Analysis of 2102Ep Cells as a Pluripotent Stem Cell Reference Standard with the BD Accuri™ C6 Flow Cytometer

Technical Bulletin

Contents

Introduction

Pluripotent human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have attracted intense scientific interest, both for their research and therapeutic potential. Pluripotent stem cells offer a window to help researchers understand the fundamental events and mechanisms regulating human development, health, and disease.

Because hESC and hiPSC lines differ, culture conditions vary, and stem cells can spontaneously differentiate, it is useful to have a stable reference standard to which cells can be compared. The human embryonal carcinoma (EC) cell line 2102Ep expresses most of the same markers as undifferentiated pluripotent cells (Table 1). This similarity makes the 2102Ep line useful for assay development, as a positive control, and as a reference standard to correlate data between experiments or laboratories.[1]

Table 1. Selected human ESC and iPSC markers that are expressed in 2102Ep.

Marker	Location	Significance
SSEA-1$^-$	Surface	Stage-Specific Embryonic Antigen-1, an antigenic epitope expressed on mouse embryonal carcinoma cells, mouse embryonic stem cells (mESCs), and mouse and human germ cells—but not on human pluripotent stem cells.
SSEA-4$^+$	Surface	Stage-Specific Embryonic Antigen-4, an established pluripotency marker for hESCs and hiPSCs.
TRA-1-60$^+$	Surface	Tumor Rejection Antigen-1-60, expressed on pluripotent stem cells such as hESCs and hiPSCs, and downregulated when they differentiate.
Nanog$^+$	Intracellular	A transcription factor specific to both mouse and human pluripotent stem cells.
Oct 3/4$^+$	Intracellular	A transcription factor expressed in hESCs and hiPSCs, required to sustain self-renewal and pluripotency.
Sox2$^+$	Intracellular	A transcription factor involved in the regulation of embryonic development and the determination of cell fate. Required for stem cell maintenance in the central nervous system.
CD147$^+$	Surface	A human cell-specific marker.

The BD Accuri C6 flow cytometer is ideally suited to the analysis of human and mouse stem cell lines. Two lasers and four fluorescence detectors allow it to measure multiple markers simultaneously. Its high resolution (24-bit digital signal processing) and advanced optics give it an extended dynamic range—more than 6 decades for both fluorescence and light scatter—without adjusting detector voltage. A fixed-voltage system simplifies instrument setup and analysis and allows use of analysis templates and preset fluorescence compensation values, reducing much of the subjective, operator-dependent variability in flow cytometric analysis. The data collection and analysis phases can be separated, reducing the risk of data loss or erroneous analysis. All of these factors minimize inter-experimental variation in results.

This technical bulletin provides guidelines, procedures, and sample data for analyzing EC 2102Ep cells on the BD Accuri C6.

Materials and Methods

Cell lines

Product Description	Clone	Vendor	Cat. No.	Cells/Vial
hESC Flow Cytometry Reference (Human Embryonal Carcinoma Cell Line 2102Ep)	4/D3	GlobalStem	GSC-2102S	2 million
			GSC-2002S	5 million

Antibodies

Marker	Fluorochrome	Clone	Vendor	Cat. No.
SSEA-1	FITC	MC480	BD Biosciences	560127
IgM isotype control	FITC	G155-228	BD Biosciences	553474
CD147	FITC	HIM6	BD Biosciences	555962
IgG_1 isotype control	FITC	MOPC-21	BD Biosciences	555748
SSEA-4	PE	MC813-70	BD Biosciences	560128
IgG_3 isotype control	PE	A112-3	BD Biosciences	559926
TRA-1-60	PE	TRA-1-60	BD Biosciences	560193
IgM isotype control	PE	G155-228	BD Biosciences	555584
Oct 3/4	Alexa Fluor® 647	40/Oct-3	BD Biosciences	560329
IgG_1 isotype control	Alexa Fluor® 647	MOPC-21	BD Biosciences	557783

Ancillary reagents and materials

Product Description	Vendor	Cat. No.
BD Pharmingen™ stain buffer (FBS)	BD Biosciences	554656
BD Perm/Wash™ buffer	BD Biosciences	554723
Spherotech 8-peak validation beads for BD Accuri cytometers	BD Biosciences	653144
Spherotech 6-peak validation beads for BD Accuri cytometers	BD Biosciences	653145

Instruments and software

All cytometer data was acquired using a BD Accuri C6 flow cytometer system with BD Accuri C6 software. The instrument was validated with Spherotech 8-peak and 6-peak validation beads. The C Comp Calculator spreadsheet was used to set compensation.

Experimental design

Table 2 shows the tube schema used for multicolor analysis in this technical bulletin. (Other experimental designs may vary, depending on the goal of the particular analysis.) As a general rule, multicolor experiments should at least contain single-color controls for each fluorochrome used (Tubes 2–5) and an unstained control (Tube 1). These controls are essential to verify fluorescence compensation values, even if using values from a prepopulated compensation matrix.

