Cat. # Y50300

For Research Use

TaKaRa

MiraCell® iPS Cell to Endothelial Cell Differentiation Kit

Product Manual

v201905

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I. Description

Primary human vascular endothelial cells are widely used in research fields such as angiogenesis and drug uptake. The properties of commercially available human endothelial cells vary between different lots depending on variation in donors. Vascular endothelial cells differentiated from human iPS cells have attracted attention as a substitute for primary human vascular endothelial cells because they are homogeneous cell populations derived from a single donor, due to the ability of iPS cells to proliferate indefinitely.

This product is a kit to induce the differentiation of human iPS cells into vascular endothelial cells. Cells produced with this kit can be used for analyzing the characteristics and functions of endothelial cells, and for performing toxicity tests.

This product was jointly developed by Takara Bio Inc. and iHeart Japan Corporation. This differentiation technique was developed by Professor Jun K. Yamashita of the Center for iPS Cell Research and Application of Kyoto University in conjunction with the iHeart Japan Corporation. This method makes it possible to prepare high-purity endothelial cells without using antibiotic or metabolic selection. In addition, this technique prevents the reduction in cell population purity that typically results from long-term cell culture.

Human iPS cells cultured under feeder-free conditions using the Cellartis® DEF-CS™500 Culture System (Cat. # Y30010) are compatible with this system.

Features

- This kit contains enough reagents to induce differentiation of vascular endothelial cells from iPS cells in one T25 flask or three wells of a 6-well plate.
- Purity (CD31-positive rate) of vascular endothelial cells is routinely >80% after inducing differentiation＊1 (Day 18).
- Induced vascular endothelial cells can be cryopreserved with a suitable cryoprotective solution, such as CELLBANKER 1 plus (Cat. # CB021; not available in all geographic locations from Takara Bio, so check availability in your area).
- Extended culture of vascular endothelial cells can be performed with MiraCell EC Culture Medium＊2.

＊1 This has not been confirmed in all iPS cell lines. Additional testing may be necessary.
＊2 Proliferative ability is different depending on the iPS cell line used.

II. Components

Reagent	Volume	Storage
Coating Reagent 1 (Day0 & 2)	0.35 ml	-80℃
Coating Reagent 2 (Day11)	1.5 ml	4℃
Supplement 1 (Day3)	0.42 ml	-80℃
Supplement 2 (Day4)	0.33 ml	-80℃
Supplement 3 (Day7)	0.3 ml	-80℃
Supplement 4 (Day8)	1.2 ml	-80℃
Supplement 5 (Day9)	1.0 ml	-80℃
Supplement 6 (Day11)	1.8 ml	-80℃
EC Differentiation Basal Medium 1 (Day3 ～ 9)	200 ml	4℃
EC Differentiation Basal Medium 2 (Day11)	50 ml	4℃

III. Storage

- Keep frozen reagents (Coating Reagent 1, Supplements 1 - 6) at -80℃ until immediately before use. As repeated freeze/thaws can decrease their activity, aliquot frozen reagents into working volumes and do not refreeze more than once after the initial thaw.
- Keep refrigerated reagents (Coating Reagent 2, EC Differentiation Basal Medium 1 and 2) at 4℃. Do not freeze them.

