

## Application Note

# Analysis of the Embryonal Carcinoma Cell line 2102Ep with the Accuri C6 Flow Cytometer<sup>®</sup>

### Introduction

The human embryonal carcinoma (EC) cell line 2102Ep is relatively nullipotent and expresses most of the same markers as undifferentiated human embryonic stem cells (hESC). It is useful for assay development, as a positive control, and as a reference standard to correlate data between experiments or laboratories. GlobalStem Inc. (Rockville, MD) has developed a product, 2102Ep Flow Cytometry Standard (GlobalStem cat# GSC-2002S), which consists of 2102Ep cells that have been dissociated to single cells, fixed with paraformaldehyde and stored frozen (-80° C). A vial of these fixed and frozen cells can be quickly thawed and stained with antibodies to common hESC epitopes of interest (e.g. SSEA4, SSEA1, TRA-1-60, OCT3/4) and TRA-1-85, a human cell-specific marker.

The C6 Flow Cytometer System is ideally suited to the analysis of EC and hESC, especially when minimizing the intra-experimental variation in flow cytometric (FC) analysis is desired. The high resolution (24-bit digital signal processing) and expanded dynamic range (greater than 6 logs for fluorescence and light scatter detectors) of the C6 allow it to function with an absolute fluorescence scale, removing the variability due to detector voltage changes. This greatly simplifies instrument set-up and analysis, and allows the use of analysis templates and pre-set fluorescence compensation values, reducing much of the subjective, operator dependent variation in FC analysis. The completely digital signal processing of the C6 allows the data collection and analysis phases to be separated, reducing the risk of data loss or inappropriate analysis.

### Materials and Methods

#### Reagents and Equipment

- 2102Ep Flow Cytometry Standard (GlobalStem, Inc.)
- FC Wash: Dulbecco's phosphate buffered saline plus 1 mg/mL BSA and 1 mg/mL NaAzide
- Antibodies: FITC conjugates: TRA-1-85 (R&D Systems), Ms IgG3 isotype, Ms IgM isotype (eBiosciences); Alexa-488 conjugates: SSEA-4, SSEA-1; PE conjugates: TRA-1-60, SSEA-4, Ms IgM isotype, Ms IgG3 isotype (eBiosciences); APC conjugates: OCT 3/4, rat IgG2a isotype (eBiosciences).
- C6 Flow Cytometer System with CFlow<sup>®</sup> Plus Software, powered on and performance validated with Spherotech 8-Peak (Accuri cat# QA-100) and Spherotech 6-Peak (Accuri cat# QA-110) Validation Beads.
- CFlow template file GlobalStem 2102Ep Analysis.c6t
- C Comp Calculator spreadsheet

### FC Experimental Design: An Example

Refer to Table 1 for an example of the samples needed for the multicolor analysis used in this Application Note. Individual experimental designs will vary, depending on the goal of particular analyses. As a general rule, however, multicolor experiments should at least contain single-color controls for each fluorochrome used (Samples 2-5) and an unstained control (Sample 1). **These controls are essential to verify fluorescence compensation values, even if using compensation values from a pre-populated C6 compensation matrix.** In the example in Table 1, there are individual single stained controls for FITC (TRA-1-85) and Alexa-488 (SSEA-4). Even though both of these fluorochromes are detected with FL1 (530 BP filter) on the C6, each of these two fluorochromes will have unique fluorescence spillover into other detectors, and therefore may require different compensation values.

All antibodies were titrated before use in this experiment to find the optimal staining conditions based on a final cell concentration of  $5 \times 10^6$  per mL.

Sample	FITC/Alexa-488	PE	Alexa-647
1	Unstained	Unstained	Unstained
2	Isotype Controls	Isotype Controls	Isotype Controls
3	SSEA-4 (Alexa-488)	Isotype Controls	Isotype Controls
4	TRA-1-85 (FITC)	Isotype Controls	Isotype Controls
5	Isotype Controls	SSEA-4	Isotype Controls
6	Isotype Controls	Isotype Controls	OCT3/4
7	SSEA-1 (Alexa-488)	SSEA-4	OCT3/4
8	TRA-1-85	SSEA-4	OCT3/4
9	SSEA-1	TRA-1-60	OCT3/4
10	Tra-1-85	TRA-1-60	OCT3/4

**Table 1.** Example of sample tube layout for FC analysis of 2102Ep cells. "Isotype Controls" indicates that a combination of titrated isotype control antibodies, conjugated to the indicated fluorochromes, were added to a sample.

