FlowCellect™ Mouse ESC Nuclear Marker Characterization Kit

25 Tests

Cat. No. FCMEC25110

(FCMEC110)

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Not for use in diagnostic procedures.

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Application

Mouse embryonic stem cells (mESCs) are unique cells. They can be maintained *in vitro* as pluripotent cells and can be induced to differentiate into many cell types by manipulating culture conditions. mESCs have been used as a model to help define differentiation and signaling pathways in human embryonic stem cells (hESCs), since hESCs are more arduous to work on. Therefore, much attention has been focused on understanding the mechanisms behind mESC differentiation.

Currently, the most utilized method to test the differentiation state of mESCs is to analyze the expression of the proteins OCT4 and the stage specific embryonic antigen, SSEA1 (1,2). The transcription factor, OCT4, is highly expressed in undifferentiated mESCs and is essential for the formation and maintenance of pluripotentcy (3,4). SSEA1 is also expressed on undiiferentiated mESCs but another stage specific embryonic antigen, SSEA4, is not (2,5,6). However, upon retinoic treatment, SSEA1 expression wanes and in some instances, accompanied by expression of SSEA4 (5,6).

The FlowCellect™ Mouse Embryonic Stem Cell Characterization Kit uses an antibody-based detection method, utilizing fluorescently conjugated antibodies to detect OCT4, SSEA4 and SSEA1. The FlowCellect™ Mouse Embryonic Stem Cell Characterization Kit is designed specifically for flow cytometers with a single blue (488 nm) laser light source and is not intended for use in microscopy applications including ICC, IHC and HTS, western blot or any other fluorometric analysis platforms. The kit does not include culture media or other materials that should be readily available in a common laboratory setting that routinely performs experiments of the type described here.

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Test Principle

Millipore's The FlowCellect™ Mouse Embryonic Stem Cell Characterization Kit is designed as an easier and quicker way to track surface marker expression of mOCT4, SSEA4 and SSEA1. The kit will also help to determine the percentage of undifferentiated mouse embryonic stem cells in culture by determining the percentage of cells that express both OCT4 and SSEA1 and not SSEA4. This quick test will allow researchers to determine the quality of their cells in culture as well as see changes in marker expression during a differentiation protocol.

Sufficient reagents are provided for 25 3-color samples and 5 isotype control tests. The kit includes all optimized fluorescently labeled antibodies and buffers necessary for analysis. A cell strainer is also included to ensure samples are devoid of large cell clumps that can alter cell analysis. Detailed assay instructions are also included to assist in analysis and to ensure the correct cell concentration is obtained for flow cytometric analysis.

Kit Components

BOX 1 (FCMEC110)

1. 10X Wash Buffer: (Part No. CS202123) 1 bottle containing 13 mL
2. Fixation Buffer: (Part No. CS202122) 1 bottle containing 13 mL
3. 5X Assay Buffer: (Part No. CS202124) 1 bottle containing 55 mL
4. 1X Permeabilization Buffer: (Part No. CS202125) 1 bottle containing 13 mL
5. 20X rat IgG2a-Alexa 488 isotype control: (Part No. CS202404) 1 vial containing 75 μL
6. 20X IgG3-PE isotype control: (Part No. CS202405) 1 vial containing 75 μL
7. 20X IgM-PE/CY5 isotype control: (Part No. CS202397) 1 vial containing 75 μL
8. 20X mOCT4-Alexa 488: (Part No. CS202394) 1 vial containing 150 μL
9. 20X SSEA4-PE: (Part No. CS202399) 1 vial containing 150 μL
10. 20X SSEA1-PE/CY5: (Part No. CS202400) 1 vial containing 150 μL

BOX 2

11. Steri-flip cell strainer (60 mm): (Catalog # SCNY00060)

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Materials Not Supplied

1. Guava EasyCyte™ Plus Flow cytometer
2. ViaCount reagent (Catalog No. 4000-0041)
3. Mouse embryonic stem cells (Catalog No. CMTI-2)
4. Tissue culture instruments and supplies (including 37°C incubator, growth media, plates, detachment buffer, etc.)
5. Test tubes for sample and buffer preparation and storage.
6. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 μL
7. Tabletop centrifuge capable of exceeding 1100 rpm
8. Vacuum pump

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 contains 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 6 months after date of receipt.

Storage

Upon receipt, all antibodies and buffers (except for Fixation buffer) should be stored at 4°C. Fixation Buffer and Steriflips 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 antibodies performance.* Discard any remaining reagents after 6 months.

Preparation of Buffers

Buffer	Volume of 10X Wash Buffer	Volume of DI water
1X Wash Buffer	11.5 mL	103.5 mL

Buffer	Volume of 5X Assay Buffer	Volume of DI water
1X Assay Buffer	50 mL	200 mL

Buffer(For 10 tests)	Volume of 10X Wash Buffer	Volume of Fixation Buffer	Volume of DI water
1X Fixation Buffer	0.5 mL	1.25 mL	3.25 mL

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

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Cell Preparation

1. Culture ES cells as desired.
2. Aspirate media and wash with 3 mL of 1XPBS
3. Add 1 mL of warm Accumax (SCR006) and incubate 5 minutes at 37ºC.
4. Add 3 mL media and pipette up and down to dislodge all cells. Transfer to 15 mL conical tube.
5. Wash wells with 3 mL of 1XPBS and transfer to same tube.
6. Spin cells for 5 minutes at 800 RPM.
7. Resuspend cells with media. ***Note:*** *Keep cells on ice for no longer than 20 minutes before starting assay.*

Assay Instructions

Note: This protocol has been optimized for CMTI-2 mouse embryonic stems cells on a Guava EasyCyte™ Plus cytometer.

