FlowCellect™ Multi-STAT Activation Profiling Kit

25 Tests

Cat. No. FCCS025550

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Not for use in diagnostic procedures.

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Application

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. STATs are involved in embryogenesis, cellular immunity, and cell proliferation (1).

Many types of cancers both solid tumor and some blood based malignancies have been found to exhibit constitutive STAT activation (2). Interestingly, the activation within these cancer cells is generally a combination of STATs, typically STAT1, STAT3, and STAT5. This has led to an increase in research revolving around the complex family of STATs.

Oncogenesis and tumour 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.

Millipore's FlowCellect STAT Activation kit includes three directly conjugated phospho specific antibodies: pSTAT1(Tyr701)-PerCP, pSTAT3(Tyr705)-Alexa 488, and pSTAT5A/B(Tyr694/Tyr699)-PE along with optimized fixation, permeabilization, wash and flow buffers to provide researchers with a complete solution for simultaneously detecting the activation of three of the most studied STAT proteins in their preferred cellular system.

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Test Principle

Millipore's FlowCellect™ Multi-STAT Activation Kit is designed to simultaneously detect the phosphorylation of the most commonly studied STAT proteins through cytokine or growth factor mediated pathway activation. The kit will also allow a researcher to quickly profile the status of constitutive activation of STAT1, STAT3, and STAT5 within a population of cells or primary tumors.

The ability to detect the activation of multiple STAT proteins simultaneously allows researchers to determine and profile the STAT signaling pathways within a cell type of interest Alterations in these signaling profiles can be used to monitor the effect of a particular upstream treatment targeting the STAT pathways.

Antibodies against phospho-STAT1, 3, and 5A/B are provided in the kit to monitor the activation state of each STAT within a given cell type. U937 cells were stimulated with Gamma Interferon, IL-6 and GM-CSF simultaneously, to activate STAT1, STAT3, and STAT5A/B, respectively. When performing a three color stain, triple positive cell populations were detected, as shown in the sample data (figure 6).

Sufficient reagents are provided for 25 3-color samples. The kit includes all optimized fluorescently labeled antibodies and buffers necessary for cell preparation and analysis. Detailed assay instructions are included to assist in analysis and to ensure the correct cell concentration is obtained during acquisition of sample data.

