FlowCellect™ STAT1 Activation Dual Detection Kit

25 Tests

Cat. No. FCCS025142

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Application

Millipore's FlowCellect™ Activation Dual Detection kits are a series of flow cytometry products which include a pair of antibodies that bind to the same protein; one to detect total protein expression and another to detect the phosphorylated form of the same target. Using two parameter analysis we can achieve target specific detection of phosphorylation and, by doing so, eliminate false positives while enhancing the signal to noise ratio.

STATs are transcription factors that can be phosphorylated normally by a cell surface receptor such as cytokine receptors, followed by dimerization and nuclear translocation where they bind to DNA and activate transcription of their target genes. Signal Transducer and Activator of Transcription 1 (STAT1) is involved in cellular immunity and cell proliferation (1). Its activation has been detected in response to viral as well as parasitic infections.

One interesting aspect of STAT1 activation is its role in antitumor biology. Macrophage stimulation with interferon gamma induces direct antimicrobial and antitumor mechanisms as well as upregulating antigen processing and presentation pathways (2). It has been shown that oncogenesis and tumor progression are supported by alterations in cell signaling. Using flow cytometry, it is now possible to track and analyze signaling events in individual cancer cells (3). Single cell resolution of signaling molecules allows for a greater understanding of the elaborate processes that occur when cells transform and begin replicating uncontrollably.

All FlowCellect kits are optimized on guava® bench top flow cytometers. FlowCellect kits can be used on any flow cytometer following the same protocol providing researchers a reliable and fully validated solution to study the STAT1 signaling pathway right in the comfort of their own lab. This kit includes optimized fixation, permeabilization, wash and flow buffers to provide researchers with a complete solution for cell signaling analysis.

Test Principle

Millipore's FlowCellect™ STAT1 Activation Dual Detection kit includes two directly conjugated antibodies, a phospho-specific Anti-phospho-STAT1 (Tyr701)-Alexa Fluor® 488 and Anti-STAT1-PerCP. This two color flow cytometry kit is designed to detect the extent of STAT1 pathway activation by measuring the STAT1 phosphorylation relative to the total STAT1 expression in any given cell population.

The levels of both the total and phosphorylated protein can be measured simultaneously in the same cell, resulting in a normalized and accurate measurement of STAT1 activation after stimulation. Moreover, simultaneous measurement of both total and phospho-STAT1 confirms target specificity of the phosphorylation event. Together, a total and phospho antibody duo performed in multiplex provides an enhanced and more reliable detection of the phospho: total ratio within a mixed population.

The antibody pair provided in this kit have been carefully titrated to ensure the ability to measure Total and phospho-STAT1 simultaneously on the same protein for accurate determination of protein level and activation. When stimulating U937 cells with Interferon gamma for ten minutes the total protein level remains constant compared to unstimulated cells, while phospho-STAT1 levels are

increased in all cells. Indicating that there is no competition between the two antibodies for their target epitopes.

Sufficient reagents are provided to perform 25 two-color tests. Detailed assay instructions are included to assist in analysis and to ensure the correct cell concentration is obtained during acquisition of sample data.