1. Prepare needed volume of Fixation Buffer: See preparation of buffers. ***Note:*** *During all steps in the assay procedure, keep Wash, Fixation and Assay Buffer on ice. Allow Permeabilization Buffer to adjust to room temperature before use.*
2. Count cells using ViaCount assay (Catalog No. 4000-0041). ***Note:*** *You will need at least 2.5 million cells to prepare controls for instrument settings adjustment.*
3. Aliquot a maximum of 5 million cells per cell line/sample to be tested. ***Note****: If you have less than 1 million cells, aliquot 500,000 cells into a 1.5 mL tube, spin down cells at 2000 rpm (400Xg) for 3 minutes and resuspend cells in 0.5 mL of wash buffer. Then skip to step 9.*
4. Spin down cells at 2000 rpm (400Xg) for 3 minutes. Discard media.

5. Completely resuspend cells in 5 mL of Wash Buffer by gently pipetting up and down.
6. Filter cells with cell strainer. Pipet cells into the center of the filter and allow the cells to pass through using gravity. Rinse top reservoir with 1 mL of Wash Buffer. A vacuum pump may be used to obtain any volume left over in the filter reservoir. ***Note:*** *Do not leave cells in Wash Buffer for longer than 20 minutes.*
7. Recount cells using ViaCount assay.
8. Aliquot 500,000 cells into a 1.5 mL tube.
9. Immediately spin cells down at 2000 rpm (400Xg) for 3 minutes. Pipet buffer out of tube and discard, leaving cell pellet intact.
10. Completely resuspend cells by gently pipetting up and down in 0.5 mL of 1X Fixation Buffer and incubate for 20 minutes at room temperature.
11. Spin cells down at 2500 rpm (600Xg) for 3 minutes. Cells will appear glassy and difficult to see after fixation, so carefully pipet out Fixation Buffer, making sure to keep cell pellet intact.
12. Add 100 µL of 1X Permeabilization Buffer to each tube and then gently resuspend cells by pipetting up and down. Incubate at room temperature for 3 minutes
13. Spin cells down cells at 2500 rpm (600Xg) for 3 minutes. Pipet buffer out of tube and discard, leaving cell pellet intact.
14. Add 0.5 mL of 1X Assay Buffer to the cells. Do not resuspend.
15. Spin cells down cells at 2500 rpm (600Xg) for 3 minutes. Carefully pipet buffer out of tube and discard, leaving cell pellet intact.
16. Completely resuspend cells by gently pipetting up and down in 100 µL of 1X Assay Buffer. Set up tubes as follows:
    1) 5 µL of rIgG2a-Alexa 488, 5 µL of IgG3-PE and 5 µL of IgM-PE/CY5
    2) 5 µL of mOCT4-Alexa 488, 5 µL of IgG3-PE and 5 µL of IgM-PE/CY5
    3) 5 µL of SSEA4-PE, 5 µL rIgG2a-Alexa 488 and 5 µL of IgM-PE/CY5
    4) 5 µL of SSEA1-PE/CY5, 5 µL of rIgG2a-Alexa 488, and 5 µL of IgG3-PE
    5) Test 1
    6) Test 2
    7) Test 3 } 5 µL of mOCT4-Alexa 488, 5 µL of SSEA4-PE and 5 µL of SSEA1-PE/CY5
    8) Test 4
    9) Test 5
17. Incubate samples on ice for one hour.
18. Rinse cells by adding 0.9 mL of 1X Assay Buffer to each tube.
19. Spin cells down at 2500 rpm (600Xg) for 3 minutes. Pipet buffer out of tube and discard, leaving cell pellet intact.
20. Resuspend cells by gently pipetting up and down in 0.5 mL of 1X Assay Buffer.
21. Transfer cells to an analysis sample tube.
22. Analyze 200µL (96-well format) or 500 µL (single tube format) on Guava EasyCyte™ Plus flow cytometer.

