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Flow cytometric analysis of the differentiation potential of human pluripotent stem cells

StemMACS™ Trilineage Differentiation Kit

Background

Thorough characterization of newly derived human embryonic (hESC) or induced pluripotent stem cell (hiPSC) lines is mandatory after reprogramming and during maintenance culture. Human pluripotent stem cell (hPSC) lines must fulfill certain criteria, such as characteristic morphology, long-term self-renewal, karyotypic stability, expression of a specific marker profile, and the capacity to differentiate into all three germ layers.

Differentiation capacity can be assessed by *in vitro* assays monitoring spontaneous or directed differentiation. Assays for spontaneous differentiation are highly variable and hard to read out, whereas assays for directed differentiation give more reproducible results due to specified media conditions. Besides, evaluation of differentiation capacity is mostly done only qualitatively by immunocytochemistry since quantification requires sophisticated microscopy tools. Here we present a flow cytometry protocol that allows quantitative analysis of the hPSC differentiation potential. The protocol is based on the StemMACS™ Trilineage Differentiation Kit which enables directed and reproducible differentiation into ectodermal, mesodermal, and endodermal lineages in only seven days.

Flow cytometry with the recombinant REAfinity™ Antibodies ensures specific detection with excellent signal-to-noise ratio and allows both qualitative and quantitative comparison of different cell lines.

Materials

Buffers and reagents

- Buffer consisting of PBS, pH 7.2, 0.5% BSA, 2 mM EDTA is prepared by diluting MACS® BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep the buffer cold (2–8 °C).
- FoxP3 Staining Buffer Set (# 130-093-142) for intracellular staining
- Propidium Iodide Solution (# 130-093-233) for dead cell exclusion

Antibodies

Table 1 specifies the lineage-specific antibodies used for detection of differentiated cells.

Lineage	Marker	Clone	Dilution	Order no.
Mesodermal lineage	CD144 (VE-Cadherin)-FITC, human	REA199	1:11	130-100-713
	CD140b-APC, human	REA363	1:11	130-105-322
Endodermal lineage	CD184 (CXCR4)-APC, human	REA649	1:11	130-109-886
	Anti-Sox17-Vio® 515, human	REA701	1:50	130-111-147
Ectodermal lineage	Anti-PAX-6-APC, human	REA507	1:11	130-107-829
	Anti-Sox2-FITC, human and mouse	REA320	1:11	130-104-993

Table 1: Marker panel for flow cytometry of differentiated cells.

Cells

Human PSCs were plated in 12-well plates, coated with Matrigel® hESC-Qualified Matrix, and differentiated using the StemMACS Trilineage Differentiation Kit, human (# 130-115-660). Differentiated cells were harvested with 0.05% trypsin/EDTA to obtain single cells for flow cytometry analysis.

Flow cytometry

The staining panel is based on a set of REAfinity™ Antibodies to ensure low background and high staining specificity. The panel consists of two marker combinations for each germ layer and includes both surface and intracellular markers. Surface markers are stained first. Subsequently, cells are fixed and permeabilized for intracellular staining. For an overview of the staining procedure refer to table 3.

Prepare FoxP3 Fixation Solution and Permeabilization Buffer freshly as indicated in the datasheet:

1. Fixation / Permeabilization Solution 1 must be diluted 1:4 with the Fixation / Permeabilization Solution 2 (e.g. 0.25 mL of Fixation / Permeabilization Solution 1 plus 0.75 mL of Fixation / Permeabilization Solution 2).
2. The 10× Permeabilization Buffer must be diluted 1:10 with deionized or distilled water before use (e.g. 1 mL of 10× Permeabilization Buffer plus 9 mL of deionized / distilled water).

Total buffer volumes should be calculated beforehand and will depend on the number of samples. To set up the instrument and compensate for spectral overlap, single stainings of CD144 (VE-Cadherin)-FITC-positive and CD140b-APC-positive differentiated cells (mesodermal lineage) and an unstained cell sample are required. The unstained control does not contain antibody. It should be treated, e.g., fixed and permeabilized, in the same way as the stained samples.

Cell staining

1. Determine cell numbers of differentiated mesodermal, endodermal, and ectodermal cells.
2. Prepare samples 1–8 as indicated in table 2. Use up to 5×10^5 cells per sample.
3. Centrifuge cell suspension at 300×g for 5 minutes. Aspirate supernatant completely.
4. Resuspend samples 1–5 in 100 µL of buffer. This will be the unstained controls and single-stained samples.
5. Resuspend sample 6 in 90 µL of buffer and samples 7 and 8 in 100 µL of buffer. This will be the samples containing the lineage-specific marker panel for analysis of the differentiation potential of hPSCs.
6. Add 10 µL of the following antibodies, detecting the surface markers, to sample 4: CD140b-APC, to sample 5: CD144 (VE-Cadherin)-FITC, to sample 6: i) CD140b-APC and ii) CD144 (VE-Cadherin)-FITC, to sample 7: CD184 (CXCR4)-APC. Do not add antibodies to samples 1–3 and 8 at this point.
7. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).