Table 2. Example sample tube layout for analysis of 2102Ep cells.

Tube	FITC	PE	Alexa Fluor® 647
1	–	–	–
2	CD147	–	–
3	–	SSEA-4	–
4	–	TRA-1-60	–
5	–	–	Oct 3/4
6	SSEA-1	SSEA-4	Oct 3/4
7	CD147	SSEA-4	Oct 3/4
8	SSEA-1	SSEA-4	Oct 3/4
9	CD147	TRA-1-60	Oct 3/4
10	IgM isotype controls	IgG_3 isotype controls	IgG_1 isotype controls
11	IgG_1 isotype controls	IgM isotype controls	IgG_1 isotype controls

Preparation and staining of 2102Ep Flow Cytometry Standard cells

A single fixed, frozen (–80°C) aliquot of 5 x 10^6 2102Ep cells was thawed quickly in a 37°C water bath, then transferred into a 50-mL conical tube containing 25 mL of stain buffer at room temperature (RT). The cells were centrifuged to pellet at 300*g*, RT, washed with 25 mL of stain buffer, and resuspended in 2 mL of stain buffer. One-hundred-microliter aliquots of cells were distributed into 12 x 75-mm polystyrene tubes.

Then, cells were washed twice with 1 mL of 1X BD Perm/Wash buffer and incubated in 1X BD Perm/Wash buffer for 10 min at RT. Antibodies were added directly to cell suspensions in 100 µL of 1X BD Perm/Wash buffer. Samples were then incubated at RT, covered, for 30 to 45 minutes. After incubation, samples were washed twice with 500 µL of stain buffer, centrifuging at 300*g*, RT. Cell pellets were snapped and resuspended in 500 µL of stain buffer.

Results

Data collection on the BD Accuri C6

BD Accuri C6 software was used for data acquisition and analysis with the standard configuration of excitation lasers and detector filters. **Lasers:** blue (488 nm) and red (640 nm). **Detectors:** FL1 (533/30 BP), FL2 (585/40), FL3 (670 LP), and FL4 (675/25 BP). FL4 detects signals excited by the red laser only. Data was acquired according to the following steps.

1. On the **Collect** tab of BD Accuri C6 software, retain the primary threshold for data collection at the default value, channel 80,000 on FSC-H.
2. On the standard FSC-H vs SSC-H plot, change the scale from linear to log/log to better accommodate the large size of 2102Ep cells (Figure 1A). You can use the Zoom tool to enhance the view.
3. Draw a polygon (P3 in Figure 1B) around the main cell population.
4. Set a Run Limit of at least 20,000 events in the light-scatter polygon and collect data for all samples in successive wells of the BD Accuri C6 software file. You can perform additional analysis and fluorescence compensation after data collection.

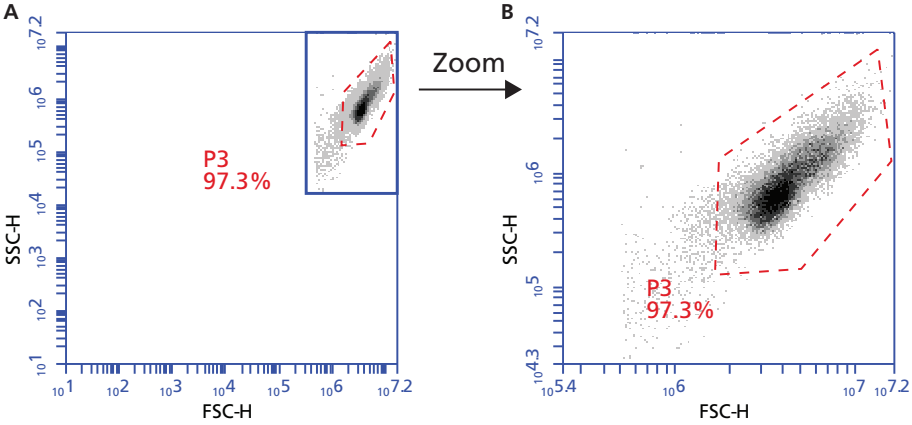

Figure 1. Light scatter distributions for 2102Ep cells after antibody staining.

A. Log FSC-H vs Log SSC-H plot showing the area to be zoomed (blue box). B. After applying zoom, a polygon (P3) was drawn around the main cell population and used to gate the overlay plots in Figure 4.

Data analysis: Setting fluorescence compensation and determining the percentage of dual-positive cells

When multiple fluorochromes are used simultaneously, it is important to run unstained and individually stained positive samples to determine and compensate for fluorescence that spills into neighboring channels.