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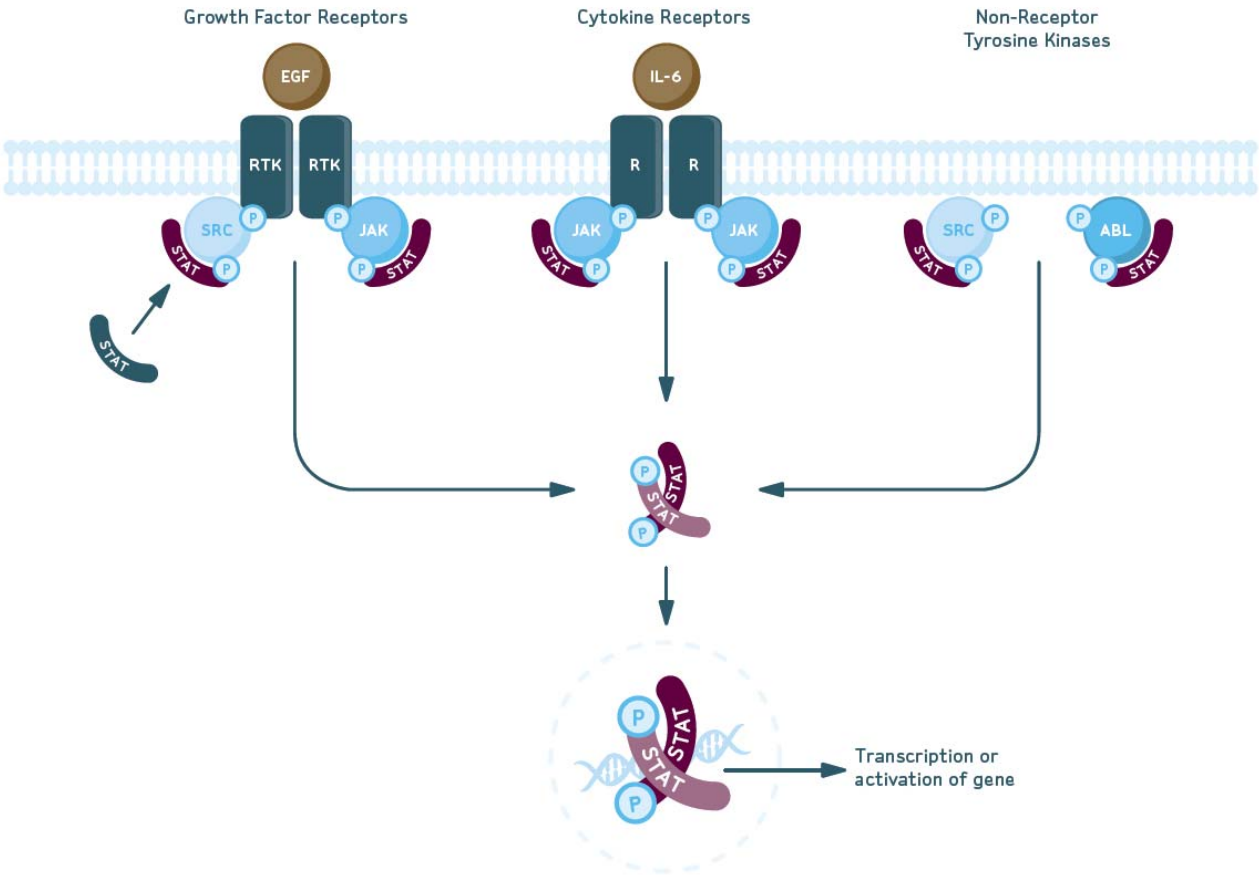

Figure 1. STAT Signaling Pathway. The STAT pathway can be activated in numerous ways. Most commonly they are activated by JAK (Janus Associated Kinase) through growth factor and cytokine receptors, however, activation may also occur by non-receptor Tyrosine Kinases such as through SRC and ABL. STAT proteins are then phosphorylated, followed by either homo or hetero-dimerization and translocated to the nucleus, where they activate transcription of their target genes.

Kit Components

- 20X Anti-phospho-STAT1(Tyr701) Alexa Fluor® 488: (Part No. CS206484) One vial containing 150 µL.
- 20X Anti-STAT1-PerCP: (Part No. CS206483) One vial containing 150 µL.
- Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL.
- 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL.
- 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL.
- 1X Permeabilization Buffer: (Part No. CS203284) Two bottles containing 14 mL.

Materials Not Supplied

1. Test tubes for sample preparation and storage
2. Tissue culture reagents, i.e. HBSS, PBS w/o Ca^{2+} or Mg^{2+}, cell dissociation buffers, etc.
3. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 µL
4. Tabletop centrifuge capable of exceeding 400 x g
5. Mechanical vortex
6. Flow Cytometer
7. Deionized water (for Buffer dilutions)
8. Human Interferon Gamma protein. (Millipore Cat. Number IF002)
9. Isotype Control: mouse IgG2a-PerCP (based on user preference)

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. Kit contains a fixation solution containing paraformaldehyde. Please refer to the MSDS sheet for specific information on hazardous materials (MSDS forms can be found by contacting Millipore technical services).
- The conjugated antibody is light sensitive and must be stored in the dark at 4°C.
- During storage and shipment, the product supplied in vials may condense within the vial. For maximum recovery of the product, centrifuge original vial prior to removing cap.
- Do not use reagents beyond the expiration date of the kit.

