

Cellomics[®] ATF-2 Activation Kit

High-Content Screening Reagents

1808.1

Number	Description
K01-0010-1	ATF-2 Activation Kit, sufficient materials for 5 × 96 wells
R01-0514-1	ATF-2 Activation Kit, sufficient materials for 50 × 96 wells

Kit Contents:	K0100101	R0105141
ATF-2 Primary Antibody (mouse)	60 µl	685 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 × 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 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 Thermo Scientific Cellomics ATF-2 Activation Kit enables quantitation of ATF-2 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 a phospho-ATF-2 specific antibody and a DyLight 488-conjugated Secondary Antibody. The nuclear region is identified by Hoechst Dye, which is also included.

The ATF-2 transcription factor is activated in response to inflammatory cytokines and cellular stress, including genotoxicity and ischemia/reperfusion. ATF-2 is activated through phosphorylation of threonines 69 and 71 by members of the SAPK family.^{1,2} Once activated, ATF-2 forms complexes with Jun family or other ATF family members. These complexes bind to the cAMP response element (CRE) present in the promoters of many genes, stimulating gene transcription.³ Activation of ATF-2 can be quantified by its translocation to the nucleus (Figure 1). The immunofluorescent assay uses a validated phospho-ATF-2 specific antibody that is non-reactive to other cAMP response element-binding protein (CREB) or ATF family members.

The ATF-2 Activation Kit, in combination with the Thermo Scientific ArrayScan[®] HCS Reader and the Cytoplasm to Nucleus Translocation or Molecular Translocation BioApplication software, enable automated plate handling, focusing, cell image acquisition, analysis and quantification of ATF-2 activation.

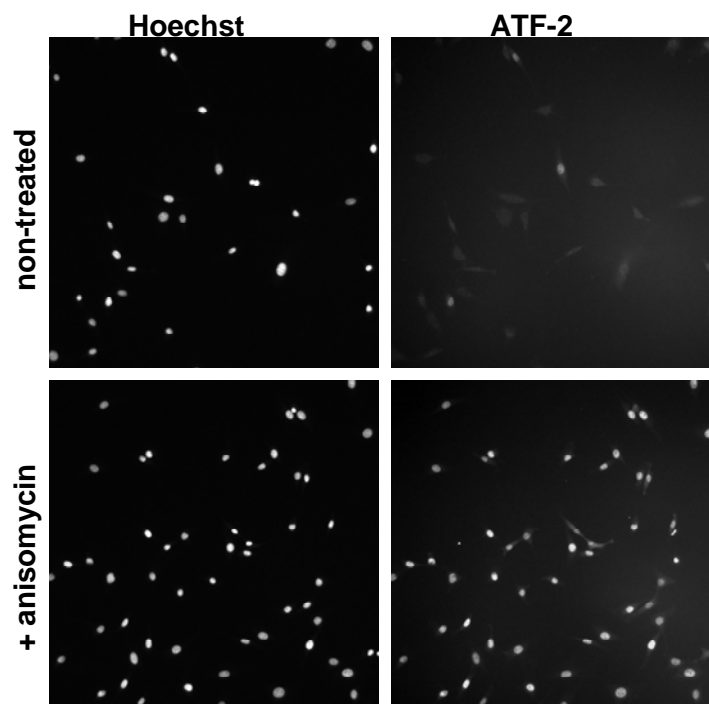


Figure 1. NIH 3T3 cells before and after ATF-2 activation by anisomycin. Cells were stimulated with 200 ng/ml anisomycin for 30 minutes. ATF-2 localization in non-treated cells (left) and stimulated cells (right).

Additional Materials Required

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

- Anisomycin (Sigma, Product No. A9789) or other ATF-2 activator
- Paraformaldehyde (16%) (Thermo Scientific 16% Paraformaldehyde, Product No. 28906)
- Black, clear-bottom microplates (Packard ViewPlate[®] Microplate, Product No. 6005182)

Cell Preparation Information

- The protocol is optimized for NIH 3T3 cells (American Type Culture Collection, Product No. CRL-1658).
- Culture NIH 3T3 cells using DMEM complete media (HyClone) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin.
- Split cells when they reach 70-80% confluency (every 3-4 days) at a dilution of 1:3 to 1:5.
- For ATF-2 activation, harvest cells with trypsin-versene mixture (BioWhittaker, Product No. 17-161F), dilute into DMEM Complete Medium and determine cell density.
- Adjust cell density to 4×10^4 cells/ml in DMEM Complete Medium and add 100 µl of the cell suspension to each well of a 96-well microplate (= 4,000 cells/well).
- Incubate cells 18-24 hours at 37°C in 5% CO₂.

