



FlowCollect™ STAT3 Activation Dual Detection kit
25 Tests

Cat. No. FCCS025143

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

Application

Millipore's FlowCelect™ 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. STAT3 (Signal transducer and activator of transcription 3) is a transcription factor that plays an important role in normal and aberrant mammalian biology (1). It becomes activated upon phosphorylation by receptor associated kinases such as JAK2, and responds to various types of upstream signaling pathways. Due to the diverse ways in which STAT3 can be activated in vivo, its biological functions vary, from regulating cell proliferation, to promoting oncogenesis, and even to act as a tumor suppressor in certain settings.

The regulation of STAT3 can be mediated through both protein expression modulation and post-translational modifications. The expression of STAT3 has been shown to be aberrantly elevated in disease states such as cervical cancer (2) and some cases of large cell lymphoma (3), where abnormal cellular proliferation and survival were believed to be linked to rapid cancer development. In these cases, phosphorylation of STAT3 was concurrently observed with increased gene and protein expression, suggesting a system of combinatorial regulation of the protein and highlighting the importance of a dual-detection approach in studying cellular signaling pathways.

FlowCelect kits are optimized on guava® bench top flow cytometers, and can also be used on any flow cytometer following the same protocol providing researchers a reliable and fully validated solution to study the STAT3 signaling pathway right in the comfort and convenience of their own lab. This kit contains optimized fixation, permeabilization, wash, and flow buffers to provide researchers with a complete solution for cell signaling analysis.

Test Principle

STAT3 is a transcription factor that is downstream of many signal transduction cascades that propagate signals generated extracellularly along intracellular interconnected transduction pathways. STAT3 pathway activation in response to receptor tyrosine kinase stimuli (e.g. EGFR stimulated by EGF) plays a significant role in the regulation of essential cellular processes. The diverse roles of STAT3 make it a key molecule in both normal and aberrant biology, as it can provide signals for cell survival, mitogenesis, as well as tumor-related stimuli for angiogenesis and metastasis.

Millipore's FlowCelect™ STAT3 Activation Dual Detection kit includes two directly conjugated antibodies, a phospho-specific Anti-phospho-STAT3 (Tyr705)-Alexa Fluor® 647 and Anti-STAT3-Alexa Fluor® 488. The antibody pair provided in this kit has been carefully titrated to ensure the ability to measure Total and phospho-STAT3 simultaneously on the same protein for accurate determination of protein level and activation.

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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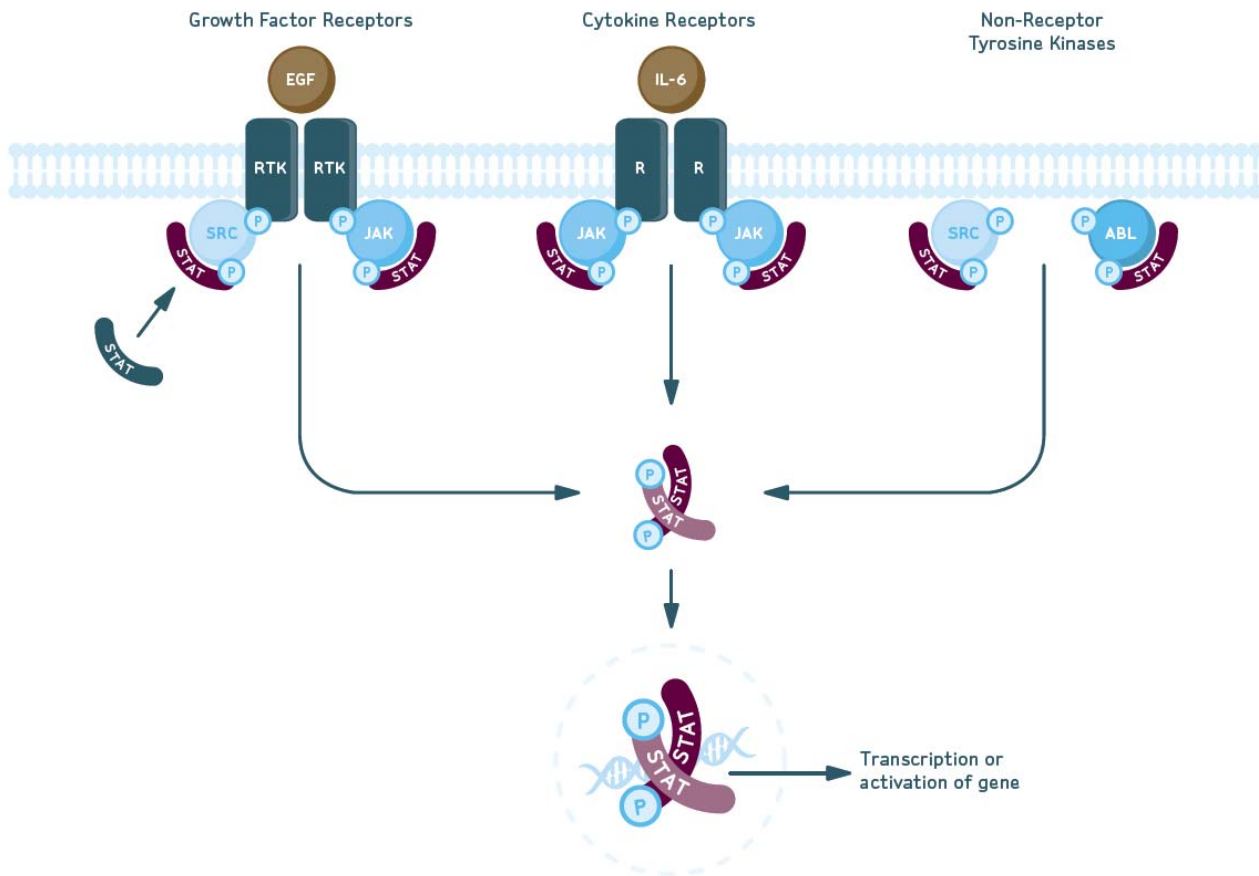