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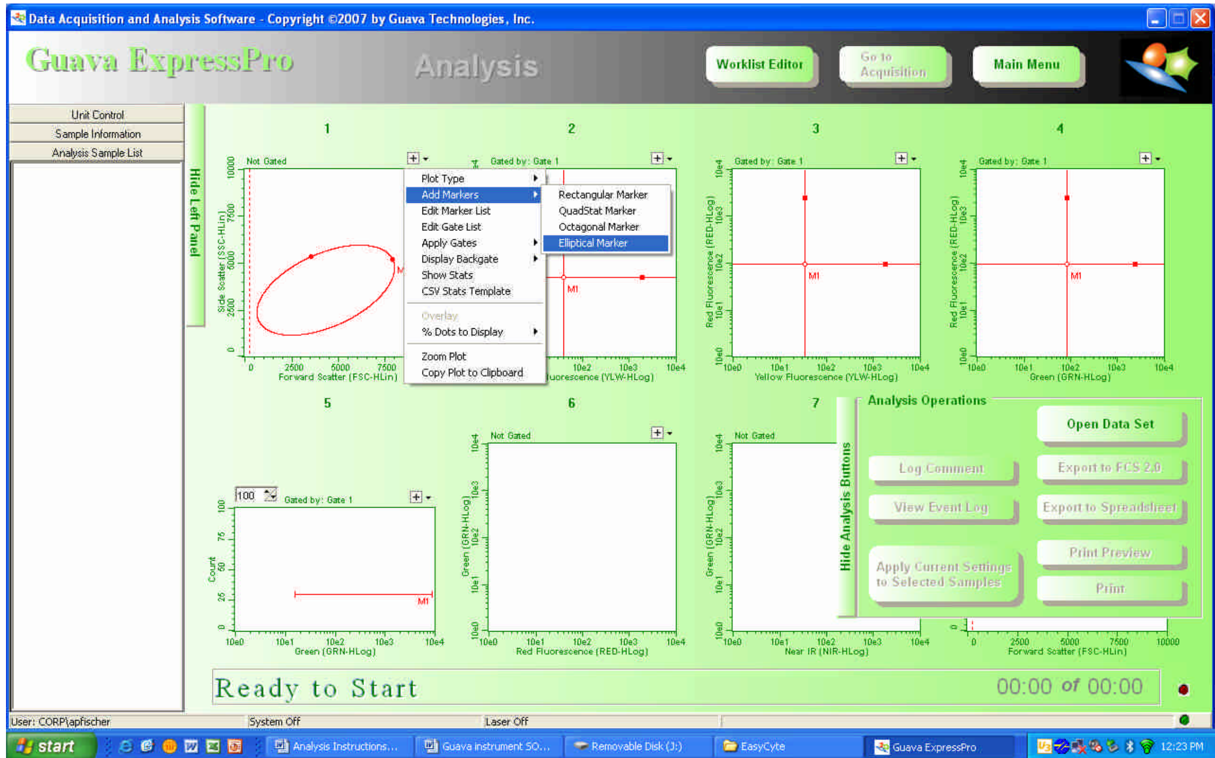
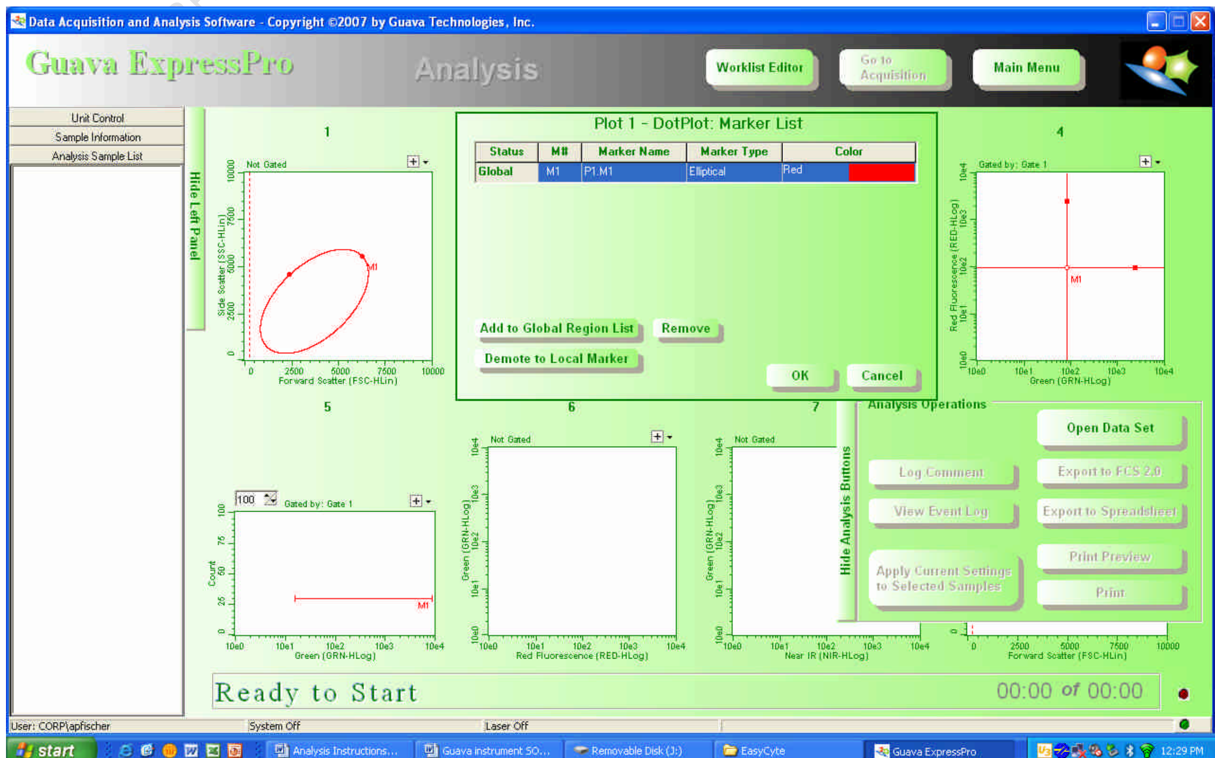
Acquiring data

