

Cellomics[®] p38 MAPK Activation Kits

Reagents for High-Content Screening

1806.1

Number	Description
K01-0004-1	p38 MAPK Activation Kit, sufficient materials for 5 × 96 wells
R01-0507-1	p38 MAPK Activation Kit, sufficient materials for 50 × 96 wells

Kit Contents:	K0100041	R0105071
p38 MAPK Primary Antibody (rabbit)	164 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	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-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 p38 MAPK Activation Kit provides the reagents and protocol necessary to quantify p38 MAPK 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 core reagents, a primary p38 MAPK antibody and DyLight 488-conjugated Secondary Antibody, are supplied. The nuclear region is identified by the nuclear dye, Hoechst, also included in the kit.

The MAPK super family comprises c-Jun N-terminal kinases (JNKs), also known as stress activated protein kinases (SAPKs), p38 kinases, and extra cellular signal regulated kinases (ERKs). The p38 signal transduction pathway has been associated with the regulation of vital cellular processes including cell growth, division, differentiation, inflammation, and death. p38 is activated by bacterial lipopolysaccharide,¹ physiochemical changes in the extra cellular milieu (heat, hyper- and hypo- osmolarity, UV irradiation, sodium arsenite, and anisomycin,²⁻⁴ and proinflammatory cytokines (e.g., IL-1 and tumor necrosis factor-alpha).⁵ p38 MAPK is in a pathway comprising a cascade of kinases, whereby p38 acts as a substrate of one or more MKK kinases. Once activated by phosphorylation, p38 phosphorylates one or more substrates, leading to transcriptional changes and other diverse cellular processes. For example, the differentiation of neuronal-like precursor cells such as PC12 cells and the myogenesis of C2C12 cells involve the activation (phosphorylation) of p38. Viral infection also induces p38 activity.

The optimized protocol is for a fixed end-point immunofluorescent assay. Inhibitors of p38 MAPK translocation are screened by stimulating cells with a control inducer, such as TNFα (Figure 1), after exposing live cells to test compounds. To identify agonists of p38 MAPK translocation, TNFα is replaced with test compounds. Translocation is directly quantified as the difference in cytoplasmic to nuclear intensity of the labeled kinase. The p38 MAPK Activation Kit, in combination with the ArrayScan HCS System and the Cytoplasm to Nucleus Translocation Application software enables automated plate handling, focusing, cell image acquisition, analysis and quantification of p38 MAPK 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.

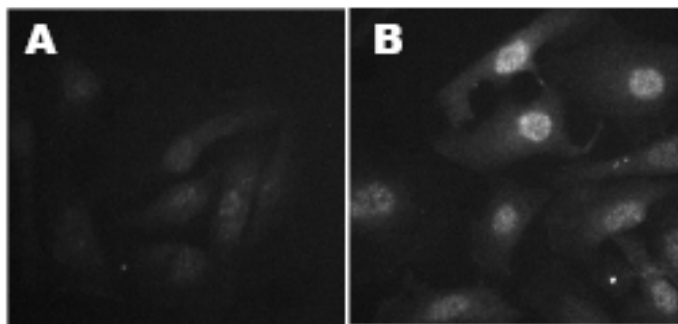


Figure 1. Images of stained HeLa cells before and after activation of p38 MAPK by TNF α (100 ng/ml for 15 minutes). Panel A: non-treated; Panel B: treated

Additional Materials Required

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

- TNF α (Sigma, Product No. T-6674) or other p38 MAPK activator
- Formaldehyde (37%) (Sigma, Product No. F1268)
- Microplates (96-well, black, clear bottom) Packard ViewPlate™ (Product No. 6005182), Greiner (Product No. 655087) or Corning Costar™ (Product No. 3603)

Cell Preparation Information

- Protocol optimized for HeLa cells (American Type Culture Collection, Product No. CCL-2).
- Culture cells in Minimum Essential Medium-Eagle (EMEM; BioWhittaker, Product No. 12-611Q) containing the following supplements (=EMEM Complete Medium): 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 solution (BioWhittaker, Product No. 13-114E), 1% sodium pyruvate (BioWhittaker, Product No. 13-115E)
- Split cells when they reach 70-80% confluency (2-3 times per week) at a dilution of 1:2 to 1:6.
- For p38 MAPK activation, harvest cells with Trypsin-Versene mixture (BioWhittaker, Product No. 17-161F), dilute into EMEM Complete Medium and determine cell density. Adjust to 5×10^4 cells/ml in EMEM Complete Medium and add 100 μ l 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.

