FlowCellect™ p38 Stress Pathway Activation Detection Kit

25 Tests

Cat. No. FCCS025132

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Application

P38 is a member of the mitogen activated kinase family that responds to stress stimuli, such as inflammatory cytokines, ultraviolet irradiation, heat shock, and osmotic shock. P38 is involved in cell differentiation and apoptosis (1). More recently, P38 has been shown to have a role in cell cycle check points and DNA damage pathways (2). The upstream kinases, MKK3, MKK6 and SEK activate p38α by phosphorylation at Thr180 and Tyr182 (3).

Once activated, p38α MAP kinase has been shown to phosphorylate and activate a number of proteins including MAPKAP kinase 2 (4) and the transcription factors ATF-2 (5), MEF2 (6) and MSK1 (7).

Test Principle

Millipore's FlowCellect™ p38 Stress Pathway Activation Detection Kit is designed to enable a researcher a quick and easy way to detect the phosphorylation state of P38α and ATF2 by flow cytometry. Millipore's FlowCellect™ p38 Stress Pathway Activation Kit was developed and tested using the activating reagent, anisomycin, in Jurkat cells but the kit can be used with other human cell lines to determine the effect of stress stimuli and chemical reagents that can induce P38 pathway signaling.

Millipore's FlowCellect™ p38 Stress Pathway Activation Detection Kit contains sufficient reagents for 25 2-color tests. The kit includes two optimized fluorescent labeled antibodies and buffers necessary for cell preparation and analysis. Detailed assay instructions are included to assist in your analysis and to ensure that the correct cell concentration is obtained during acquisition of sample data.

Kit Components

1. 20X Anti-pP38(T180/Y182) AlexaFluor®488 Antibody: (Part No. CS205783) 1 vial containing 150 µL
2. 20X Anti-pATF2(T69/71) AlexaFluor®647 Antibody: (Part No.CS205784) 1 vial containing 150 µL
3. Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL buffer.
4. 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL buffer.
5. 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL buffer.
6. 1X Permeabilization Buffer: (Part No. CS203284) Two bottles containing 14 mL buffer.

Materials Not Supplied

1. Flow Cytometer
2. Cells of interest and/or Jurkat cells as positive control
3. Media for cell line of interest
4. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 µL
5. Tabletop centrifuge capable of 2500 rpm.
6. Sample tubes capable of holding 1 mL
7. Anisomycin or other activating reagent

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. The kit includes a fixation solution containing formaldehyde. Please refer to the MSDS sheet which can be found at www.millipore.com for specific information on hazardous materials.
- All fluorochrome conjugated antibodies are light sensitive and must be stored in the dark at 2-8°C.
- During storage and shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For maximum recovery of product, centrifuge vial briefly prior to removing cap.
- Do not use reagents beyond 4 months from date of receipt.

Storage

Upon receipt, all antibodies and buffers should be stored at 2-8°C. ***Caution:*** *Fluorochrome conjugated antibodies should always be stored at 2-8°C. Do not freeze fluorescent antibodies. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.*

Preparation of Buffers

- Make 100 mL of 1X Assay Buffer: Mix 20 mL of 5X Assay Buffer with 80 mL of deionized water.
- Place Permeabilization Buffer on ice. *Permeabilization buffer must be ice cold for optimal results.*

Note: Prepared 1X Assay Buffer is stable up to four months if stored at 2-8ºC, and can be prepared when you receive the kit or when you start the first assay. 10X Wash Buffer is included in the kit but is not used in the assay.

Example Cell Treatment Protocol

Note*: This assay protocol has been optimized for Jurkat cells treated with anisomycin. However, this kit is suitable for flow cytometric analysis of a variety of human cell types and treatments but treatments may need to be optimized.*

Cell Preparation for Ansiomycin Treatment

1. Seed cells into 2 T-25 flasks ($6X10^6$ cells in 6 mL of media per flask).
2. Label one flask **Untreated** and label the other flask **Treated**.
3. To the **Treated** flask and add 9.6 µL of 1.25 mg/mL anisomycin (in DMSO) for a final concentration of 2 µg/mL. To the **Untreated** flask and add 9.6 µL of DMSO.
4. Incubate the flasks in a 37°C, 5% CO_2 incubator for 30 minutes.
5. Count cells.

