

Cellomics[®] ERK MAPK Activation Kits

High-Content Screening Reagents

1810.1

Number	Description
K01-0007-1	ERK MAPK Activation Kit , sufficient materials for 5 × 96 wells
R01-0509-1	ERK MAPK Activation Kit , sufficient materials for 50 × 96 wells

Kit Contents:	K0100071	R0105091
ERK Primary Antibody (rabbit)	144 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	--
Blocking Buffer (10X)	85 ml	4 × 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 Antibody 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 ERK MAPK Activation Kit provides the reagents and protocol necessary to quantify ERK activation by directly measuring its translocation from the cytoplasm to the nucleus. This fixed end-point assay is performed on live cells growing on standard high-density microplates. Inhibitors of ERK translocation are screened by stimulating cells with a control inducer, such as phorbol 12-myristate 13-acetate (PMA) after exposing cells to the test compounds. The kit is supplied with an anti-ERK primary antibody and a DyLight 488-conjugated secondary antibody. The nuclear region is identified by the nuclear dye, Hoechst, also included in the kit.

Mitogen-activated protein kinase (MAPK) pathways regulate an extensive range of cellular processes including gene transcription, cytoskeletal organization, metabolic homeostasis, cell growth and apoptosis.^{1,2,3} MAPKs are activated by concomitant Tyr and Thr phosphorylation via a complex multi-step signal transduction cascade. The extracellular signal-regulated kinases (ERK) 1 and 2, also known as p44 and p42 MAPK, accumulate in the nucleus after stimulation with mitogens.⁴ Thus the MAPK/ERK cascade constitutes a functional signaling unit that links surface receptor-mediated signals to nuclear events.

The ERK MAPK Activation Kit enables ERK quantitation by its translocation to the nucleus (Figure 1). This kit in combination with the ArrayScan HCS Reader and the Cytoplasm to Nucleus Translocation BioApplication software allows automated plate handling, focusing, cell image acquisition and analysis of ERK MAPK activation. For a more detailed description of the image processing algorithm, see the Cytoplasm to Nucleus Translocation BioApplication Guide that accompanies the Cytoplasm to Nucleus Translocation BioApplication software.

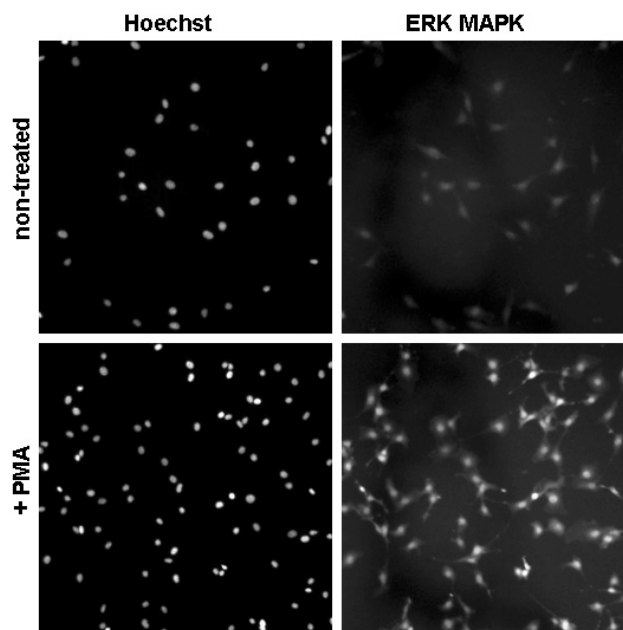


Figure 1. NIH 3T3 cells before and after ERK MAPK activation. Cells were stimulated with 100 ng/ml PMA for 30 minutes. ERK localization in non-treated cells (top panels) and stimulated cells (bottom panels).

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.

- Phorbol 12-myristate 13-acetate (PMA, Sigma, Product No. P1585)
- Paraformaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Black, clear-bottom microplates (Packard ViewPlate[®], Product No. 6005182)

Cell Preparation Information

- This 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 ERK 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 5×10^4 cells/ml in DMEM 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% CO₂.