Setting up plots

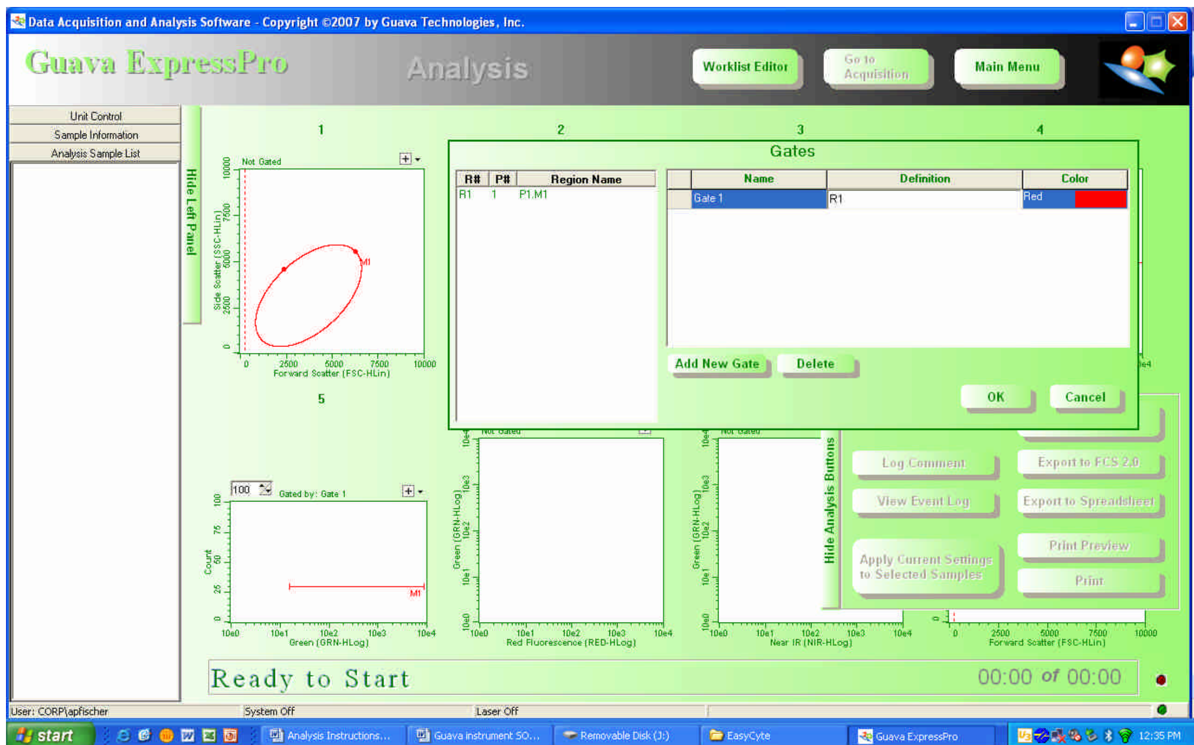
1. Place an elliptical gate in plot 1 (SSC by FSC plot) by clicking on the plus sign (+) in the upper-right corner above the plot or right-click the plot to open the plot menu. Choose **Add Markers** and then choose **Elliptical Marker**.



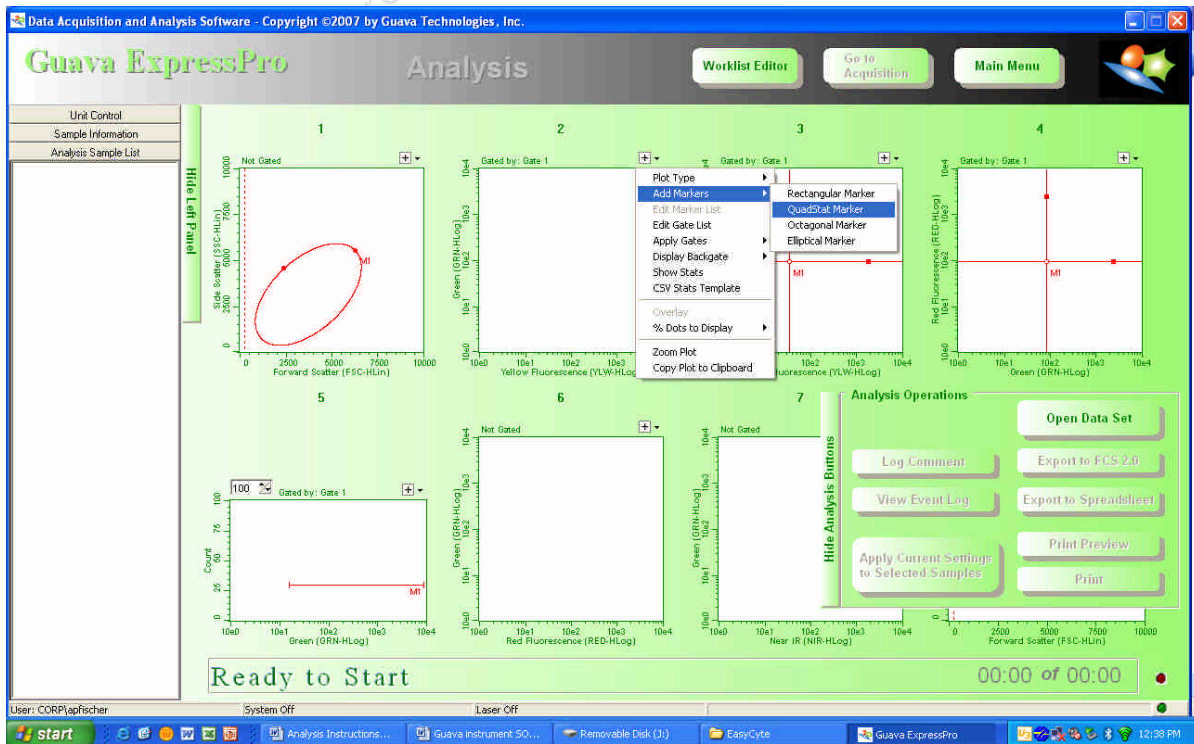
2. Select elliptical marker to global list. Go to plot menu and select **Edit Marker List.** Choose **Add to Global Region** and then press **OK.**