Example Cell Staining Protocol:

- ***Note*****:** *You will need $0.5X10^6$ cells for one test on Guava cytometer and $1X10^6$ cells for one test on other flow cytometers. Permeabilization Buffer must be ice cold prior to assay.*

1. Aliquot $0.5X10^6$ cells in media into a microfuge tube for each test to be performed. If using the assay for the first time, you will need to set up additional tubes to use for adjusting instrument settings as follows: **Untreated** cells will be labeled tube 3. **Treated** cells will be labeled tube 1, 2, and 4 (see table below).
2. Add 0.25 mL of Fixation Buffer by gently pipetting up and down.
3. Incubate for 20 minutes at room temperature.

4. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

5. Resuspend cells in 0.5 mL 1X Assay Buffer by gently pipetting up and down.

6. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

7. Resuspend cells in 0.5 mL of **ice cold** 1X Permeabilization Buffer by gently pipetting up and down.

8. Incubate samples on ice for 10 minutes. (If necessary, cells can be stored in 1X Permeabilization Buffer overnight at 4 degrees and the assay can be continued the next day.)

9. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

10. Resuspend cells in 0.5 mL 1X Assay Buffer by gently pipetting up and down.

11. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

12. Resuspend cells in tubes 1 and 2 in 95 μL 1X Assay Buffer by pipetting up and down. Resuspend cells in tubes 3 and 4 in 90 μL 1X Assay Buffer by pipetting up and down.

13. Add 5 μL of 20X antibody to tubes using the flowing chart.

Tube #	Description
1	Treated cells stained with pP38(T180/Y182)-AlexaFluor®488
2	Treated cells stained with pATF2(T69/71)-AlexaFluor®647
3	Untreated cells stained with both antibodies
4	Treated cells stained with both antibodies

14. Incubate tubes in the dark on ice for 60 minutes.

15. Add 900 μL of 1X Assay Buffer to each tube then pellet the cells at 2500 rpm for 3 minutes and discard buffer.

16. If using a Guava cytometer, resuspend cells in 500 μL of 1X Assay Buffer. If using another cytometer, resuspend cells in 1 mL of 1X Assay Buffer.

17. Transfer 200 μL of cells to a 96-well plate or transfer 500 μL- 1 mL to a sample tube and analyze on a Flow Cytometer.

Note: Samples 1 and 2 are used for adjusting compensation. Once the initial compensation for a given cell type/treatment has been performed the settings file can be saved and used for subsequent data collection without the need for samples 1 and 2.

Sample Data

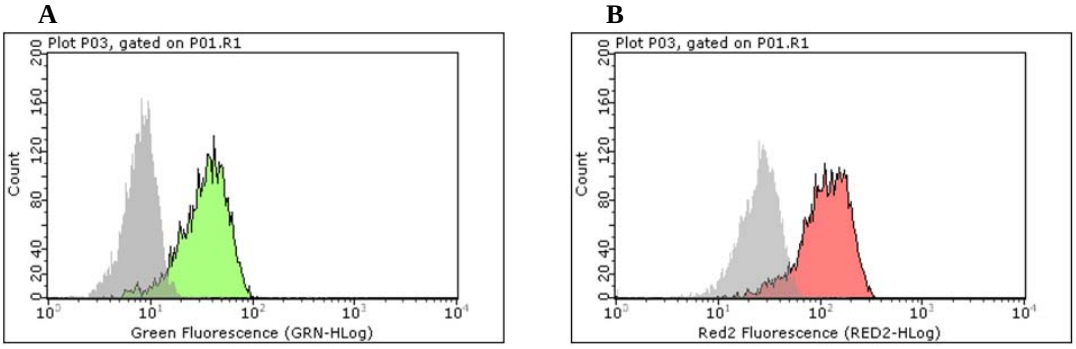

Figure 1: A) Single color histogram analysis of P38 phosphorylation. Jurkat cells treated with ansiomycin for 30 mins (green shaded histogram) or untreated Jurkat cells (grey shaded histogram) and stained with anti-pP38 (T180/Y182)-AlexaFlour®488. B) Single color histogram analysis of ATF-2 phosphorylation. Jurkat cells treated with ansiomycin for 30 mins (orange shaded histogram) or untreated Jurkat cells (grey shaded histogram) and stained with anti-pATF-2(T69/71)-AlexaFlour®647.