ERK MAPK Activation Kit Protocol

A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml ultrapure water. 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 7.5 ml 10X Blocking Buffer to 67.5 ml of ultrapure water (75 ml final volume). 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 27.5 µl of ERK MAPK antibody to 5.5 ml 1X Blocking Buffer.
Secondary Antibody Staining Solution	Add 3.0 µl of Hoechst Dye and 12.0 µl of the DyLight 488 Goat Anti-Rabbit to 6.0 ml of 1X Blocking 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.

1. Dilute PMA stock solution to 500 ng/ml into culture medium. Add 25 µl/well and mix thoroughly. Incubate 30 minutes at 37°C. For an agonist screen, compound replaces the 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 a fume hood at room temperature for 10 minutes. Pre-warming fixative is critical to maintaining cell integrity.
3. Aspirate Fixation Solution and wash plate once with 100 µl of 1X Blocking Buffer.
4. Aspirate Blocking Buffer, add 100 µl of 1X Permeabilization Buffer, and incubate for 15 minutes.
5. Aspirate Permeabilization Buffer and wash plate once with 100 µl of 1X Blocking Buffer.
6. Aspirate Blocking Buffer and add 50 µl Primary Antibody Solution per well. Incubate 1 hour.
7. Aspirate Primary Antibody Solution and wash twice with 100 µl of 1X Blocking Buffer.
8. Aspirate Blocking Buffer and add 50 µl of Staining Solution per well. Incubate for 1 hour protected from light.
9. Aspirate Staining Solution and wash twice with 100 µl of 1X Blocking Buffer.
10. Aspirate Blocking Buffer and add 200 µl of 1X Wash Buffer to each well.
11. Seal plate and evaluate on ArrayScan HCS Reader.
12. Store sealed plates in the dark at 4°C.

Additional Information

A. Performance Data

To produce a dose-response curve, NIH 3T3 cells were stimulated with PMA for 30 minutes. For the time-course experiment, NIH 3T3 cells were incubated at 37°C with 100 ng/ml PMA and sampled at 5-minute intervals. Cells were analyzed using the ArrayScan HCS Reader with the Cytoplasm to Nucleus Translocation BioApplication Software.

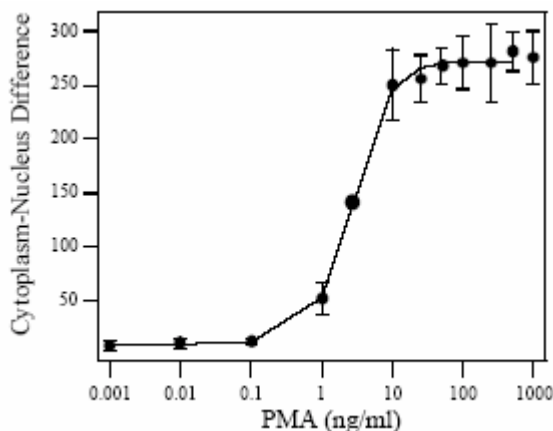


Figure 2A. Dose-response curve of NIH 3T3 cells treated with PMA. $EC_{50} = 2.7$ ng/ml

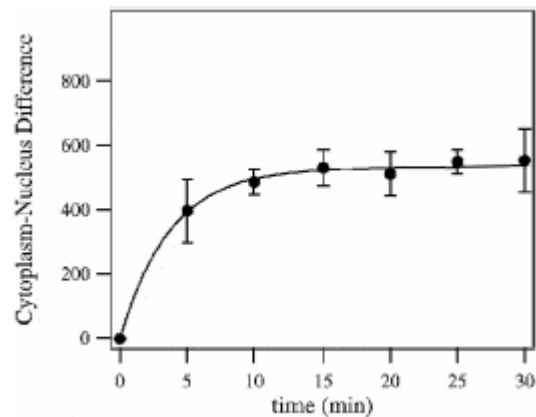


Figure 2B. Time course experiment of NIH 3T3 cells treated with PMA. Maximum stimulation was evident after 30 minutes. The $t_{1/2}$ was 3.7 minutes.

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

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5. 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.
6. 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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