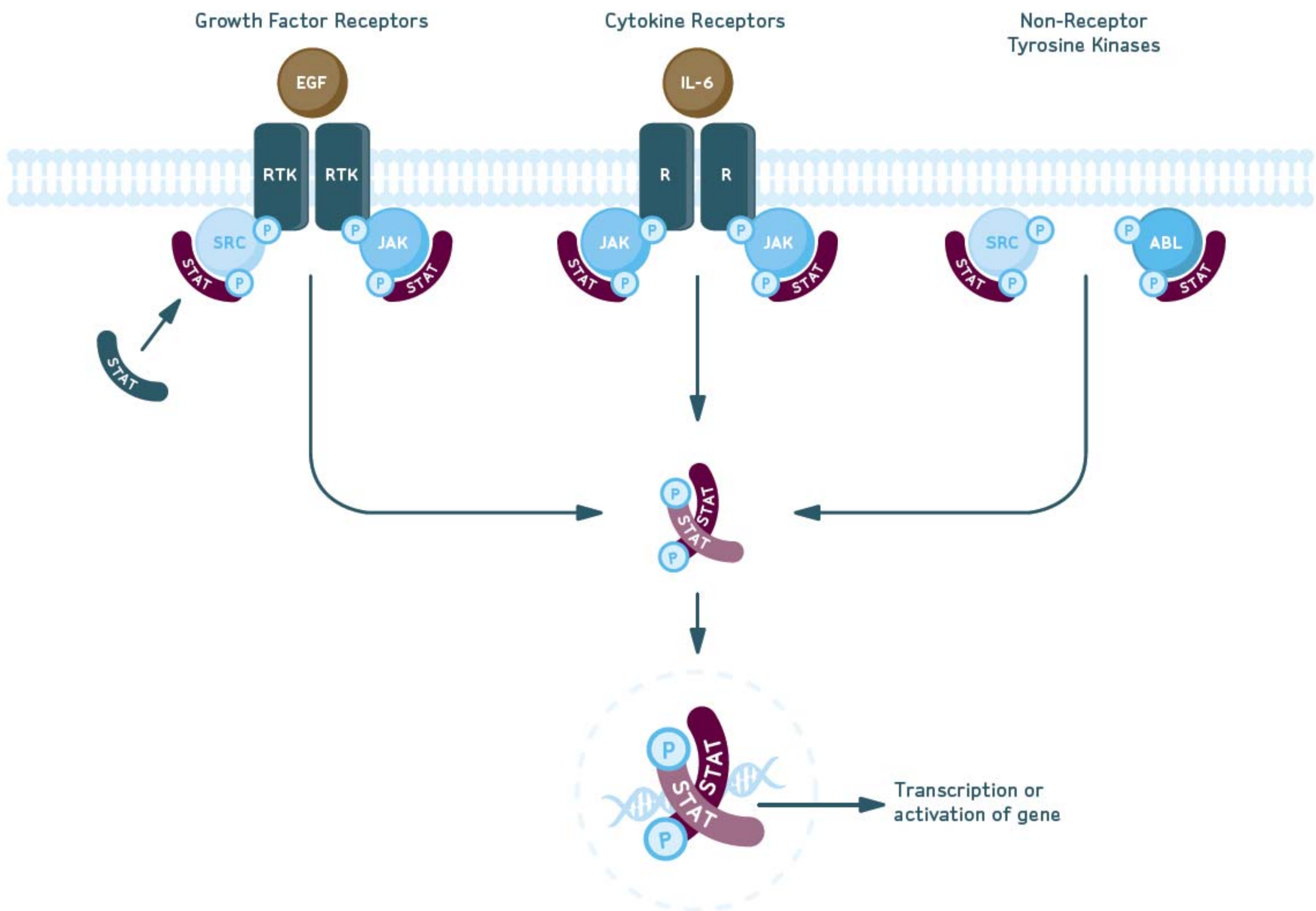

Figure 1. STAT signaling Pathway. The STAT pathway can be activated in numerous ways. Most commonly they are activated by JAK through growth factor and cytokine receptors; however, activation may also occur by non-receptor Tyrosine Kinases such as SRC and ABL. STAT proteins are then phosphorylated, followed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

Kit Components

1. 10X Wash Buffer: (Part No. CS202123) 1 bottle containing 13 mL buffer.
2. Fixation Buffer: (Part No. CS202122) 1 bottle containing 13 mL buffer.
3. 5X Assay Buffer: (Part No. CS202124) 1 bottle containing 55 mL buffer.
4. 1X Permeabilization Buffer: (Part No. CS203284) 2 bottles, each containing 14 mL.
5. 20X pSTAT1 (Y701)-PerCP Monoclonal Antibody: (Part No. CS203319) 1 vial containing 150 μL antibody.
6. 20X pSTAT3 (Y705)-Alexa 488 Monoclonal Antibody: (Part No. CS203316) 1 vial containing 150 μL antibody.
7. 20X pSTAT5A/5B (Y694/Y699)-PE Monoclonal Antibody: (Part No. CS203313) 1 vial containing 150 μL antibody.

Materials Not Supplied

1. Guava EasyCyte™ Plus Flow Cytometer
2. ViaCount™ reagent (Catalog No. 4000-0041)
3. Cell line of interest or U937 cells for example protocol
4. Media for cell line of interest or RPMI + 10% FCS and Pen/Strep for example protocol
5. Tissue culture instruments and supplies (including 37°C incubator, growth media, plates, detachment buffer, etc.)
6. Test tubes for sample and buffer preparation and storage.
7. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 μL
8. Tabletop centrifuge capable of exceeding 2500 rpm.
9. Milli-Q™ Distilled Water or di water.
10. Cell Line stimulants: γ-IFN, GM-CSF, and IL-6

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 formaldehyde solution (fixation solution). Please refer to the MSDS sheet for specific information on hazardous materials.
- All fluorochrome conjugated antibodies are light sensitive and must be stored in the dark at 4°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.