ATF-2 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 ml 16% paraformaldehyde to 9 ml 1X Wash Buffer. Warm to 37°C before use. Prepare solution just before each assay.
Primary Antibody Solution	Add 11 µl of ATF-2 antibody to 5.5 ml of 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 µl per well volume unless indicated otherwise. This protocol requires ~3 hours to perform once compound incubation has been completed.

- To activate ATF-2, add 25 µl of prewarmed anisomycin working solution (3 µg/ml) to wells. To non-stimulated wells, add 25 µl DMEM Complete Medium. Incubate 20 minutes at 37°C. (For an agonist screen, replace the compound with stimulator. For an antagonist screen, add compound before adding the stimulator.)
- Aspirate culture medium and add 100 µl prewarmed Fixation Solution to each well. Incubate plate in a fume hood at room temperature for 10 minutes. Pre-warming fixative is critical for maintaining cell integrity.
- Aspirate Fixation Solution and add 100 µl/well of 1X Blocking Buffer.
- Aspirate Blocking Buffer, add 100 µl/well of 1X Permeabilization Buffer and incubate for 15 minutes.
- Aspirate Permeabilization Buffer and wash twice with 100 µl/well of 1X Blocking Buffer.
- Aspirate Blocking Buffer, add 50 µl/well of Primary Antibody Solution and incubate for 1 hour.
- Aspirate Primary Antibody Solution and wash twice with 100 µl/well of 1X Blocking Buffer.
- Aspirate Blocking Buffer and add 50 µl/well of Staining Solution. Incubate for 1 hour protected from light.
- Aspirate Staining Solution and wash twice with 100 µl/well of 1X Blocking Buffer.
- Aspirate Blocking Buffer and add 200 µl/well of 1X Wash Buffer.
- Seal plate and run on ArrayScan HCS Reader.
- Store sealed plates at 4°C, protected from light.

Additional Information

A. Assay Performance

NIH3T3 cells were stimulated with anisomycin for 30 minutes ranging from 5 to 250 ng/ml (Figure 2). For the time-course experiment, NIH3T3 cells were incubated at 37°C with anisomycin for various time intervals ranging from 0 to 50 minutes. Cells were analyzed using the ArrayScan HCS Reader with the Cytoplasm to Nucleus Translocation BioApplication Software.

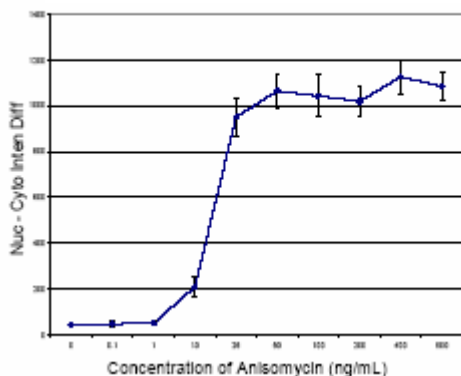


Figure 2. Dose response of NIH3T3 cells treated with anisomycin. $EC_{50} = 15.5$ ng/ml

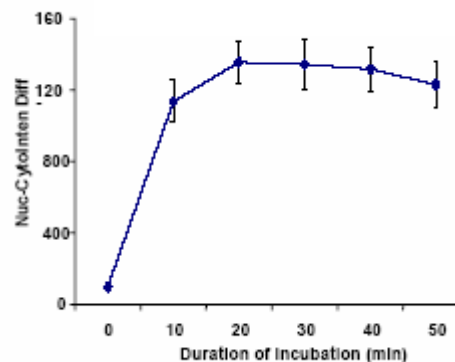


Figure 3. Time course of NIH 3T3 cells treated with anisomycin. Maximum stimulation resulted after incubating for 20 minutes.

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 CO₂ 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.

<u>Kit Component</u>	<u>96-Well Plates</u> (<u>µl/well</u>)	<u>384-Well Plates</u> (<u>µl/well</u>)	<u>24-Well Plates</u> (<u>µl/well</u>)
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

- Livingstone, C., *et al.* (1995). ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J* **14**(8):1785-97.
- Van Dam, H. *et al.* (1995). ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J* **14**(8):1798-1811.
- Gupta, S., *et al.* (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267**:389-93.
- 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.
- 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.

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