Figure 1. STAT Signaling Pathway. The STAT pathway can be activated in numerous ways. Most commonly they are activated by JAK through growth factor and cytokine receptors; however, activation may also occur by non-receptor Tyrosine Kinases such as SRC and ABL. STAT proteins are then phosphorylated, followed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

Kit Components

- 20X Anti-phospho-STAT3 (Tyr705) Alexa Fluor[®] 647: (Part No. CS206488) One vial containing 150 µL.
- 20X Anti-STAT3-Alexa Fluor[®] 488: (Part No. CS206476) 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 Ca²⁺ or Mg²⁺, 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 Epidermal Growth Factor protein. (Millipore Cat. Number 01-107)
9. Isotype Control: mouse IgG1-Alexa Fluor[®] 488 (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 - 8°C. The 10X Wash Buffer (Part # CS202123) and Fixation Buffer (Part # CS202122) can be stored at either 2 - 8°C or at room temperature upon receipt. **Caution:** The fluorochrome conjugated antibody should always be stored at 2 - 8°C 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 - 8°C.

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 - 8°C.

Assay Instructions

Note: This assay protocol has been optimized for human A431 cells. However, this kit is suitable for measuring the extent of STAT3 target-specific detection of 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. Seed 6 million A431 cells per T-175 flask overnight in a 37°C incubator with 5% CO₂. Cells should be at about 70% confluent the next day.
2. Aspirate growth media from T-175 flask and gently wash with 10 mL of PBS to remove any residual growth media.
3. Add 2 mL Accutase or other cell-detachment enzyme to the flask. Incubate for 5 minutes.
4. Gently tap the sides of the flask to dislodge cell monolayer and then immediately add 10 mL of growth media to collect cells. Transfer cell suspension into a 50 mL conical tube. Pipet the cells several times to achieve a single cell suspension.
5. Determine cell numbers by using ViaCount or a hemacytometer and note cell viability. Healthy cells should be above 90% viable.
6. After cell numbers have been determined, centrifuge cells at 670 x g for 5 minutes to pellet cells.
7. Aspirate to remove the supernatant and resuspend cell pellet at 2 million cells per mL in fresh media.

8. Divide cells equally 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.
9. Place both conical tubes in a 37°C water bath for 20 to 30 minutes to bring cells back to 37°C.
10. Add 100 ng/mL of EGF into the tube labeled “treated”, and the same volume of media to the untreated tube. Incubate both tubes at 37°C for 5 minutes.

II. Fix and Permeabilize Cells

11. Immediately after stimulation, fix cells by adding an equal volume of ice-cold 2X Fixation Buffer into both tubes for 20 minutes at room temperature.
12. Centrifuge fixed cells at 670 x g for 5 minutes in a 4°C centrifuge and aspirate off supernatant.
13. Wash cells once with 5 mL of 1x Wash Buffer and repeat step #12 above.
14. 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.
15. Centrifuge cells at 670 x g for 5 minutes in a 4°C centrifuge and dump out supernatant.
16. 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.
17. Centrifuge cells at 670 x g for 5 minutes in a 4°C centrifuge and dump out supernatant.
18. Wash cells with 200 µL of a 1x Wash Buffer and repeat step #17 above.