Note: Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.

8. Wash cells by adding 1 mL of buffer per sample and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
9. Resuspend samples 1, 4, 5, and 6 in 500 µL of buffer and store at 2–8 °C for later analysis.
10. Resuspend samples 2, 3, 7, and 8 in 300 µL freshly prepared FoxP3 Fixation Solution.
11. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).
12. Wash cells by adding 1 mL of buffer per sample and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
13. Resuspend samples 2, 3, 7 in 100 µL and sample 8 in 90 µL of FoxP3 Permeabilization Buffer.
14. Add antibodies detecting intracellular markers as indicated in table 3: to sample 7 add 2 µL of Anti-Sox17-Vio® 515, to sample 8 add 10 µL of i) Anti-PAX-6-APC and ii) Anti-Sox2-FITC. Do not add antibodies to samples 2 and 3, these are the unstained, fixed controls.
15. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C).
16. Wash cells by adding 1 mL of FoxP3 Permeabilization Buffer and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
17. Resuspend samples 2, 3, 7, and 8 in 500 µL of buffer for analysis by flow cytometry.
18. Proceed to instrument setup.

	Sample	Cell type
1.	Unstained mesoderm	mesoderm
2.	Unstained endoderm	endoderm
3.	Unstained ectoderm	ectoderm
4.	Single-color staining APC	mesoderm
5.	Single-color staining FITC	mesoderm
6.	Panel mesoderm	mesoderm
7.	Panel endoderm	endoderm
8.	Panel ectoderm	ectoderm

Table 2: Cell samples used for flow cytometry.

	Sample	Buffer	Cell surface staining			Intracellular staining			
			CD140b-APC	CD144 (VE-Cadherin)-FITC	CD184 (CXCR4)-APC	FoxP3 Perm Buffer	Anti-Sox17-Vio 515	Anti-PAX-6-APC	Anti-Sox2-FITC
1.	Unstained mesoderm	100 µL	-	-	-	-	-	-	-
2.	Unstained endoderm	100 µL	-	-	-	100 µL	-	-	-
3.	Unstained ectoderm	100 µL	-	-	-	100 µL	-	-	-
4.	Single-color staining APC	100 µL	10 µL	-	-	-	-	-	-
5.	Single-color staining FITC	100 µL	-	10 µL	-	-	-	-	-
6.	Panel mesoderm	90 µL	10 µL	10 µL	-	-	-	-	-
7.	Panel endoderm	100 µL	-	-	10 µL	100 µL	2 µL	-	-
8.	Panel ectoderm	100 µL	-	-	-	90 µL	-	10 µL	10 µL

Table 3: Pipetting scheme for surface and intracellular staining.

Instrument setup and sample acquisition

For convenience, the instrument setup is done only with cells from the mesodermal differentiation. These cells are most easily processed since only surface markers are stained. Cells from the endo- and ectodermal differentiation can then be measured using the same settings.

1. Set up the scatter settings and voltages for all channels with the unstained cell sample (sample 1) (fig. 1A and B). Specify the trigger.
2. Use single-color stainings (samples 4 and 5) to adjust the voltage of the FITC and the APC channel (fig. 1C; left: CD140b-APC single staining, right: CD144 (VE-Cadherin)-FITC single staining). Usually no compensation is necessary for these two channels.
3. Add PI solution to samples 1 and 6.
4. Acquire samples 1–3 and 6–8.