IV. Materials Required but not Provided

- Dulbecco' s PBS with Ca^{++} & Mg^{++} [D-PBS (+/+)]
- Dulbecco's PBS without Ca^{++} & Mg^{++} [D-PBS (-/-)]
- Accumax Cell Dissociation Solution (Innovative Cell Technologies, Inc., Cat. # AM105)
- Cellartis DEF-CS 500 Culture System (Cat. # Y30010)
- MiraCell EC Culture Medium (Cat. # Y50053)* or EGM-2 Endothelial Growth Medium-2 BulletKit (LONZA, Cat. # CC-3136)
- DMEM (Merck, Cat. # D5796)
- Fetal bovine serum
- Penicillin-streptomycin (Thermo Fisher Scientific, Cat. # 15140122 or equivalent)
- TrypLE Select Enzyme (1X), no phenol red (Thermo Fisher Scientific, Cat. # 12563011)
- Trypan Blue Solution, 0.4% (Thermo Fisher Scientific, Cat. # 15250-061 or equivalent)
- Hemocytometer
- Cryopreservation solution
- BICELL (Freezing container) (Nihon Freezer), or equivalent cryopreservation container
- 37℃ , 5% CO_2 incubator
- Clean bench or safety cabinet
- Electric pipette controller and plastic pipettes
- Micropipettor and sterilized tips (with filters)
- 50-ml centrifuge tubes
- 15-ml centrifuge tubes
- 1.5-ml tubes
- T25 flasks: 25 cm^2 Rectangular Canted Neck Cell Culture Flask with Vent Cap (Corning, Cat. # 430639)
- T75 flasks: 75 cm^2 U-Shaped Canted Neck Cell Culture Flask with Vent Cap (Corning, Cat. # 430641U)
- 6-well plates: Costar 6 Well Clear TC-Treated Multiple Well Plates, Individually Wrapped, Sterile (Corning, Cat. # 3516)

* Not available in all geographic areas from Takara Bio. Check for availability in your area.

V. Preparation before use

1. As Coating Reagent 1 (Day0 & 2) is prone to turning into a gel, thaw it at 4℃, add it to cold media, mix well, and then warm it up prior to use.
2. For frozen reagents other than Coating Reagent 1 (Day0 & 2), thaw at room temperature and add into prewarmed media.
3. To prevent culture medium inactivation, after the initial thaw, aliquot the medium into working volumes and only thaw necessary aliquots for the experiment planned.
4. Avoid warming up the culture medium for more than 1 hour.

VI. Protocol

VI-1. Workflow

[≥2 weeks before use: acclimating cultured iPS cells in DEF-CS system *1]

*1 When using cells that have been cultured in other medium systems or thawed from frozen stocks, we recommend 3 to 5 passages in the Cellartis DEF-CS system.

[Day 0: Harvesting and seeding of iPS cells]

Differentiation induction 1

Day 1: Medium change
Day 2: Medium change
Day 3: Medium change
Day 4: Medium change

Day 7: Medium change

[Day 8: Subculture*2 (no coating reagent necessary*3)]

*2 For example, transfer cells in one T25 flask to three T75 flasks, or one well of a 6-well plate to three T25 flasks.

*3 Use uncoated tissue culture flasks or dishes.

Differentiation induction 2

Day 9: Medium change

[Day 11: Subculture*4]

*4 Transfer to the same size of culture vessel that was used on Day 8. (For example, transfer cells from a T75 flask to a new T75 flask, or a T25 flask to a new T25 flask.).

Endothelial cell culture

Day 14 and after: Medium change and subculture

VI-2. Culture Schedule

Schedule	Handling	Medium volume (for T25 flask)	Main reagents/kits
≥2 weeks before use	iPS cell pre-culture	–	Cellartis DEF-CS 500 Culture System* (includes coating)
Day 0	Seeding	7.5 ml	Coating Reagent 1 (Day0 & 2), Cellartis DEF-CS 500 culture medium*
Day 1	Medium change	7.5 ml	Cellartis DEF-CS 500 culture medium*
Day 2	Medium change	10 ml	Coating Reagent 1 (Day0 & 2), Cellartis DEF-CS 500 culture medium*
Day 3	Medium change	10 ml	EC Differentiation Basal Medium 1 (Day3 ～ 9), Supplement 1 (Day3)
Day 4	Medium change	10 ml	EC Differentiation Basal Medium 1 (Day3 ～ 9), Supplement 2 (Day4)
Day 7	Medium change	10 ml	EC Differentiation Basal Medium 1 (Day3 ～ 9), Supplement 3 (Day7)
Day 8	Passage	4 ml	EC Differentiation Basal Medium 1 (Day3 ～ 9), Supplement 4 (Day8)
Day 9	Medium change	4 ml	EC Differentiation Basal Medium 1 (Day3 ～ 9), Supplement 5 (Day9)
Day 11	Passage	4 ml	Coating Reagent 2 (Day11), EC Differentiation Basal Medium 2 (Day11), Supplement 6 (Day11)
Day 14	Medium change	4 ml	MiraCell EC Culture Medium*

* Medium/kit must be purchased separately.

