cell image acquisition, analysis, and quantification. For more detailed description of the image processing algorithm, see the Cytoplasm to Nucleus Translocation Application Guide that accompanies the software.

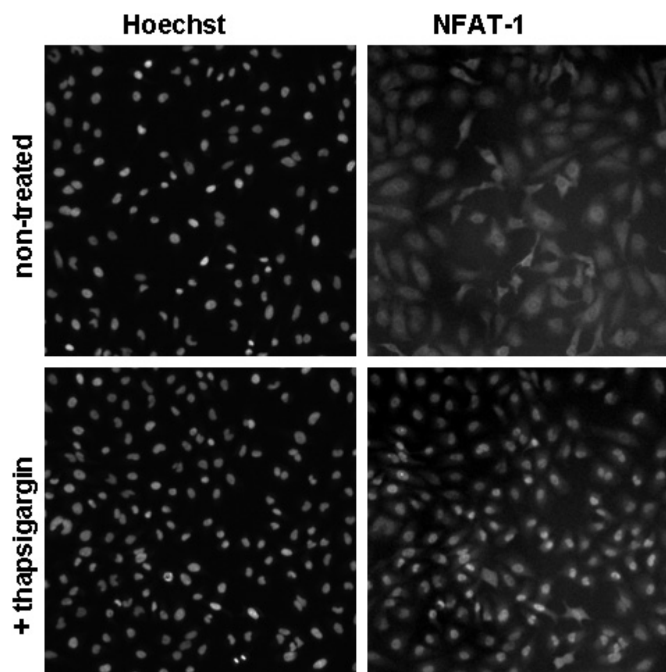

Figure 1. HeLa cells before and after NFAT-1activation. Cells were stimulated with 60 nM thapsigargin for 40 minutes. NFAT-1 localization in non-treated (top), and treated cells (bottom).

Additional Materials Required

Note: For the screening size kit, Wash Buffer, Permeabilization Buffer, and Blocking Buffer are available separately (please contact customer service for more information).

- Thapsigargin or other NFAT-1 activator (Calbiochem, Product No. 586005)
- Paraformaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Black, clear-bottom microplates (Packard ViewPlate®, Product No. 6005182)

Cell Preparation Information

- Protocol optimized for HeLa cells (American Type Culture Collection, Product No. CCL-2).
- Perform cell culture in Minimum Essential Medium-Eagle (EMEM; BioWhittaker, Product No. 12-611Q) containing the following supplements (=EMEM Complete Medium) with, 10% fetal calf serum (BioWhittaker, Product No. 14-503F), 1X penicillin/streptomycin (BioWhittaker, Product No. 17-602E), 1% L-glutamine (BioWhittaker, Product No. 17-605E), 1% non-essential amino acids (BioWhittaker, Product No. 13-114E), 1% sodium pyruvate (BioWhittaker, Product No. 13-115E)
- Split cells when they reach 70-80% confluency (every 3-4 days) at a dilution of 1:2 to 1:6.
- For NFAT-1 Activation, harvest cells with trypsin-versene mixture (BioWhittaker, Product No. 17-161F), dilute into EMEM Complete Medium and determine cell density.
- Adjust cell density to 5×10^4 cells/ml in EMEM Complete Medium and add 100 µl of the cell suspension to each well of a 96-well microplate (=5,000 cells/well).
- Incubate cells for 18-24 hours at 37°C in 5% CO_2.

NFAT-1 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 Blocking Buffer	Add 10 ml 10X Blocking Buffer to 90 ml ultrapure water for a final volume of 100 ml. Store buffer at 4°C for up to 7 days.
Fixation Solution	Add 3.0 ml 16% paraformaldehyde to 9.0 ml 1X Wash Buffer. Warm to 37°C before use. Prepare solution just before each assay.
Primary Antibody Solution	Add 12 µl of NFAT-1 antibody to 6.0 ml 1X Wash Buffer.
Secondary Antibody Staining Solution	Add 3.0 µl of Hoechst Dye and 12.0 µl of the DyLight™ 488 Goat Anti-Mouse to 6.0 ml of 1X Wash Buffer. Prepare solution just before each assay.