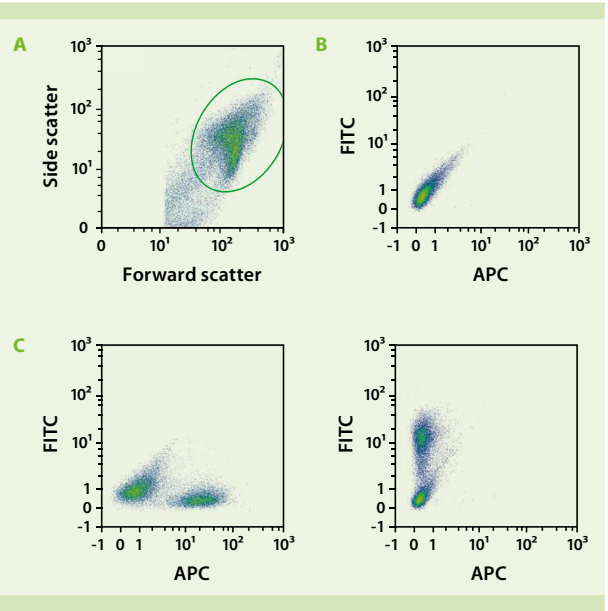

Figure 1: Setting up the flow cytometer for analysis of stained cells. (A and B) Adjustment of scatter and voltage using an unstained sample. (C) Adjustment of channel voltage.

Gating strategy

Figure 2 specifies gating strategies for meso-, endo-, and ectoderm, including dead cell exclusion for differentiated mesodermal cells. First, debris is excluded in the FSC / SSC plot for meso-, endo- and ectoderm (fig. 2A). For the mesoderm cell samples, propidium iodide (PI) is added shortly before flow cytometric analysis, and dead cells are excluded in the PI / FSC plot (fig. 2B). Note that no dead cell exclusion is done for endo- and ectoderm, since these cells are fixed. For mesoderm samples CD140b$^+$ and CD144$^+$ cells are gated, for ectoderm Sox2$^+$PAX-6$^+$ cells and for endoderm Sox17$^+$CD184 (CXCR4)$^+$ cells (fig. 2C).