VI-3. Differentiation into Endothelial Cells

- Use human iPS cells that have been cultured for ≥2 weeks with the Cellartis DEF-CS 500 Culture System (sold separately).
- Use the medium and additive reagents supplied in the Cellartis DEF-CS 500 Culture System on Days 0, 1, and 2.
- Use MiraCell EC Culture Medium (sold separately) for culture on Day 14 and after.
- Please consult the Cellartis DEF-CS Culture System User Manual for details.

[Day 0]

Seed culture vessel(s) using 80 to 90% confluent iPS cells that have been passaged 3 - 5 times after thawing.

1. Thaw Coating Reagent 1 (Day0 & 2) at 4℃ and add it (dilute 1 : 60) into cold DMEM. Mix well, add to culture vessels, and incubate at 37℃ for at least 1 hour. Store the remainder of the Coating Reagent 1 at 4℃ to use on Day 2.

Culture vessel	T25 flask	6-well plate (per well)
DMEM (not sold by Takara Bio)	2.5 ml	1 ml
Coating Reagent 1 (Day0 & 2)	42 µl	17 µl

[Note] As Coating Reagent 1 (Day0 & 2) tends to turn into a gel when left at room temperature, keep the solution cold while aliquoting and mixing.

2. Prepare appropriate volumes of Cellartis DEF-CS 500 Basal Medium containing GF-1 (dilute 1 : 333), GF-2 (dilute 1 : 1,000), and GF-3 (dilute 1 : 1,000). Warm medium to 37℃.
3. Aspirate supernatant from cultured iPS cells and rinse cells with D-PBS (-/-).
4. Add an appropriate amount of TrypLE Select, according to the table below. Incubate at 37℃ for 5 to 8 minutes. Ensure the cells have detached.

[Note] Cell detachment time will need to be optimized based on your iPS culture workflow.

Culture vessel	T25 flask	6-well plate (per well)
DEF-CS medium for resuspension	2 ml	1.7 ml
TrypLE Select (1X)	500 µl	300 µl

5. Collect cells by adding the medium prepared in Step 4. Count cells using the hemocytometer, then adjust cell concentration to 2 x 10^5 cells/ml.
6. Aspirate coating solution (prepared in Step 1) from the culture vessel. Following the table below, seed cells at 6 x 10^4 cells/cm^2 and culture them at 37℃, 5% CO_2 for 1 day.

Culture vessel	T25 flask	6-well plate (per well)
Cell suspension (2 x 10^5 cells/ml)	7.5 ml	3 ml

[Note 1] Ensure even distribution of cells to avoid clumping.
[Note 2] Increase seeding density if your iPS cells grow slowly. Cells should be seeded so that they are 70 to 100% confluent by Day 2.

[Day 1]

1. Prepare medium by adding GF-1 (dilute 1 : 333) and GF-2 (dilute 1 : 1,000) to Cellartis DEF-CS 500 Basal Medium. Warm medium to 37℃.
2. Aspirate the culture medium from culture vessels, and add the medium from Step 1.

Culture vessel	T25 flask	6-well plate (per well)
DEF-CS medium	7.5 ml	3 ml

3. Culture cells at 37℃, 5% CO_2 for 1 day.

[Day 2]

Make sure that the cells are 70 to 100% confluent. If cells are less than 70% confluent, please start over. Thaw a new vial from a bank of DEF-CS-adapted cells into an appropriate culture dish and, when confluent, passage the cells at 6 x 10^4 cells/cm^2 (or appropriate cell density you tested) into coated T25 flasks or 6-well plates, as described in [Day 0].

1. Prepare medium by adding GF-1 (dilute 1 : 333) and GF-2 (dilute 1 : 1,000) to Cellartis DEF-CS 500 Basal Medium. Keep prepared medium cold.
2. Add Coating Reagent 1 (Day0 & 2) (stored at 4℃ on Day 0; diluted 1 : 60) into cold medium from Step 1 and mix well. Warm the medium to 37℃.