III. Cell Staining and Flow Analysis

19. For single color staining, resuspend the cells in 95 µL of assay buffer and add 5 µL of either Anti-phospho-STAT3-Alexa Fluor[®] 647 **or** Anti-STAT3-Alexa Fluor[®] 488 to each sample well.
20. For multiplexing, resuspend the cells in 90 µL of assay buffer and add 5 µL of Anti-phospho-STAT3-Alexa Fluor[®] 647 **and** add 5 µL of Anti-STAT3-Alexa Fluor[®] 488 to each sample well.
21. Incubate cells for one hour on ice in the dark.
22. Add 100 µL of 1x Wash Buffer to each well and centrifuge at 670 x g for 5 minutes at 4°C. Discard supernatant.
23. 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.

24. Resuspend cells in each well with 200 μ L of 1x ice-cold Assay Buffer.

25. Perform flow cytometry analysis

Sample Data

Single parameter Data:

Total STAT3 antibody performance

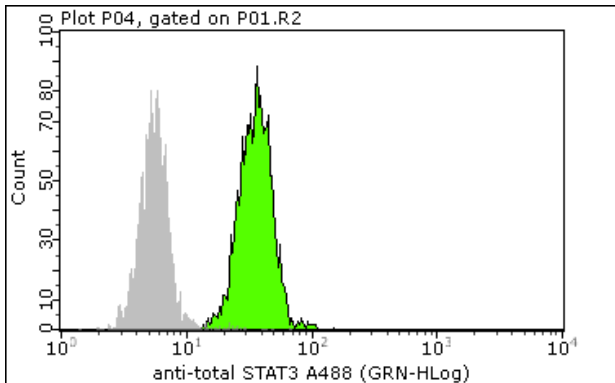


Figure 3. Analyzed Single Parameter Data for Anti-STAT3 (total STAT3) on A431 cells
A431 cells were stained for Anti-STAT3-Alexa Fluor[®] 488 (green) antibody versus an isotype control (grey).

Phospho-STAT3 antibody performance

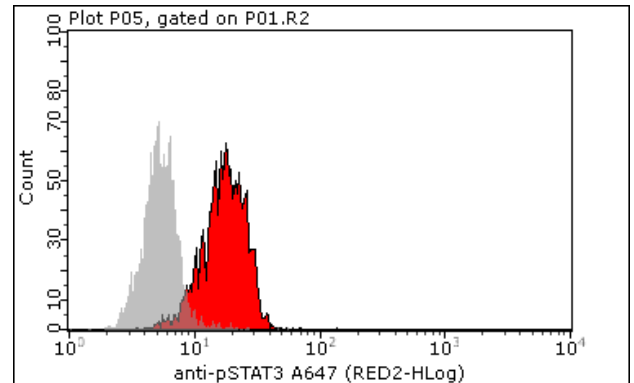


Figure 2. Analyzed Single Parameter Data for Anti-phospho-STAT3 (Tyr705) A431 cells were treated with 100 ng/mL of EGF and then stained with pSTAT3-Alexa Fluor[®] 647 (red). Untreated A431 cells (grey) were also stained and results are shown overlaid in each plot.

Two parameter analysis using total and phospho antibodies:

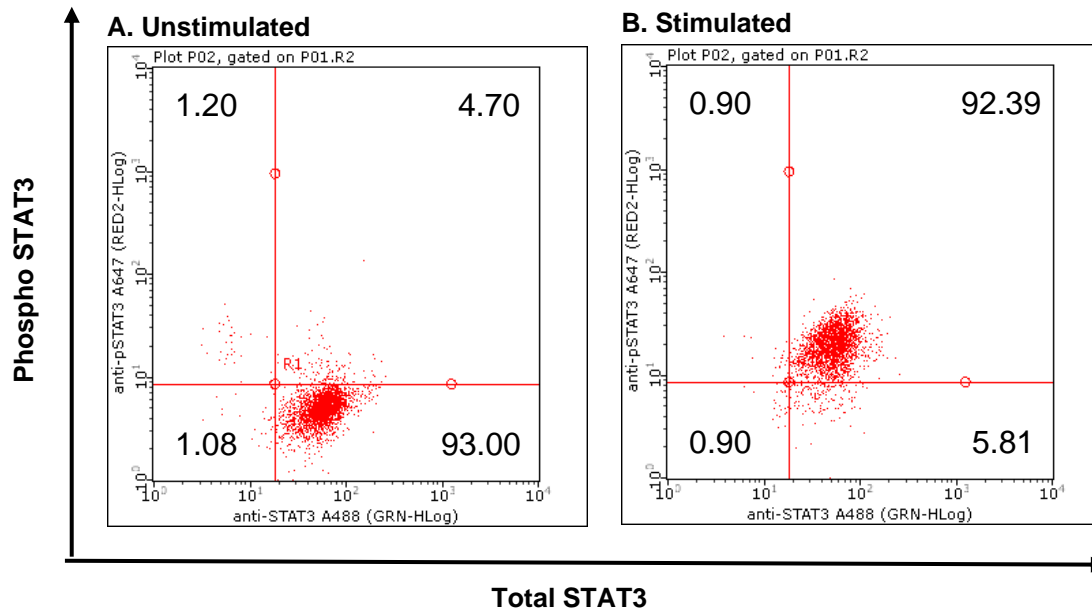


Figure 4. Dual Parameter Analysis of Total and Phospho STAT3 on A431 Cells (A) Unstimulated A431 cells stained with total and phospho STAT1. 93% of the cells are positive for total STAT3 (based on Isotype control from single parameter data above) and negative for phospho-STAT3. Following stimulation with 100 ng/mL of EGF plot **(B)** shows 92.39% of the cells are positive for phospho-STAT3 while their expression levels for total STAT3 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	<ul style="list-style-type: none">• If storing at -20°C, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37°C 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	<p>This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following:</p> <ul style="list-style-type: none">• 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 stream takes place.
Cellular Analysis	A loss or lack of signal	<ul style="list-style-type: none">• 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.

Assay Step	Potential Problem	Experimental Suggestions
Cellular Analysis	Background and/or non-specific staining of cells	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> 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 <i>Analytical Sensitivity and Detection Limits Section for Guava Check standards</i>)

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

References

1. Ohkmae K. Park, Timothy S. Shaefer, and Daniel Nathans. *In vitro* activation of Stat3 by epidermal growth factor receptor kinase. *Proc. Natl. Acad. Sci. USA.* 93: 13704-13708 (1996).
2. Shirish Shukla, Gauri Shishodia, Sutapa Mahata, Suresh Hedau, Arvind Pandey, Suresh Bhambhani, Swaraj Batra, Seemi F Basir, Bhudev C Das and Alok C Bharti. Aberrant expression and constitutive activation of STAT3 in cervical carcinogenesis: implications in high-risk human papillomavirus infection. *Mol. Cancer.* 9: 282 (2010).
3. Mona Anand, Raymond Lai, and Pascal Gelebart. Beta-catenin is constitutively active increases STAT3 expression/activation in ALK-positive anaplastic large cell lymphoma. *Haematologica.* Epub ahead of print; (Oct 22 2010).

Related Products

1. FlowCollect™ Bivariate Cell Cycle Kit for DNA Replication Analysis (Catalog No. FCCH025102)
2. FlowCollect™ Bivariate Cell Cycle Kit for G2/M Analysis (Catalog No. FCCH025103)
3. FlowCollect™ Multi-Color DNA Damage Response Kit (Catalog No. FCCH025104)
4. FlowCollect™ DNA Damage (ATM) and Cell Cycle Analysis Kit (Catalog No. FCCH025143)
5. FlowCollect™ p38 Stress Pathway Activation Detection Kit (Catalog No. FCCS025132)
6. FlowCollect™ EGFR/MAPK Pathway Activation Kit (Catalog No. FCCS025101)
7. FlowCollect™ EGFR/STAT3 Pathway Activation Kit (Catalog No. FCCH025111)
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9. FlowCollect™ Multi-STAT Activation Profiling Kit (Catalog No. FCCS025550)
10. FlowCollect™ PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection kit (Catalog No. FCCS025100)
11. FlowCollect™ PI3K Activation Dual Detection Kit (Catalog No. FCCS025105)
12. FlowCollect™ MAPK Activation Dual Detection Kit (Catalog No. FCCS025106)
13. FlowCollect™ EGFR RTK Activation Dual Detection Kit (Catalog No. FCCS025107)
14. FlowCollect™ PLC- γ 1 Activation Dual Detection Kit (Catalog No. FCCS025145)
15. FlowCollect™ STAT1 Activation Dual Detection Kit (Catalog No. FCCS025142)

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