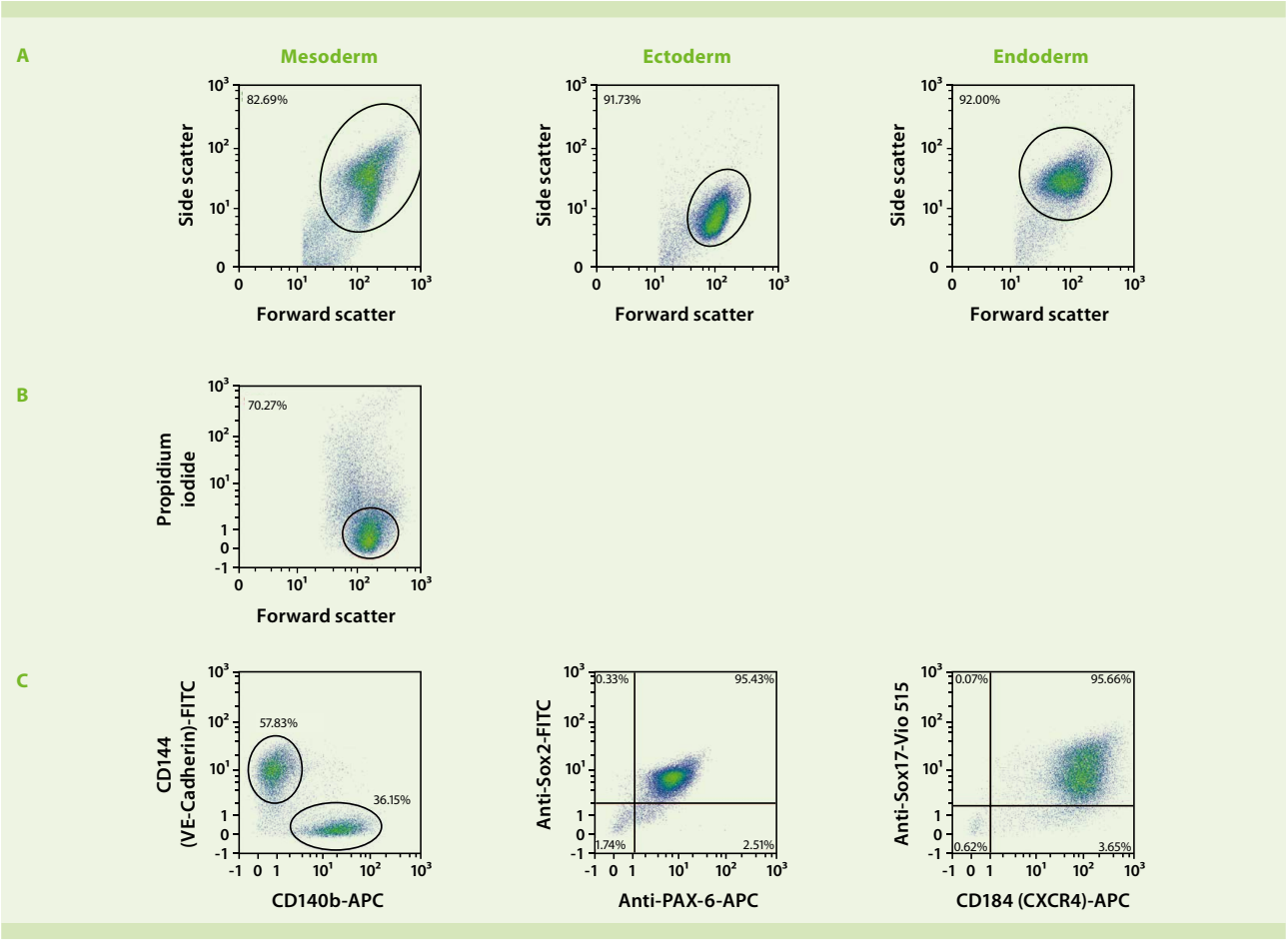

Figure 2: Gating strategy for meso-, ecto-, and endoderm.

Results

The StemMACS™ Trilineage Differentiation Kit in combination with the REAfinity Antibody panel described above enables the quantitative assessment of the PSC differentiation potential. Figure 3 shows representative results for four independent human iPSC lines.

The assay revealed different propensities for each line to differentiate into the three germ layers: Clone 2 and clone 3 showed a high capacity for differentiation into Sox2$^+$PAX-6$^+$ neuroectoderm cells (89–90%), while clone 1 and clone 4 had a slightly lower efficiency (81–84%).

Endodermal differentiation capacity was higher for clone 1 (91%) and clone 4 (83%) compared to clones 2 and 3, which showed a lower tendency to differentiate into CXCR4$^+$Sox17$^+$ definitive endoderm cells (70–71%).

The overall capacity to differentiate into the mesodermal lineage was high for all four clones (94–99%), but differences could be detected in the ratio of CD140b$^+$ and CD144$^+$ cells.

In summary, the assay revealed differences in the differentiation potential between the four human iPSC clones tested (fig. 4) and thus facilitates the functional characterization and comparison of human PSC lines.