Culture vessel	T25 flask	6-well plate (per well)
DEF-CS medium	10 ml	4 ml
Coating Reagent 1 (Day0 & 2)	167 μl	67 μl

3. Aspirate the culture medium from culture vessels, and add the medium from Step 2.
4. Culture cells at 37℃, 5% CO_2 for 1 day.

[Day 3]

Perform the following medium change in the morning.

1. Add Supplement 1 (Day3) (dilute 1 : 33) to EC Differentiation Basal Medium 1 (Day3 ～ 9) that has been warmed to 37℃.

Culture vessel	T25 flask	6-well plate (per well)
Supplement 1 (Day3)	300 μl	120 μl
EC Differentiation Basal Medium 1 (Day3 ～ 9)	10 ml	4 ml

2. Aspirate the culture medium from culture vessels, and add the medium from Step 1.
3. Culture cells at 37℃, 5% CO_2 for 24 ± 1 hours.

[Day 4]

Perform the following medium change **24 ± 1 hours after the total medium change on Day 3**.

1. Add Supplement 2 (Day4) (dilute 1 : 50) to EC Differentiation Basal Medium 1 (Day3 ～ 9) that has been warmed up to 37℃.

Culture vessel	T25 flask	6-well plate (per well)
Supplement 2 (Day4)	200 µl	80 µl
EC Differentiation Basal Medium 1 (Day3 ～ 9)	10 ml	4 ml

2. Aspirate the culture medium from culture vessels, and add the medium from Step 1.
3. Culture cells at 37℃, 5% CO_2 for 72 ± 1 hours.

[Day 7]

Perform the following medium change **72 ± 1 hours after the medium change on Day 4**.

[Note] Medium may be colored yellow and/or cells may be over-confluent; this will not affect the outcome of differentiation.

1. Add Supplement 3 (Day7) (dilute 1 : 50) to EC Differentiation Basal Medium 1 (Day3 ～ 9) that has been warmed to 37℃.

Culture vessel	T25 flask	6-well plate (per well)
Supplement 3 (Day7)	200 µl	80 µl
EC Differentiation Basal Medium 1 (Day3 ～ 9)	10 ml	4 ml

2. Aspirate the culture medium from culture vessels, and add the medium from Step 1.

[Day 8]

Perform the following subculture **between 28 and 32 hours after the medium change on Day 7**. We recommend subculturing cells from one T25 flask to 2 - 3 T75 flasks, or from one well of a 6-well plate to 2 - 3 T25 flasks.

[Note] As cells are fragile following detachment, handling should be gentle.

1. Add Supplement 4 (Day8) (dilute 1 : 50) in EC Differentiation Basal Medium 1 (Day3 ～ 9) that has been warmed to 37℃.

Culture vessel	Per 3 T75 flasks	Per 3 T25 flasks
Supplement 4 (Day8)	1 ml	320 µl
EC Differentiation Basal Medium 1 (Day3 ～ 9)	50 ml	16 ml

2. Remove the culture medium from the vessel and gently rinse cells twice with D-PBS (-/-) in order to remove dead cells.
3. Add 2.5 or 1 ml of Accumax Cell Dissociation Solution into a T25 flask or one well of a 6-well plate, respectively, and incubate at 37℃ for 30 minutes＊.

＊ Adjust the reaction time depending on the cells.

4. Confirm that cells are detached by gently rocking or tapping culture vessel. Collect cells by adding 2.5 or 1 ml of DMEM medium containing 10% FBS and 1% Penicillin-streptomycin to a T25 flask or well (6-well plate), respectively, and put it in a 15-ml centrifuge tube.
5. Add 2.5 or 1 ml of DMEM medium containing 10% FBS and 1% Penicillin-streptomycin into the same T25 flask or well, respectively. Tap the culture vessels and collect and transfer cells remaining in the vessels into the 15-ml centrifuge tube.
6. Centrifuge at 200*g* for 5 minutes.
7. Remove supernatant, leaving about 0.2 ml of medium, and loosen the cells by tapping gently.
8. Add 6 ml (for a T25 flask) or 2.5 ml (for one well of a 6-well plate) of medium prepared in Step 1, and prepare cell suspension by gently pipetting.
9. After counting cells, prepare cell suspension at 1 x 10^6 cells/ml. Seed 3 ml of the cell suspension into 12 ml of the culture medium in a T75 flask. Seed 1 ml of the cell suspension into 4 ml of the medium in a T25 flask.