B. Procedure

Note: Use 100-200 µl per well volume unless indicated otherwise. This protocol requires ~5 hours post-compound incubation to perform.

1. Add 25 µl/well of pre-warmed thapsigargin (300 nM in EMEM Complete Medium) to obtain final concentration of 60 nM. To wells which are to be unstimulated, add 25 µl EMEM Complete Medium. Incubate 40 minutes (15-40 minutes) at 37°C. (For an agonist screen, compound replaces stimulator. For an antagonist screen, add compound before adding the stimulator).
2. Aspirate culture medium and add 100 µl prewarmed Fixation Solution to each well. Incubate in fume hood at room temperature for 1 hour (10-60 minutes). Prewarming fixative is critical for maintaining cell integrity.
3. Aspirate Fixation Solution and add 1X Permeabilization Buffer. Incubate for 15 minutes.
4. Aspirate Permeabilization Buffer and wash twice with 1X Blocking Buffer.
5. Aspirate Blocking Buffer and add 50 µl of Primary Antibody Solution. Incubate for one hour.
6. Aspirate Primary Antibody Solution and wash twice with 1X Blocking Buffer.
7. Aspirate Blocking Buffer and add 50 µl of Staining Solution. Incubate for 1 hour, protected from light.
8. Aspirate Staining Solution and wash twice with 1X Blocking Buffer.
9. Immediately aspirate Blocking Buffer and add 200 µl of 1X Wash Buffer for storage.
10. Seal plate and run on ArrayScan HCS System.
11. Store sealed plates in the dark at 4°C.

Additional Information

A. Assay Performance

To generate the dose-response curve, HeLa cells were stimulated with thapsigargin for 40 minutes at a range of 0.1 to 200 nM, processed as described in the protocol and analyzed using the ArrayScan HCS Reader with the Cytoplasm to Nucleus Translocation Application (Figure 2A). For the time-course experiment, HeLa cells were incubated at 37°C with thapsigargin (60 nM) at time intervals ranging from 0 to 60 minutes, processed as described in the kit protocol and analyzed using the ArrayScan HCS Reader with the Cytoplasm to Nucleus Translocation Application (Figure 2B).

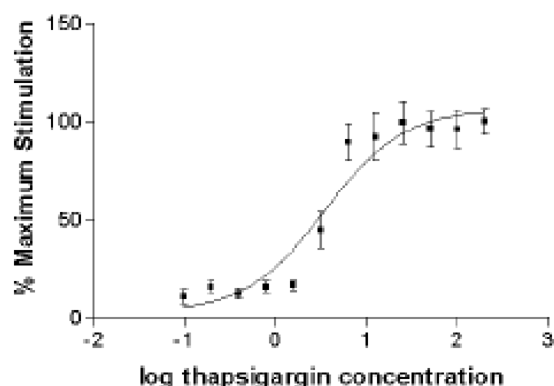

Figure 2A. Dose-response curve of thapsigargin. This representative dose-response curve has an average EC_{50} of 3.35 nM (+1.34).

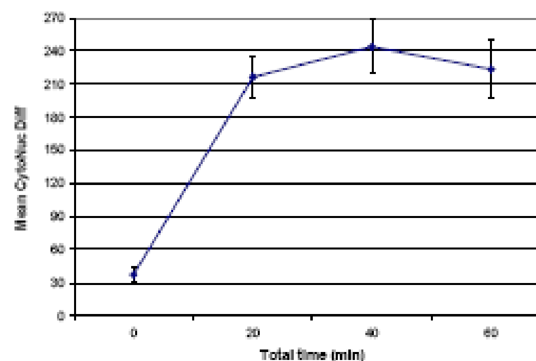

Figure 2B. Time-course experiment of NFAT-1 activation. Optimal stimulation was at ~40 minutes.

B. 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

C. 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 Blocking 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

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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.

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