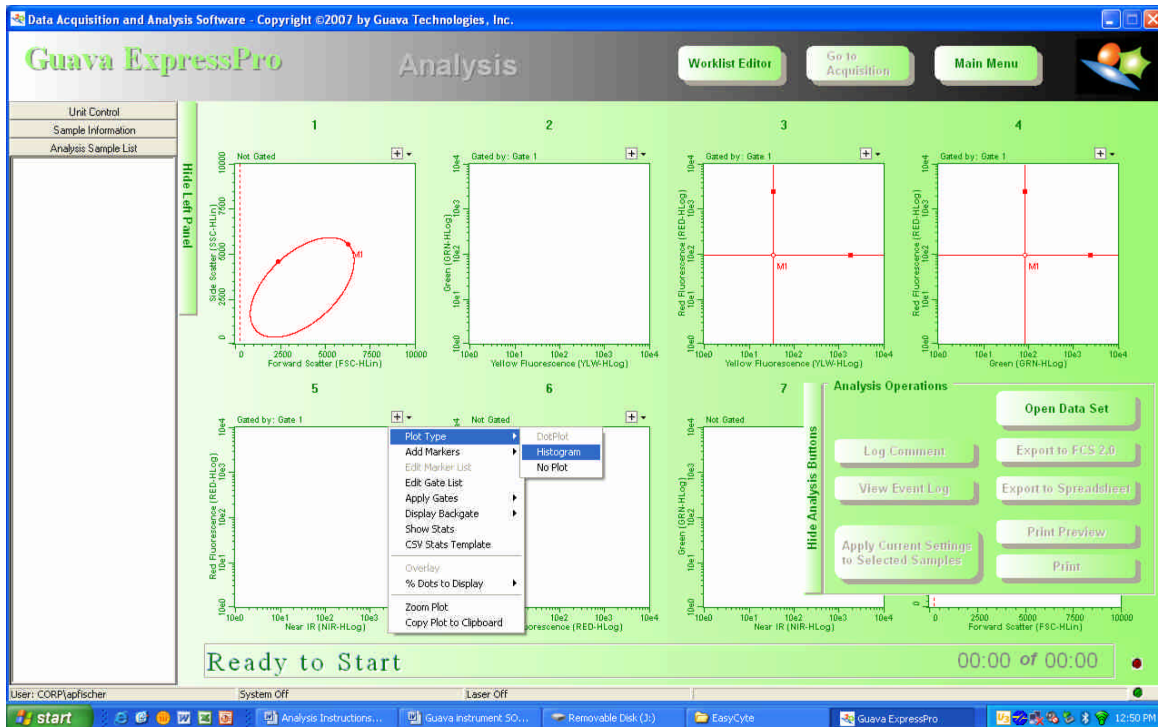
3. Define marker. Go to plot menu and select **Edit Gate List.** Type R1 in to the Definition cell then press **OK.**