   [Note] Seed cells directly into tissue culture flasks or dishes. Coating is not necessary for these culture vessels.

[Day 9]

1. Add Supplement 5 (Day9) (dilute 1 : 50) in EC Differentiation Basal Medium 1 (Day3 ～ 9) that has been warmed to 37℃.

Culture vessel	T75 flask	T25 flask
Supplement 5 (Day9)	240 μl	80 μl
EC Differentiation Basal Medium 1 (Day3 ～ 9)	12 ml	4 ml

2. Aspirate the culture medium from culture vessels, and add the medium from Step 1.

[Day 11]

Perform subculture in a new culture vessel of equal size.

* At this point, cells should be roughly 10 to 30% confluent. Higher cell numbers may result in a lower ratio of endothelial cells, so we recommend checking purity using flow cytometry with an anti-human CD31 antibody.

1. Add Coating Reagent 2 (Day11) (dilute 1 : 20) to D-PBS (+/+). After mixing, add to new culture vessels and incubate at 37℃ for at least 20 minutes.

Culture vessel	T75 flask	T25 flask
D-PBS (+/+)	7.5 ml	2.5 ml
Coating Reagent 2 (Day11)	375 µl	125 µl

2. After warming EC Differentiation Basal Medium 2 (Day11) to 37℃, add Supplement 6 (Day11) (dilute 1 : 28). [For example, add 36 µl of Supplement 6 (Day11) per 1 ml of EC Differentiation Basal Medium 2 (Day11).]

Culture vessel	Per 3 T75 flasks	Per 3 T25 flasks
Supplement 6 (Day11)	1.44 ml	504 µl
EC Differentiation Basal Medium 2 (Day11)	40 ml	14 ml

3. Remove the culture medium from the culture vessel, then rinse with D-PBS (-/-).
4. Add 1.5 or 0.5 ml of Accumax Cell Dissociation Solution to a T75 or T25 flask, respectively. Incubate at 37℃ for 5 minutes.
5. Confirm that cells are detached by rocking or gently tapping the culture vessel.
6. Collect cells by adding 7.5 or 2.5 ml of DMEM medium containing 10% FBS and 1% Penicillin-streptomycin to a T75 or T25 flask, respectively, and transfer to a centrifuge tube.
7. After cell collection, add 7.5 or 2.5 ml of DMEM medium containing 10% FBS and 1% Penicillin-streptomycin into the same T75 or T25 flask, respectively. Gently tap the culture vessel, collect all remaining cells, and transfer into the centrifuge tube.
8. Centrifuge at 200*g* for 5 minutes.
9. Remove supernatant and add 1.5 ml (for one T75 flask) or 0.5 ml (for one T25 flask) of the medium prepared in Step 2. After gentle pipetting, count the number of cells.
10. Remove the coating solution from the new vessels and seed the cells into the new vessels at a concentration of 7.5 x 10^5 to 1.5 x 10^6 cells/12 ml for a T75 flask, or from 2.5 to 5 x 10^5 cells/4 ml for a T25 flask (1 - 2 x 10^4 cells/cm^2).

[Day 14]

Either perform a total medium change or subculture cells using MiraCell EC Culture Medium (Cat. # Y50053). Seed cells at a density of 1 - 2 x 10^4 cells/cm^2. Perform subsequent cell culture following the protocol for MiraCell EC Culture Medium. The subculture protocol is the same as that for [Day 11]. Use 1 mg/ml fibronectin (derived from human blood plasma; Merck, Cat. # F0895) for coating culture vessels.