1. On the **Collect** tab of BD Accuri software, click the **Set Color Compensation** button to open the **Compensation Settings** dialog.
2. Open the C Comp Calculator spreadsheet file available in the Support > Resources > BD Accuri C6 System section of bdbiosciences.com.
3. Copy and paste the suggested Compensation Settings values for FITC and PE from the C Comp Calculator spreadsheet into the appropriate wells of the **Compensation Settings** dialog.
4. In the dialog, click **Preview**. Compare the median fluorescence channel values of the unstained and single-stained controls using the FL1-A vs FL2-A plot.
5. Adjust the values in the **Compensation Settings** dialog as needed to equalize the medians (Figure 2A–C).
6. Advance to the dual-stained FITC and PE data wells to determine the percentage of double-stained cells (Figure 2D). In this case, 99.7% of the dual-stained sample was SSEA-4$^+$CD147$^+$, consistent with GlobalStem's reported pluripotency standards.[2]
7. Repeat this process as needed with any other fluorochrome combinations used.

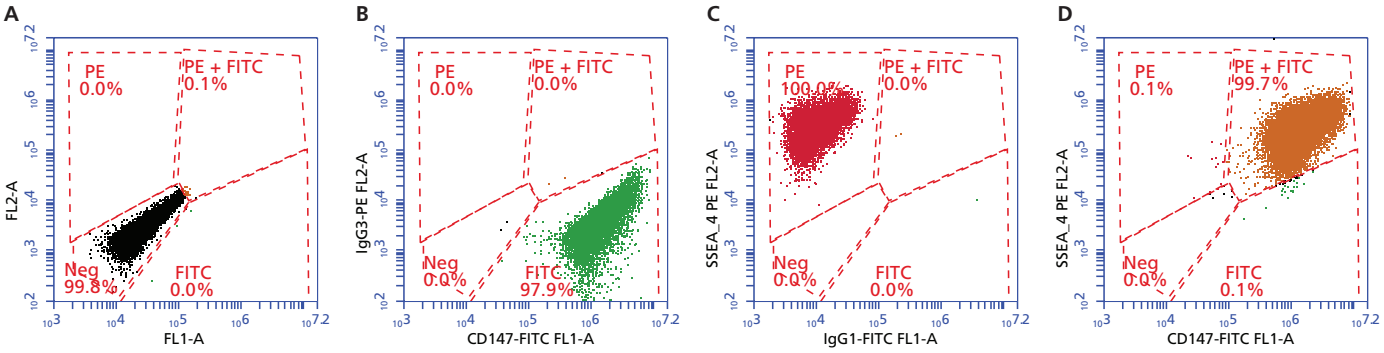

Figure 2. Results of 2012Ep cell staining with SSEA-4 and CD147 antibodies.

*Properly compensated **(A)** unstained, **(B, C)** single-stained, and **(D)** double-stained 2102Ep flow cytometry reference cells. All plots were gated on the light-scatter polygon (P3 in figure 1B).*

Data analysis: Creating overlay plots

After applying fluorescence compensation to each sample, you can create single-color overlay histograms.

1. On the **Analyze** tab of BD Accuri C6 software, click the Overlay Tool (Figure 3).

Figure 3. The Overlay tool.

2. Click the x-axis parameter name and select the detector to display.
3. Click the gray gate box above the plot and select the light-scatter polygon.
4. Select up to six samples to display in the overlay (Figure 4).

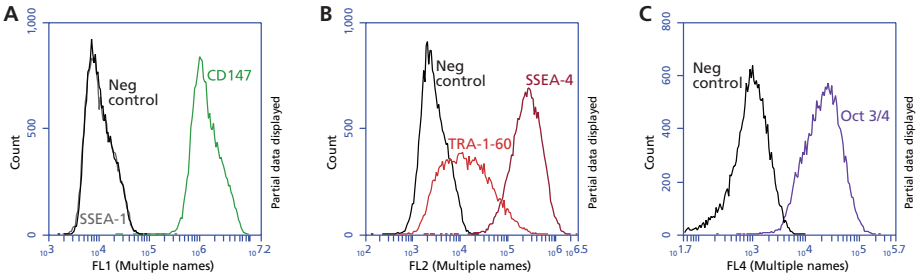

Figure 4. Overlay plots of hESC markers in stained 2012Ep cells.

*Overlays of single-parameter histograms after fluorescence compensation. **A.** SSEA-1 and CD147 vs negative controls on FL1 (FITC, 533/30 BP). The curve for SSEA-1 (a negative pluripotency marker) is almost identical to the control. **B.** SSEA-4 and TRA-1-60 vs negative controls on FL2 (PE, 585/40 BP). **C.** Oct 3/4 vs negative controls on FL4 (Alexa Fluor® 647, 575/25 BP). All plots were gated on the light-scatter polygon (P3 in Figure 1B).*

References

1. Josephson R, Ording C, Liu Y, et al. Quantification of embryonal carcinoma 2102Ep as a reference for human embryonic stem cell research. *Stem Cells.* 2007;25:437-446.
2. GlobalStem, Inc. hESC Flow Cytometry Reference-2M. Available at: http://www.globalstem.com/assays/pluriq-standards/flow-cytometry-standard-2m. Accessed February 16, 2012.

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