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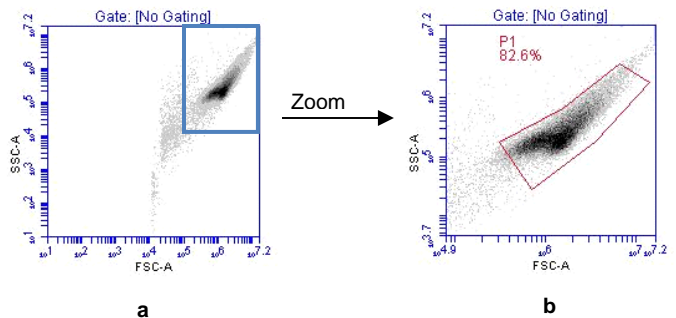
## Preparation and staining of 2102Ep Flow Cytometry Standard cells

A single fixed, frozen (-80°C) aliquot of  $5 \times 10^6$  2102Ep cells was thawed quickly in a 37°C waterbath, then transferred into a 50 mL conical tube containing 25 mL of FC Wash at room temperature (RT). The cells were centrifuged to pellet at 300xg, RT, and resuspended in 1 mL FC Wash. 100µL aliquots of cells were distributed into 12x75 mm polystyrene tubes. Antibodies were added directly to cell suspensions using appropriate volumes determined in previous titration experiments. Samples were incubated on ice, covered, for 30 minutes. After incubation, 500µL FC Wash was added to each tube and samples were centrifuged at 300xg, 4° C. Cell pellets were snapped and resuspended in 300 µL of FC Wash.

## Flow Cytometric Data Collection

A C6 Flow Cytometer System with CFlow<sup>®</sup> Plus Software was used for the analysis. The excitation lasers and detector filters used were the standard configuration, as follows. Lasers: blue (488nm) and red (640nm); Detectors: FL1 (530/30 BP), FL2 (585/40), FL3 (670 LP), FL4 (675/25 BP). FL4 detects red laser excited signals only.

1. Open the CFlow Template called “**GlobalStem 2102Ep Analysis**”.
2. The **primary threshold** for data collection should be set to the CFlow default value of **channel 80,000 on FSC-H**.
3. The standard linear FSC-A versus linear SSC-A default CFlow plot should be changed to a **Log FSC-A/ Log SSC-A** view to better accommodate the large size of 2102Ep cells (Figure 1a). The Zoom tool can be used to enhance the view. Draw a **polygon P1**, around the main cell population (Figure 1b).
4. Set a **Run Limit of at least 20,000 events in P1** and collect data for all samples in successive wells of the CFlow file. Additional analysis and fluorescence compensation can be performed post-data collection.



**Figure 1.** Light scatter distributions for 2102Ep Flow Cytometry Standard cells analyzed, after antibody staining. **a)** Log FSC-A versus Log SSC-A plot showing area for Zoom (blue box). **b)** After Zoom is applied, polygon P1 has been drawn around the main cell population, and is used to gate the overlay plots in Figure 4a to d (next page).