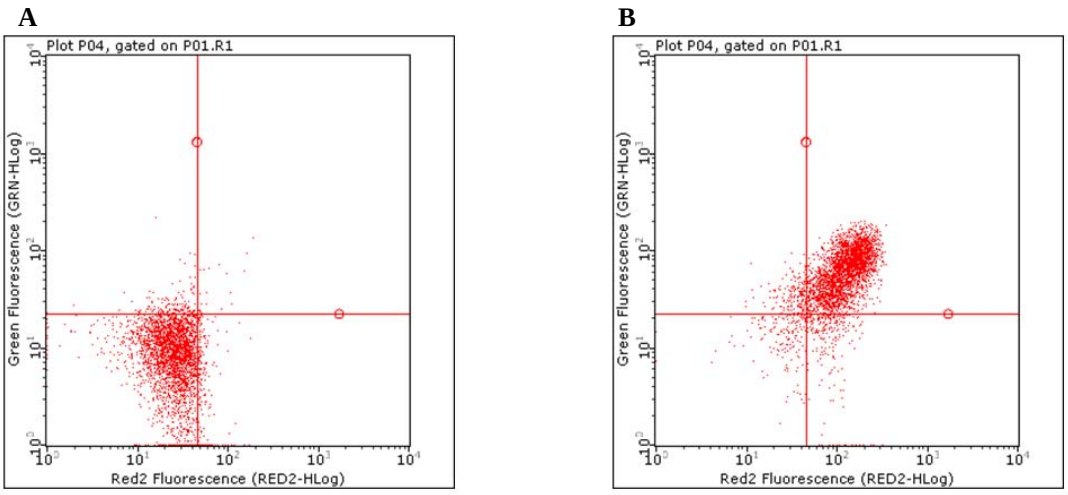

Figure 2: Multi-color dot blot analysis of P38 and ATF2 phosphorylation upon stimulation with anisomycin treatment. Untreated Jurkat cells (A) and Jurkat cells treated with anisomycin for 30 mins (B) and then were stained with anti-phospho P38(T180/Y182)-AlexaFlour®488 and anti-phospho ATF2(T69/71)-AlexaFlour®647.

Technical Hints

- Permeabilization Buffer must be ice cold prior to assay
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- Certain cell cultures cell pellets may become hazy or transparent following the fixation step, making it difficult to see. After the washing steps they will become easier to visualize.
- If sampling a small collection of cells for flow analysis, it is recommended that all steps be performed in a smaller collection tube (e.g. centrifuge tube)
- Do not mix or interchange reagents from various kit lots.
- If minor precipitate is detected in the 10X Wash Buffer place the bottle in a warm water bath for 30 minutes, followed by mixing the contents on a mechanical vortex.

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically Instrument clogging Too many cells	• Cell concentration too high - Decrease the number of cells per microliter by diluting sample to 300 – 500 cells per microliter. Guava cytometers give the most accurate data when the flow rate is less 500 cells per microliter. • Run three Quick Cleans (for Guava cytometers) to rinse out the flow cell. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.
Too few cells	• Spin down cells and resuspend in a smaller volume. The assay instructions are optimized to give you a range of cells between 100-500 cells/μL in the final sample volume. However, cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to difficulty in adjusting settings. Make sure to leave the cell pellet intact when discarding buffer. If the cells are not generating a compact pellet after centrifugation, increase the time to 5 minutes and/or increase the speed by 500 rpm until a compact and visible cell pellet forms.
Background staining and/or non-specific staining of cells	• This assay was optimized using Jurkat cells. Therefore, further antibody titrations may be necessary for other cell types and conditions to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure.

Variability in day to day experiments	• Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any changes in culture conditions or viability can influence experimental results. • Make sure that a quality check on the flow cytometer to be used (e.g. calibration) is performed on a daily basis prior to use.
Staining is weak	• Some cell lines may require higher concentrations of fixation buffer to stain correctly. Use twice as much Fixation Buffer.
Staining is weak	• Some cell lines may require higher concentrations of conjugated antibodies. Try using twice as much antibody for your staining.
Staining is too bright	• Some cell lines may require lower concentrations of conjugated antibodies. Try using half as much antibody for your staining.

***For further support, please contact Millipore's Technical services at +1(800) 437-7500*

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