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Storage

Upon receipt, all antibodies and buffers (except for Fixation Buffer) should be stored at 4°C. Fixation Buffer should be stored at room temperature. ***Caution:*** *Fluorochrome conjugated antibodies should always be stored at 4°C. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.*

Preparation of Buffers

Buffer Preparation for 10 samples (Scale up or down as necessary).

1. Make 10 mL of 1X Wash Buffer: Add 1 mL of 10X Wash Buffer and 9 mL of di water.
2. Make 6 mL of 1X Fixation buffer: Add 600 μL of 10X Wash Buffer, 1.125 mL of Fixation buffer, and 4.275 mL of di water.

3. Make 8.0 mL of 1X Assay Buffer. Add 1.6 mL of 5X Assay Buffer and 6.4 mL of di water.

4. Place Permeabilization Buffer on ice. (Permeabilization buffer must be ice cold for optimal performance).

Note: *Prepared 1X Fixation Buffer is stable up to one month if stored at 4ºC, therefore should only be made before starting assay. Prepared 1X Wash and Assay Buffers are stable up to four months if stored at 4ºC, and can be prepared when you receive the kit or when you start the first assay.*

Example Cell Staining Protocol:

This protocol is for U937 cells which are grown in RPMI + 10% Fetal Calf Serum and Pen/Strep. The cells are a suspension cell line which should not exceed 1.0 X 10^6 cells per mL of media at any time. As cells become too dense they may decrease receptor expression on the cell surface which can adversely affect experimental results. The viability of the cells should be above 90-95% for optimal results.

1. U937 cells are counted, pelleted at 2500 rpm for 3 minutes and $5X10^6$ cells are resuspended in 5 mL of Media.

2. 2.5 mL of the resuspended cells are added to a T-25 flask labeled Untreated.

3. 2.5 mL of the resuspended cells are added to a T-25 flask labeled **Treated**.

4. 125 ng of γ-IFN, 25 ng GM-CSF, and 50 ng IL-6 is added to the treated flask and both flasks are incubated at 37 degrees for 10 minutes.

5. Each sample is then pelleted in a 15 mL conical tube at 2500 rpm for 3 minutes and the supernatant is removed.

6. Resuspend the cells completely in 2.5 mL of 1X Wash Buffer by gently pipetting up and down several times followed by pelleting at 2500 rpm for 3 minutes and removal of the supernatant.

7. Resuspend the cells completely in 2.5 mL of 1X Fixation Buffer by gently pipetting up and down several times.

8. Incubate at room temperature for 10 minutes.

9. Pellet the samples at 2500 rpm for 3 minutes. Remove the supernatant and resuspend in 1.25mL of ice cold Permeabilization Buffer and mix (vortexing at high speed for 20 seconds) to permeabilize the cells.

10. Incubate in the Permeabilization Buffer on ice for at least 10 minutes. (Cells can be stored at -20°C for several weeks at this point with minimal loss of signal).

11. Aliquot 250 μL (contains 500,000 cells in Permeabilization Buffer) of each cell type to be tested into individual microfuge tubes. Keep any unused cells at -20°C for future or repeat testing.