Storage

This kit must be stored at 2 - 8ºC. The 10X Wash Buffer (Part # CS202123) and Fixation Buffer (Part # CS202122) can be stored at either 2 - 8ºC or at room temperature upon receipt. **Caution:** The fluorochrome conjugated antibody should always be stored at 2 - 8ºC and stored in the dark.

All kit components are stable up to six (6) months from date of receipt if stored and handled correctly. **Please avoid repeated changes in temperature as this will affect the integrity of the product.**

Preparation of Reagents

1. **Wash Buffer**

   Wash Buffer is supplied at 10X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Wash Buffer is stable up to one year. Store at 2 - 8ºC.

   Note: 10X Wash Buffer can be stored at RT to avoid any potential precipitate formation

2. **Assay Buffer**

   Assay Buffer is supplied at 5X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Assay Buffer is stable up to one year. Store at 2 - 8ºC.

Assay Instructions

Note: This assay protocol has been optimized for human U937 cells. However, this kit is suitable for measuring the extent of STAT1 target-specific detection and activation via phosphorylation on a variety of human cell types. Alternate species reactivity must be confirmed by the end user.

I. Cell Culture and Stimulation

1. U937 cells should not exceed 1 million cells per mL in culture as they may down regulate STAT proteins. Determine cell numbers by using ViaCount or a hemacytometer and note cell viability. Healthy cells should be above 90% viable.

2. After cell numbers have been determined, centrifuge cells at 670 x g for 5 minutes to pellet cells.

3. Aspirate to remove the supernatant and resuspend cell pellet at 1 million cells per mL in fresh media.

4. Divide cells into two separate 50 mL conical tubes. Note volume of cell solution-One tube will serve as the “treated” or experimental sample while the other will serve as the “untreated” or control.

5. Place both conical tubes in a 37°C water bath for 20 to 30 minutes to bring cells back to 37°C.

6. Add 50 ng/mL of Human Interferon Gamma into the tube labeled “treated” and the same volume of media to the tube labeled “untreated” for 5 minutes at 37°C.

II. Fix and Permeabilize Cells

7. Immediately after stimulation, centrifuge treated and untreated cells at 670 x g for 5 minutes in a 4°C centrifuge and aspirate off supernatant.

8. Resuspend cells in enough 1X fixation buffer to yield 1 million cells per mL into both tubes an incubate 20 minutes at room temperature.

9. Centrifuge fixed cells at 670 x g for 5 minutes in a 4°C centrifuge and aspirate off supernatant.

10. Wash cells once with 5 mL of 1x Wash Buffer and repeat step #9 above.

11. Resuspend each cell pellet to 5 million cells per mL and add 100 µL of cells into a “V” bottomed 96-well plate. (see guava manual for instrument compatible plates) NOTE: Plate must be placed on ice before adding cells.

12. Centrifuge cells at 670 x g for 5 minutes in a 4°C centrifuge and dump out supernatant.

13. Permeabilize cells by adding 100 µL of ice-cold Permeabilization Buffer and incubate on ice for 20 minutes. Gently resuspend each sample twice during this 20 minute incubation.

14. Centrifuge cells at 670 x g for 5 minutes in a 4°C centrifuge and dump out supernatant.

15. Wash cells with 200 µL of a 1x Wash Buffer and repeat step #14 above.

III. Cell Staining and Flow Analysis

16. For single color staining, resuspend the cells in 95 µL of assay buffer and add 5 µL of either Anti-phospho-STAT1-Alexa Fluor® 488 **or** Anti-STAT1-PerCP to each sample well.

17. For multiplexing, resuspend the cells in 90 µL of assay buffer and add 5 µL of Anti-phospho-STAT1-Alexa Fluor® 488 **and** add 5 µL of Anti- STAT1-PerCP to each sample well.