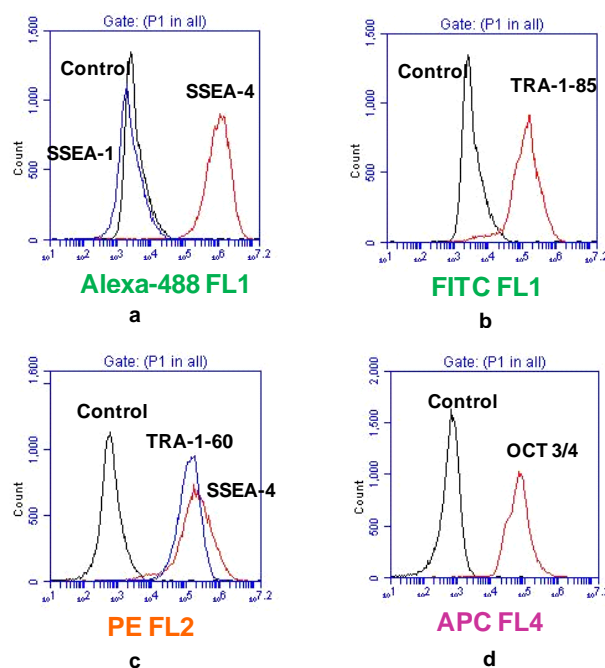
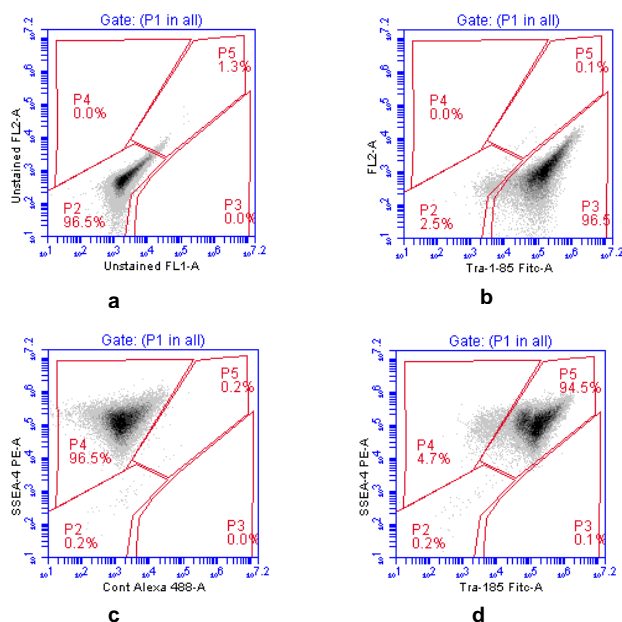
## Flow Cytometric Data Analysis

### Setting Fluorescence Compensation and determining percentage of dual positive cells

If multiple fluorochromes are used simultaneously, unstained and individually stained positive samples should be run in order to determine the amount of fluorescence spilling into neighboring channels (Table 1).

1. In the Collect Tab, Click on the **Set Color Compensation** button.
2. Fill in the suggested Compensation Settings values for FITC and PE, from the Accuri **C Comp Calculator**, spreadsheet in the appropriate wells of the dialog box.
3. Click **Preview** in the dialog box, and compare the median fluorescence channel numbers of the unstained and single stained controls using the FL1-A versus FL2-A plot.
4. Adjust the values in the Compensation Settings dialog box as needed to equalize medians (Figure 2 a-c).
5. Advance to the dual stained FITC and PE data wells to determine percentage of double stained cells (Figure 2d).
6. Repeat this process as needed with any other fluorochrome combinations used.

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**Figure 2.** Properly compensated unstained (a), single stained (b,c) and dual stained (d) 2102Ep Flow Cytometry Standard cells (GlobalStem, Inc.) on the C6 using the **C Comp Calculator** spreadsheet and the CFlow Template **GlobalStem 2102Ep Analysis.c6t**, both provided by Accuri Cytometers.

**Figure 4.** Overlays of single parameter plots after proper fluorescence compensation has been applied. Labeling on plots was added in presentation software after plots were dragged and dropped in from the CFlow file.

## Create Overlay Plots

Once fluorescence compensation has been properly applied to each sample, single color overlays can be created with CFlow.

1. Open the Analyze Tab, to the right of the Collect Tab.
2. Click on the Overlay Tool under the words "Make a new plot" (Figure 3).



**Figure 3.** The Overlay Tool

3. Click on the X-axis parameter name and choose the desired parameter to be displayed from the drop-down menu.
4. Click on the grey gate box above the plot and select P1.
5. Select up to six samples to display in the overlay (Figure 4).

## References

Qualification of embryonal carcinoma 2102Ep as a reference for human embryonic stem cell research. *Stem Cells* 2007, 25:437-446, Josephson R et al.

The Accuri C6 Flow Cytometer – A Small Revolution. Rich C, Howes G. In Press, 2009.

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