12. Example

    Tube 1 = Untreated cells stained with pSTAT1-PerCP

    Tube 2 = Treated cells stained with pSTAT1-PerCP

Tube 3 = Untreated cells stained with pSTAT3-Alexa 488

Tube 4 = Treated cells stained with pSTAT3-Alexa 488

Tube 5 = Untreated cells stained with pSTAT5-PE

Tube 6 = Treated cells stained with pSTAT5-PE

Tube 7 = Untreated cells stained with all three antibodies

Tube 8 = Treated cells stained with all three antibodies

13. Pellet the cells for each sample at 2500 rpm for 3 minutes and remove the supernatant.
14. Wash the cells 2X with 500 μL of Assay Buffer followed by pelleting at 2500 rpm for 3 minutes and removal of the supernatant.
15. Fully resuspend the pellets of all samples in 95 μL of Assay buffer by pipetting up and down.
16. Add 5 μL of 20X STAT5-PE antibody to Tubes 5, 6, 7 and 8. (Should be 100 μL total staining volume per tube). Set tubes 1, 2, 3, and 4 aside in the dark until step 20.
17. Incubate in the dark at room temp for 60 minutes.
18. Add 900 μL of Assay Buffer to each tube then pellet the cells for each sample at 2500 rpm for 3 minutes. Remove the supernatant.
19. Add 500 μL of Assay Buffer to Tubes 5 and 6 and keep in the dark until step 25.
20. Fully resuspend the pellets for tubes 7 and 8 in 90 μL of Assay buffer by pipetting up and down.
21. Add 5 μL of 20X STAT1-PerCP antibody to Tubes 1, 2, 7 and 8. Add 5μL of 20X STAT3-Alexa 488 antibody to tubes 3, 4, 7, and 8 (all tubes should be 100 μL total staining volume).
22. Incubate in the dark at room temp for 30 minutes.
23. Add 900 μL of Assay Buffer to each tube then pellet the cells at 2500 rpm for 3 minutes. Remove the supernatant and resuspend in 500 μL of Assay Buffer.
24. Set up the following in sample tubes to analyze on the Guava Flow Cytometer:

    Sample 1 = 250 μL of Tube 1 and 250 μL of Tube 2.

    Sample 2 = 250 μL of Tube 3 and 250 μL of Tube 4.

    Sample 3 = 250 μL of Tube 5 and 250 μL of Tube 6.

    Sample 4 = 500 μL of Tube 7

    Sample 5 = 500 μL of Tube 8

    Note: Samples 1, 2, and 3 are mixtures of treated and untreated cells which 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, 2, and 3.

Data Acquisition

Software Setup:

- > **Select the Express Pro module**
- > **Click Show Acquisition Buttons**
- > **Open the Worklist Editor**
- > **Select Create Worklist**
- > **Setup and save your worklist**
- > **Click Start This Worklist**
- > **Load samples into the sample tray as outlined in your worklist.**
- > **Choose a location to save the data file and make note of its location.**
- > **Click Adjust Settings**
- > **Setup plots and gating to suit your needs (Sample data below shows examples).**

Adjusting Settings

1. To adjust instrument settings start with sample 4 (Untreated three color stain) to set up your live gate and adjust voltages.
2. Adjust forward and side scatter parameters: Once cells are acquiring, increase or decrease forward and side scatter PMTs until your cells of interest are within the scale of the plot. Adjust **threshold gate** to exclude **debris** form being collected. Move and adjust the size of the elliptical gate to encompass the **live cell** population.