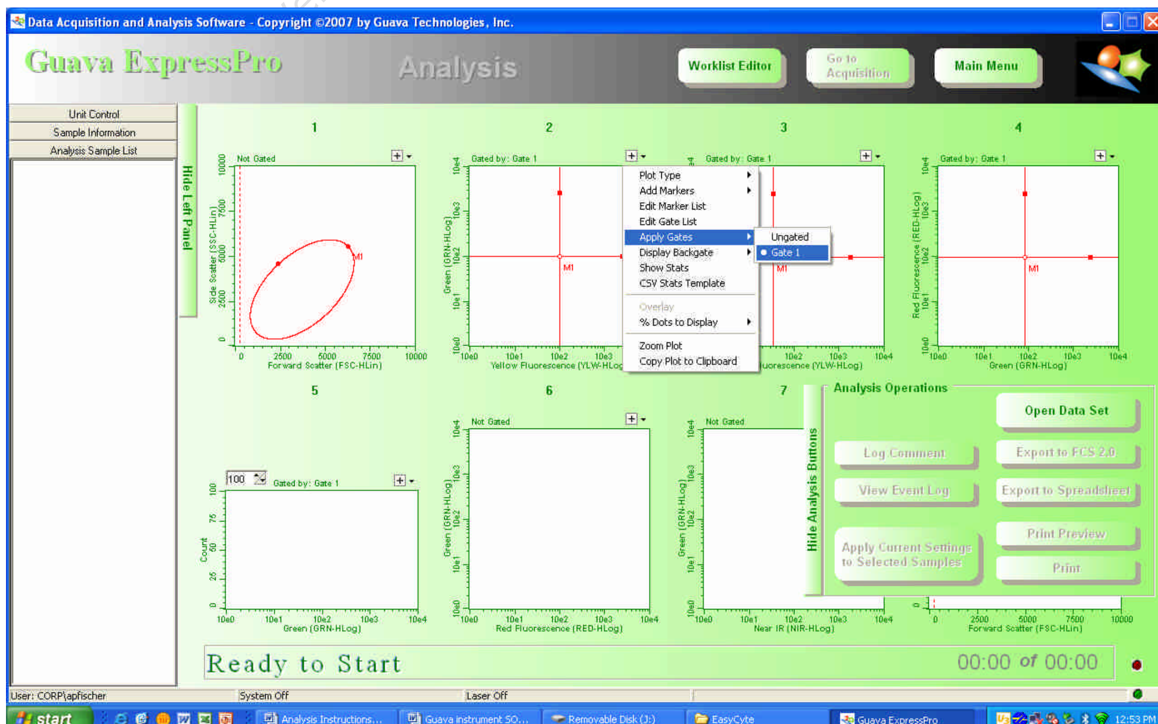
4. Add quadrant gates to plots 2, 3 and 4. Go to plot menu and select **Add Markers** and then select **QuadStat Marker**.



5. Change plot 5 to a histogram plot. Go to plot menu and select **Plot Type** and then select **Histogram**. Repeat for plots 6 and 7. Choose Green Fluorescence for plot 5, Yellow Fluorescence for plot 6 and Red Fluorescence for plot 7.

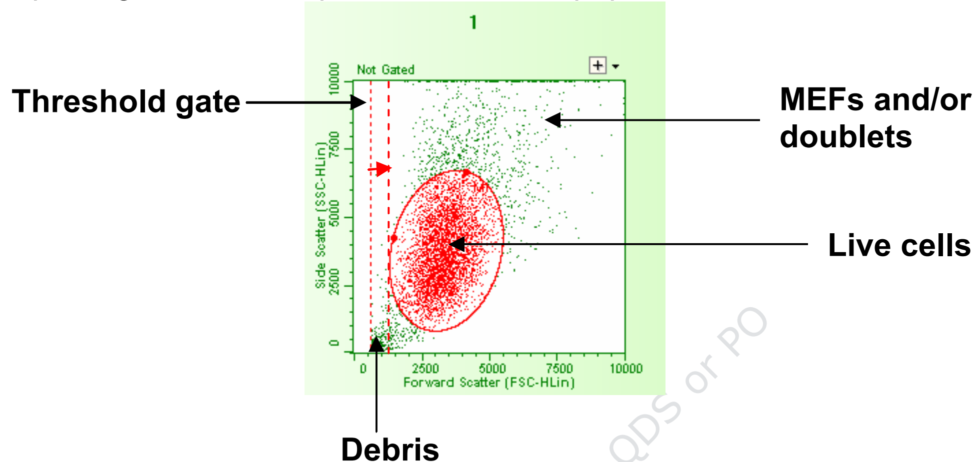


6. Select Gate 1 for plots 2-5. Go to plot menu and select **Apply Gates** and then select **Gate 1**. By selecting gate 1 to plots 2-7, they will now only collect data from cells falling within the elliptical gate (live cell gate) in plot 1.