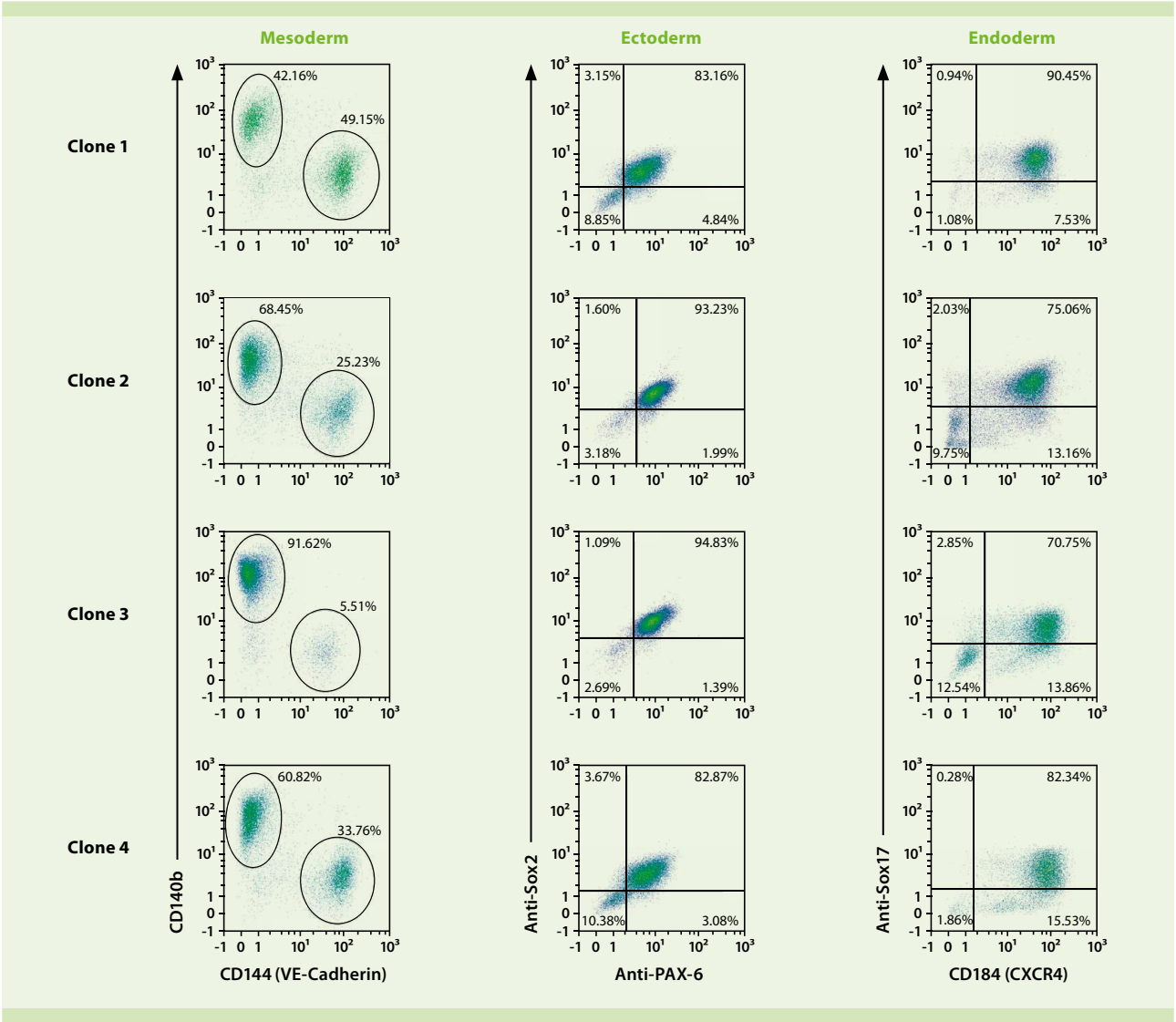

Figure 3: Differentiation of four different iPSC lines into the three germ layers. The pluripotent differentiation potential was assessed with the StemMACS Trilineage Differentiation Kit.

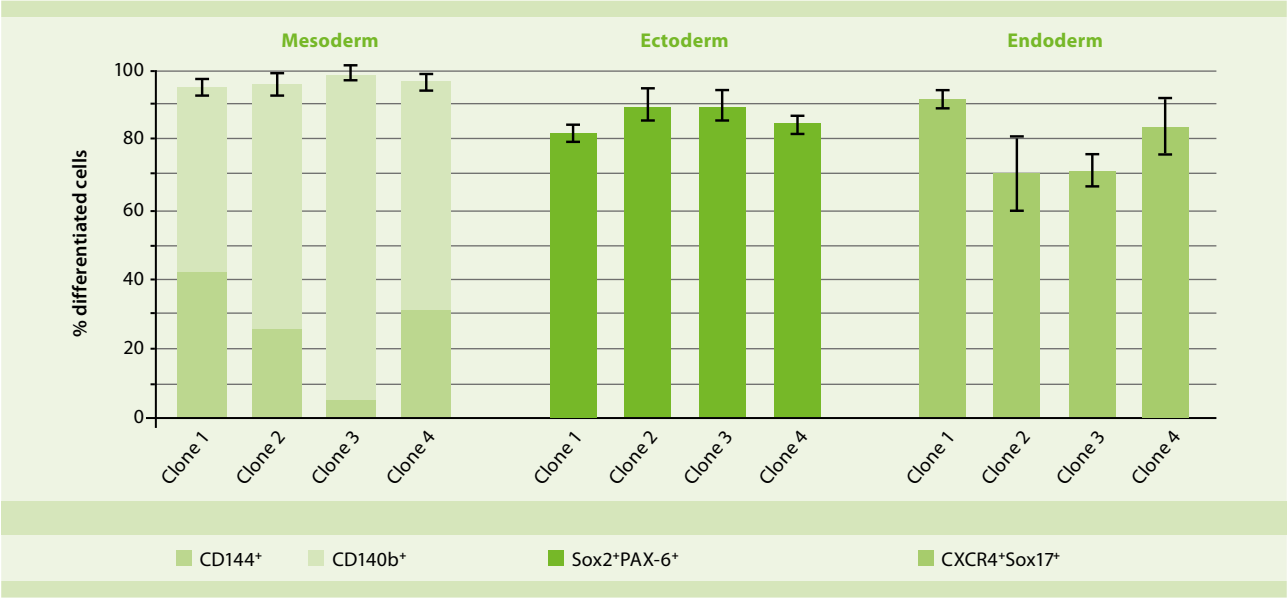

Figure 4: Expression of various differentiation markers in four iPSC lines. Data indicate mean +/– SD with n = 3.

Conclusion

- The StemMACS™ Trilineage Differentiation Kit enables directed and reproducible differentiation of PSCs into ectodermal, mesodermal, and endodermal lineages in only seven days.
- The StemMACS Trilineage Differentiation Kit in combination with a panel of the recombinantly engineered REAfinity Antibodies enables the quantitative assessment of the hPSC differentiation potential.
- The assay facilitates the functional characterization and comparison of human PSC lines.

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