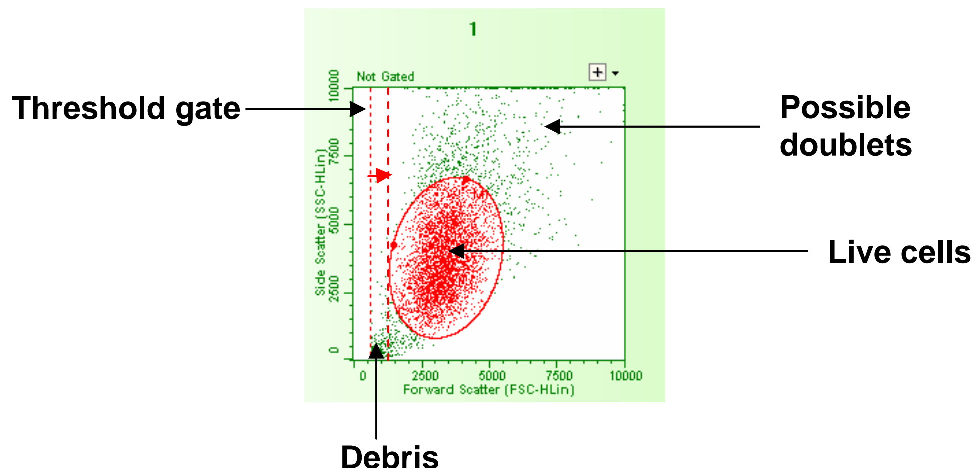


Figure 1. Example of forward and side scatter plot showing gating of the population of interest.

3. Adjust the red fluorescence parameter: Adjust the red voltage until most of the cells fall in the lower left hand Quadrant on the dot plots. Use histogram plots as a guide to determine the number of cells falling on the axis. Increase PMTs until the cells collecting at the axis are at a minimum. Repeat for the Green and Yellow voltages as well. See **Fig. 2** for example plots.

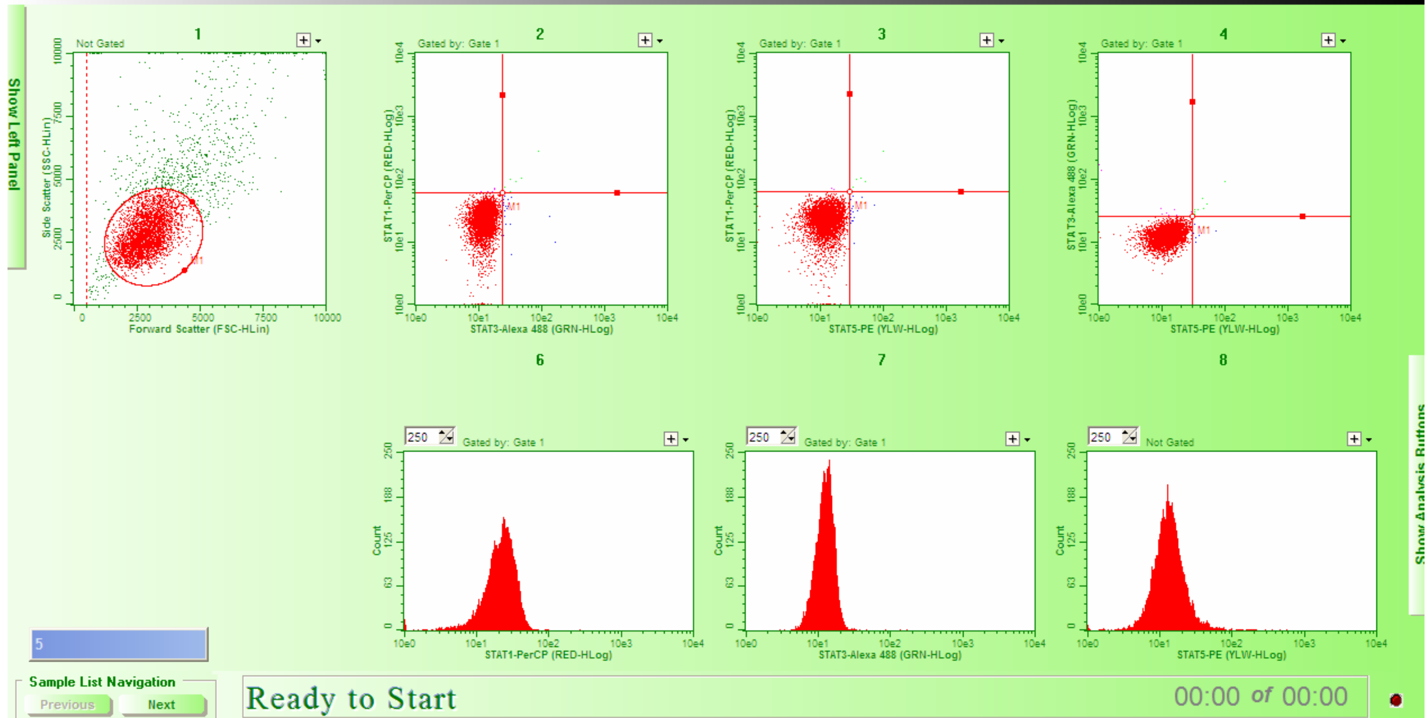
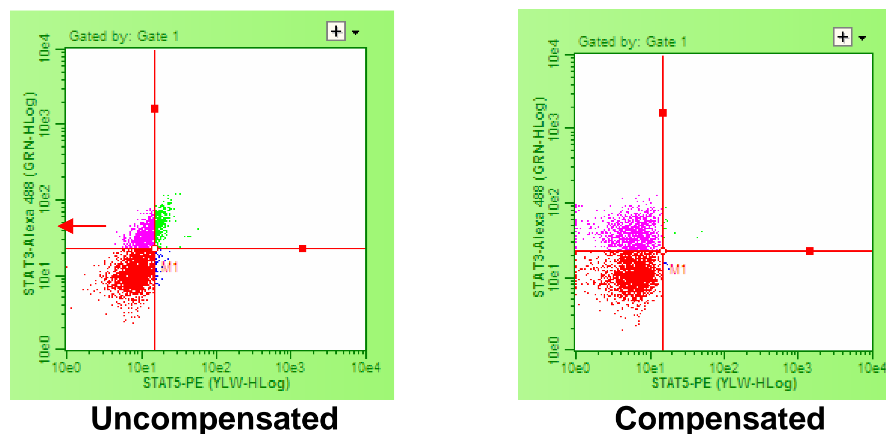