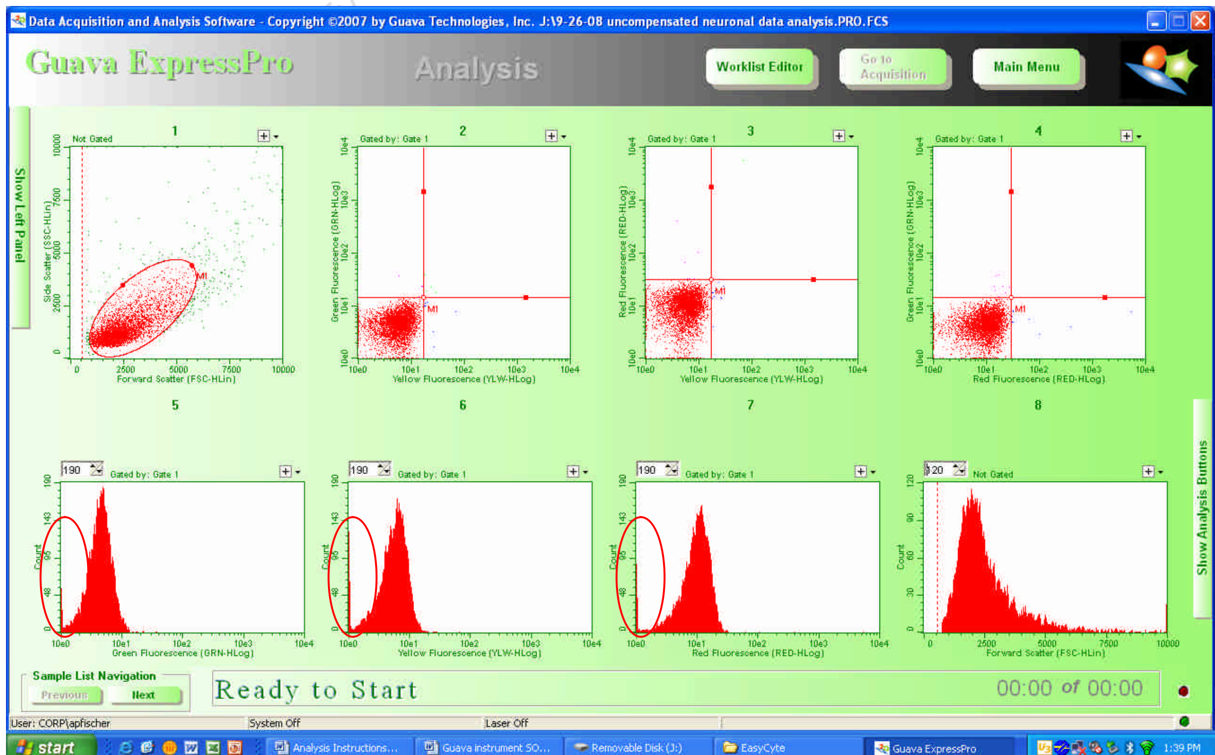


Adjusting Settings

7. To adjust instrument settings tubes 1-4 will be used. Place all tubes to be analyzed into tray and create a work list to be used. Start the work list and select a file to save the data. Press **Settings** and then **Adjust Settings.**
8. Adjust forward and side scatter parameters. Select Tube 1 (all three isotype controls) to set your PMT voltages for side scatter and forward scatter. Once cells are acquiring, increase or decrease forward and side scatter PMTs until your cells of interest are in the middle 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 and exclude the MEFs and/or doublets.



9. Adjust green, yellow and red fluorescence parameters. Decrease green, yellow and red PMTs until most of the cells labeled with isotype control antibodies fall in the lower right hand corner of each dot plot. Use histogram plots as a guide to determine the number of cells falling on the axis. Increase PMTs until the number of cells collecting at the axis is less 100.

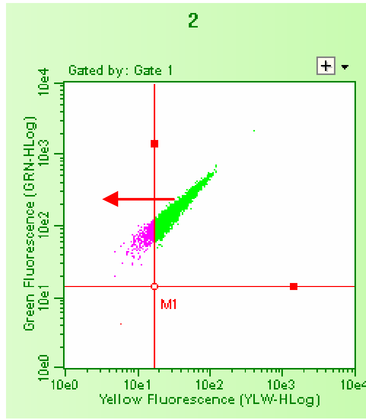


10. Select **Next Step**.

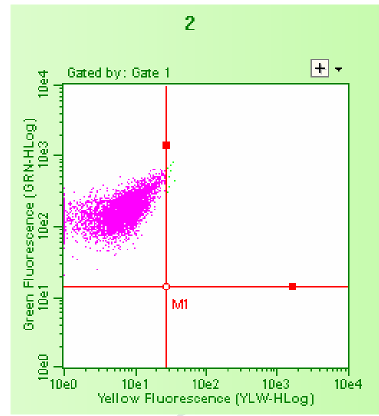
11. Adjust compensation for green fluorescence parameter. Press **Settings** and then **Adjust Settings.**

12. Select Tube 2.

13. Once cells are acquiring, click on compensation window 1 and adjust %YLW-GRN. Start at 10% and 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.



Uncompensated



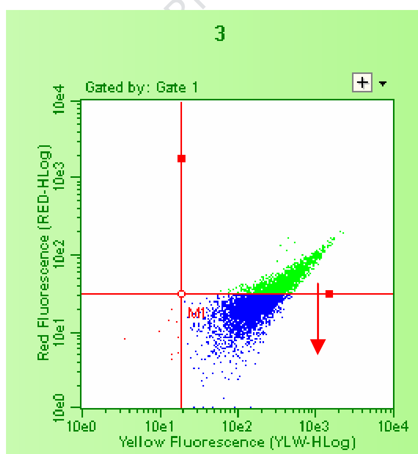
Compensated

14. Select **Next Step**.

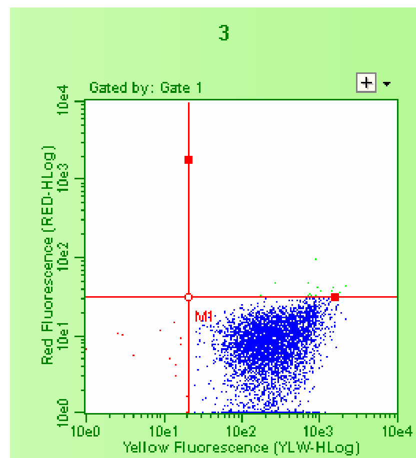
15. Adjust compensation for yellow fluorescence parameter. Press **Settings** and then **Adjust Settings.**

16. Select Tube 3.

17. Once cells are acquiring, click on compensation window 1 and adjust %RED-YLW. Start at 10% and increase or decrease until cells are no longer present in red 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.