18. Incubate cells for one hour on ice in the dark.

19. Add 100 µL of 1x Wash Buffer to the 100 µL of diluted antibodies already in the wells and centrifuge at 670 x g for 5 minutes at 4°C. Discard supernatant.

20. Wash with 200 µL of 1x Wash Buffer and centrifuge cells at 670 x g for 5 minutes at 4°C. Discard supernatant. Repeat once more.

21. Resuspend cells in each well with 200 µL of 1x ice-cold Assay Buffer.

22. Perform flow cytometry analysis

Sample Data

Single parameter Data:

Total STAT1 antibody performance

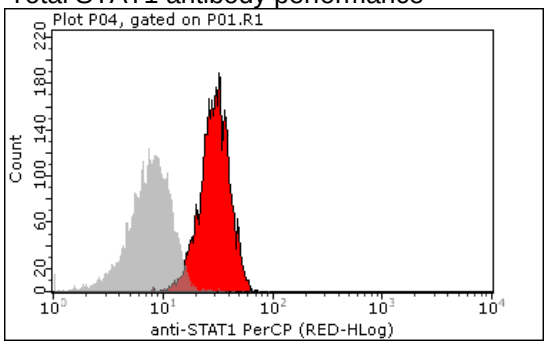

Figure 2. Analyzed Single Parameter Data for Anti-STAT1 Untreated U937 cells were stained with anti-STAT1-PerCP (Red) or Stained with Isotype control (grey).

Phospho-STAT1 antibody performance

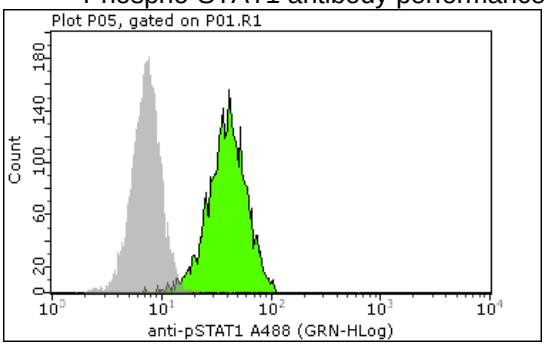

Figure 3. Analyzed Single Parameter Data for Anti-phospho-STAT1 (Tyr701) U937 cells were treated with 50 ng/mL of Interferon Gamma and then stained with phosphor-STAT1 (Tyr701) Alexa Fluor 488 (green). Untreated U937cells (grey) were also stained and results are shown overlaid in each plot.

Two parameter analysis using total and phospho antibodies:

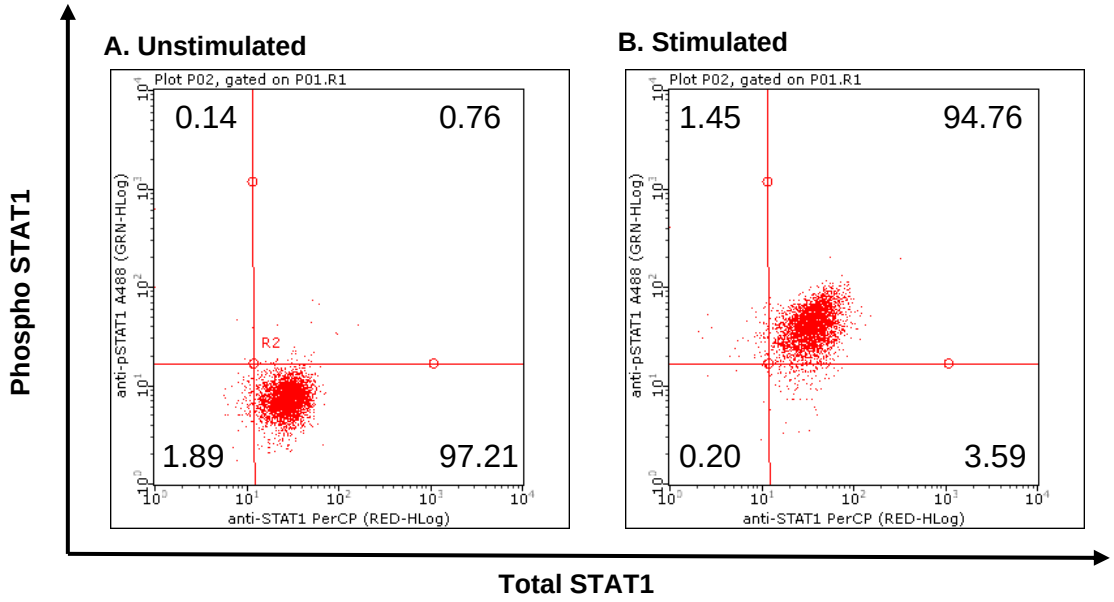

Figure 4. Dual Parameter Analysis of Total and Phospho STAT1 on A431Cells (**A**) Unstimulated U937 cells stained with total and phospho STAT1. 97% of the cells are positive for Total STAT1 (based on Isotype control from single parmeter data above) and negative for phospho-STAT1. Following stimulation with 50 ng/mL of Interferon gamma plot (**B**) shows 94.76% of the cells are positive for phospho-STAT1 while their expression levels for total STAT1 remains unchanged.

Technical Hints

- 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.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- For certain cell cultures cell pellets may become hazy or transparent following the fixation step, making it difficult to see. 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.

Troubleshooting

Assay Step	Potential Problem	Experimental Suggestions
Reagent Preparation	Precipitation found in 10X Wash buffer	• If storing at -20ºC, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37ºC water bath, swirling the contents occasionally. If this does not remove the precipitate completely, allow 10X Wash Buffer to sit at room temperature overnight.
Acquisition	Acquisition rate decreases dramatically	This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following: • Decreasing number of cells for analysis. Guava flow cytometers have the capacity of analyzing a steady stream of 300 – 500 cells per microliter. Any cell densities in excess can essentially block the normal flow, causing disruption during the assay. Decrease the number of cells being analyzed by diluting the sample to approximately 0.5 million cells per milliliter. • Adherent or sticky cells can result in cellular clumping. Use a more aggressive enzyme for dissociation such as trypsin during cell harvesting should help keep cells in single suspension. Alternatively, using a cell strainer can help disrupt cell clumping if needed (Catalog No. SCNY00060; 60 μM) • After many uses, it is possible that the fluid system on any standard flow cytometer will require cleaning. Run standard cleaning procedures to clean the fluid system during or after an assay. This will prevent any material from forming where the steady flow steam takes place.
Cellular Analysis	A loss or lack of signal	• Cell numbers may need to increase. Cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to a loss of signal. Make sure that cell density remains at approximately 0.5 million cells per milliliter during analysis. • Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. A lack of signal may indicate that excess antibody will need to be used during the staining procedure.

Cellular Analysis	Background and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types 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.
Cellular Analysis	Variability in day to day experiments	• Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results. • When using the guava easyCyte™ Plus instrument for flow analysis, make sure that a quality check on the instrument (e.g. calibration) is performed on a daily basis prior to use. (**See Analytical Sensitivity and Detection Limits Section for Guava Check standards*)

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

References

1. Shizuo Akira. Functional Roles of STAT Family Proteins: Lessons from Knockout Mice. Stem Cells, Vol. 17, No. 3, 138-146, May 1999

2. Kate Schroder, Paul J. Hertzog, Timothy Ravasi, David A. Hume. Interferon-Y: an overview of signals mechanisms and functions. Journal of Leukocyte Biology. 163-189, 2004

3. Jonathan M. Irish, Nikesh Kotecha and Garry P. Nolan. Mapping normal and cancer cell signalling networks: towards single-cell proteomics. 2006 Nature Reviews Cancer Vol. 6, 146.

Related Products

1. FlowCellect™ Bivariate Cell Cycle Kit for DNA Replication Analysis (Catalog No. FCCH025102)
2. FlowCellect™ Bivariate Cell Cycle Kit for G2/M Analysis (Catalog No. FCCH025103)
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15. FlowCellect™ STAT3 Activation Dual Detection Kit (Catalog No. FCCS025143)

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11/2010
Revision A