Figure 2. Three color stain of untreated U937 cells showing proper gating and voltage settings.

4. Select **Next Step**.
5. Adjust compensation for the green fluorescence parameter. Press **Settings** and then **Adjust Settings.**
6. Select Sample 2.
7. Once cells are acquiring, click on compensation window 1 and adjust %YLW-GRN. Increase until The population of untreated cells is perpendicular to the population of treated cells. See below. Do not allow too many cells to collect on the axis. There should still be a visible space between the axis and the cells.



Uncompensated **Compensated**

Figure 3. Example of uncompensated data versus properly compensated data. Compensation was achieved by removing yellow from the green channel.

8. Select **Next Step**.

9. Adjust compensation for yellow fluorescence parameter: Press **Settings** and then **Adjust Settings**. Select Sample 3. Repeat Step 7 but this time adjust the %RED-YLW to compensate for bleed over into the Red channel.

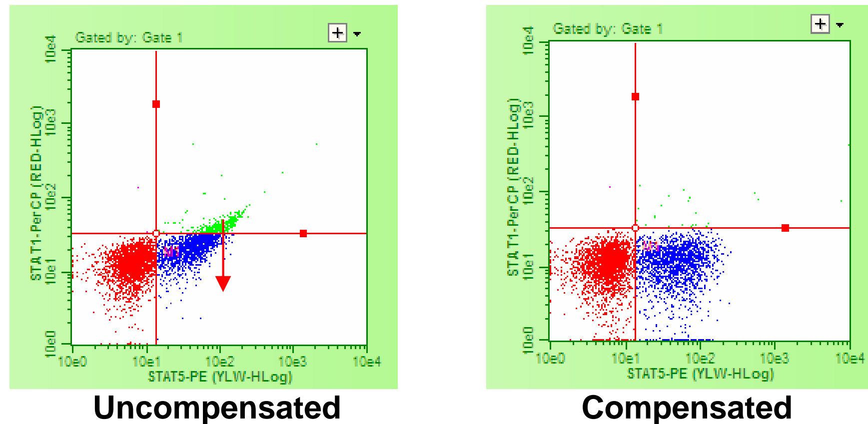


Figure 4. Example of uncompensated versus properly compensated data. Compensation was achieved by removing red from the yellow channel.