Uncompensated



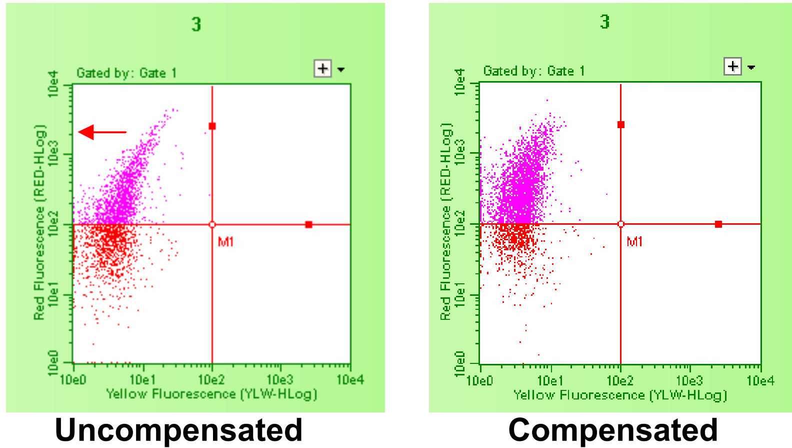
Compensated

18. Select **Next Step**.

19. Adjust compensation for yellow fluorescence parameter. Press **Settings** and then **Adjust Settings.**

20. Select Tube 4.

21. 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.



22. Select **Next Step**.
23. Fine tune compensation for all parameters. Press **Settings** and then **Adjust Settings.**
24. Select tube with cells stained with all three antibodies. (For example: Tube 5)
25. Once cells are acquiring, click on compensation window 1 and adjust compensation, so that you get the desired result, i.e. something very similar to the data presented in Figure 1 of this manual.
26. Select **Next Step**.
27. Save settings. Press **Settings** and then **Save Settings.**
28. Click **Resume** to acquire data.
29. If using the same cells and same conditions you can use the same settings for future experiments without the use of isotype controls. However, some adjustments may be necessary to obtain the desired results.

Sample Data

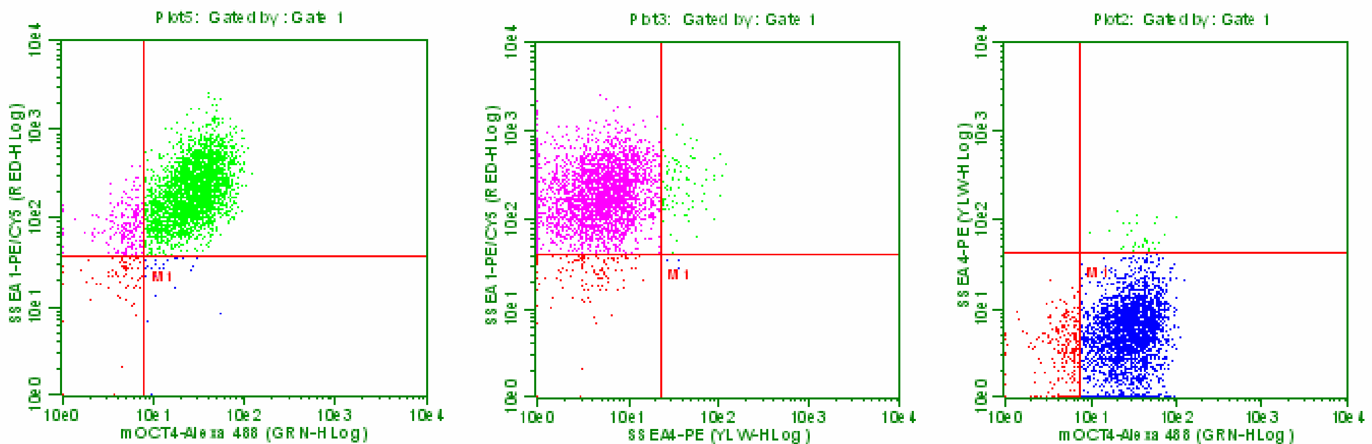


Figure 1. Representative data of CMTI-2 mouse embryonic stem cells stained with mOCT4-Alexa 488, SSEA4-PE and SSEA1-PE/CY5.

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	• Too many cells per microliter- Decrease the number of cells 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 rpms until a compact and cell visible 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 mouse embryonic cell lines may require higher concentrations of fixation buffer to stain correctly. Use the fixation buffer at 2X rather than 4X.

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Staining is weak	• Some mouse embryonic cell lines may require higher concentrations of conjugated antibodies. Use the antibodies at 10X rather than 20X.
Staining is too bright	• Some mouse embryonic 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

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