p38 MAPK Activation Kit Protocol

A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml of ultrapure water. Store this solution at 4°C for up to 7 days.
1X Permeabilization Buffer	Add 10 ml of 10X Permeabilization Buffer to 90 ml ultrapure water. Store this solution at 4°C for up to 7 days.
1X Blocking Buffer	Add 10 ml of 10X Blocking Buffer to 90 ml of ultrapure water. Prepare just before each assay.
Fixation Solution	Add 1.1 ml 37% formaldehyde to 9.9 ml 1X Wash Buffer. Keep warmed to 37°C until use. Prepare just before each assay.
Primary Antibody Solution	Add 25.5 µl of p38 MAPK antibody to 5.5 ml 1X Wash Buffer. Prepare just before each assay.
Secondary Antibody Staining Solution	Add 12 µl of the Secondary Antibody and 2.75 µl of the Hoechst Dye to 5.5 ml of 1X Wash Buffer. Mix well. Prepare just before each assay.

B. Procedure

Note: Use 100 µl per well unless indicated otherwise. This protocol requires ~3 hours post-compound incubation to perform.

1. Dilute stock TNF α to 500 ng/ml in EMEM Complete Medium to make the Positive Control Working Solution. Warm solution to 37°C, 5% CO₂.
2. Add 25 µl of the prewarmed Positive Control Working Solution to wells for p38 MAPK activation. To unstimulated wells add 25 µl of EMEM Complete Medium.
3. Incubate plate for 15 minutes at 37°C.
4. Aspirate culture medium and add 100 µl 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; low-velocity fluid dispensing is recommended.
5. Aspirate Fixation Solution and add 100 µl of Blocking Buffer.
6. Aspirate Blocking Buffer, add 100 µl of 1X Permeabilization Buffer and incubate for 15 minutes.
7. Aspirate Permeabilization Buffer and wash with 100 µl of Blocking Buffer.
8. Aspirate Blocking Buffer, add 50 µl of Primary Antibody Solution and incubate for 1 hour.
9. Aspirate Primary Antibody Solution and wash twice with 100 µl of Blocking Buffer.
10. Aspirate Blocking Buffer and add 50 µl of Staining Solution. Incubate 1 hour, protected from light.
11. Aspirate Staining Solution and wash twice with 100 µl of Blocking Buffer.
12. Aspirate Blocking Buffer and add 200 µl of 1X Wash Buffer.
13. Seal plate and run on ArrayScan HCS System.
14. Store sealed plates at 4°C in the dark.

Additional Information

A. Dose Response Curve and Time Course

HeLa cells were stimulated with TNF α for 15 minutes, and stained as described in the protocol. An increase in the cytoplasmic to nuclear intensity of labeled p38 MAPK was observed with increasing TNF α concentrations (Figure 2A). For the time-course experiments, HeLa cells were incubated at 37°C with TNF α at a maximal dose (100 ng/ml) and sampled at 5-minute intervals (Figure 2B).

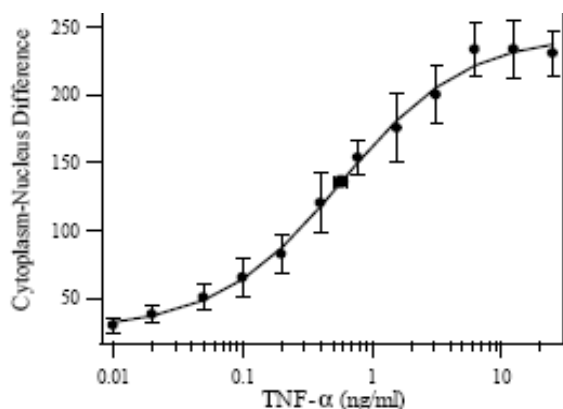


Figure 2A. Dose response curve of TNF α . Mean value from 8 wells \pm SD is plotted for each concentration. The EC₅₀ was \sim 0.58 ng/ml.

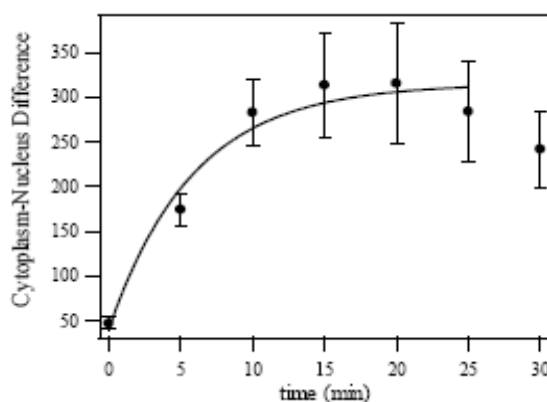


Figure 2B. Time course of p38 MAPK activation. Maximum stimulation was evident after 15 minutes, where complete translocation from cytoplasm to nucleus was observed. Mean value from 8 wells \pm SD is plotted for each time point. $t_{1/2}$ was \sim 5.7 minutes.

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