10. Select **Next Step**.
11. Adjust compensation for red fluorescence parameter. Press **Settings** and then **Adjust Settings.**
12. Select Tube 1.
13. Repeat Step 7 but this time adjust the %YLW-RED to compensate for bleed over into the Yellow channel.
14. Once cells are acquiring, click on compensation window 1 and adjust %YLW-RED. Increase until cells are no longer present in yellow fluorescence parameter. See below. Do not allow too many cells to collect on the axis. There should still be a visible space between the axis and the cells.

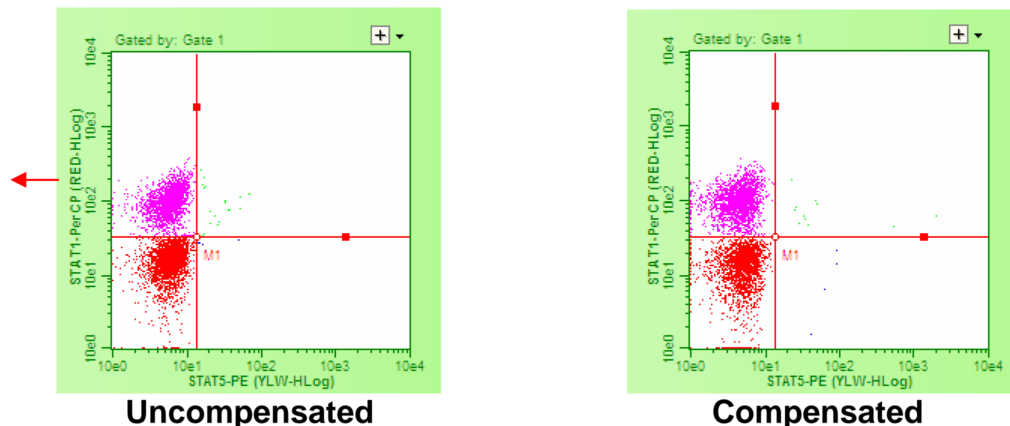


Figure 5. Example of uncompensated versus properly compensated data. Compensation was achieved by removing yellow from the red channel.

15. Fine tune compensation for all parameters. Press **Settings** and then **Adjust Settings.**
16. Select tube with cells stained with all three antibodies.
17. Once cells are acquiring, click on compensation window 1 and adjust compensation, so that you get the desired result, i.e. something similar to the data presented in Figure 6 of this manual.
18. Select **Next Step**.

19. Save settings. Press **Settings** and then **Save Settings.** Name the new settings file and note its location on your computer for future use.

    Note: If using the same cells and same conditions you can use the same settings for future experiments with very little adjustments needed.

20. Click **Resume** to acquire data.

Sample Data

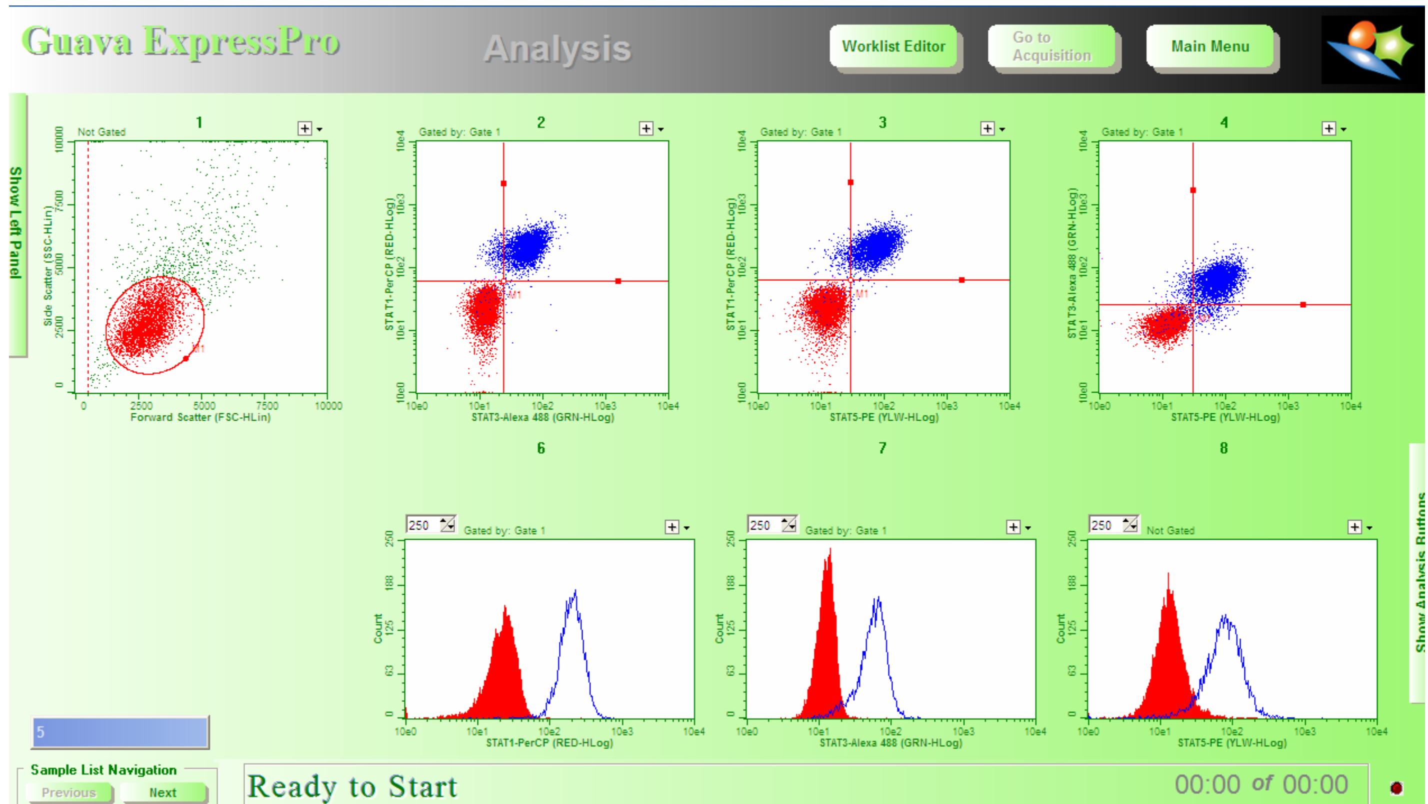


Figure 6. Analyzed Example Protocol Data: Untreated (red) and Gamma Interferon, IL-6, and GM-CSF treated samples (blue) are shown overlaid for comparison purposes. The dot plots 2, 3, and 4 show double positive activation of each of the STAT markers (blue dots) as compared to the untreated (red dots). The histogram plots 6, 7, and 8 show the individual activation of each of the stats (Blue histogram) as compared to the untreated sample (red histogram).

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

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. The Guava EasyCyte™ Plus gives the most accurate data when the flow rate is less 500 cells per microliter. • Run three Quick Cleans 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	• 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.
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. • 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.
Staining is weak	• Some cell lines may require higher concentrations of fixation buffer to stain correctly. Use the Fixation Buffer at 2X rather than 4X.
Staining is weak	• Some cell lines may require higher concentrations of conjugated antibodies. Use the antibodies at 10X rather than 20X.
Staining is too bright	• Some cell lines may require lower concentrations of conjugated antibodies. Use the antibodies at 40X rather than 20X.

******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. Ralf Buettner, Linda B. Mora, and Richard Jove. Activated STAT Signaling in Human Tumors Provides Novel Molecular Targets for Therapeutic Intervention. Clinical Cancer Research Vol. 8, 945–954, April 2002

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.

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