VII. Applications

VII-1. Cryopreservation of Endothelial Cells

Endothelial cells can be cryopreserved beginning on Day 16. Suspend the cells in CELLBANKER 1 plus (Nippon Zenyaku Kogyo Ltd, Cat. # CB021) at >1.5 x 10^6 cells/ml and store them at -80℃ using a freezing container (BICELL, etc.). For cell thawing, follow the MiraCell EC Culture Medium thawing protocol.

VII-2. Tube Formation Test

iPS-derived endothelial cells produced by this kit can form capillary tube structures. Tube formation capability can be evaluated using the Angiogenesis Assay Kit (PromoCell GmbH, Cat. # PK-CA577-K905). See Figure 1.

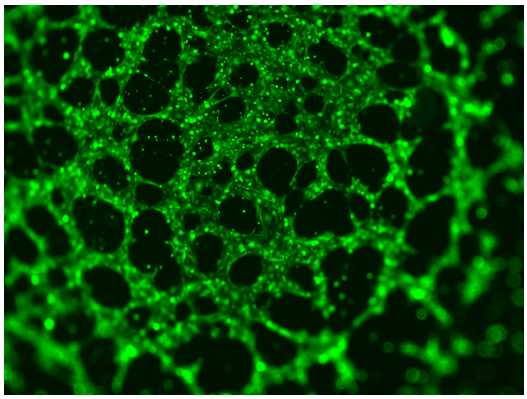


Figure 1. Endothelial cells were differentiated from ChiPSC21. Cells were cryopreserved on Day 16. At 8 days after thawing the cryopreserved cells, the assay was performed.

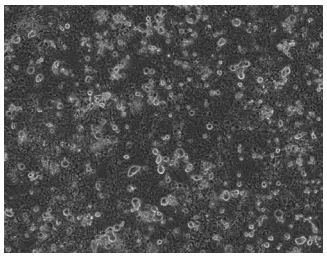
VIII. Troubleshooting

	Issue	Recommended action
Days 4 - 8	A large amount of cells are dead and cells are detaching.	Increase seeding density on Day 0 as cell death increases with low seeding density.
	Yellow-colored medium and/or over-confluent/ floating cells.	This is normal and will not affect the outcome.
Day 9	Large amounts of cell death and no attached cells.	• There is no problem if cells are 10 to 30% confluent. • If few adherent cells are observed, ensure you are using the recommended culture vessels (Note: no coating is necessary on Day 8.) • Delay subculture timing slightly on Day 8. • If you have a large number of samples, perform subculture by dividing into several samples. • Ensure pipetting and tapping of vessels is done gently.
Day 11	Cells did not proliferate after Day 9.	There is no issue; subculture cells even if cell density is low on Day 11.
Day 11 and after	Ratio of differentiated cells is low.	Perform medium changes once every 2 - 3 days with MiraCell EC Culture Medium on Day 14 and after.
		• Decrease seeding density and delay subculture timing on Day 8. • If the above change shows no improvement, ensure you are using the appropriate seeding density on Day 0.

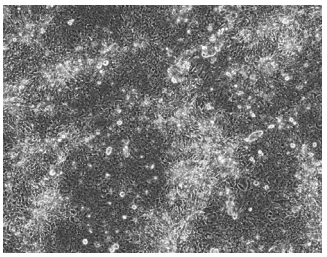
IX. Appendix: Example of Differentiation Induction with Cellartis Human iPS Cell Line 12 (ChiPSC12)

IX-1. Cell Morphology

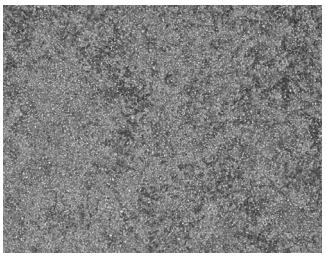
[Day 2]



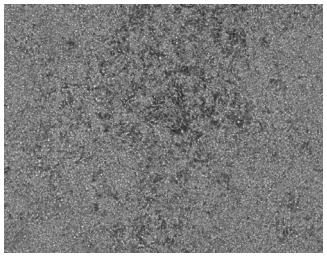
[Day 3]



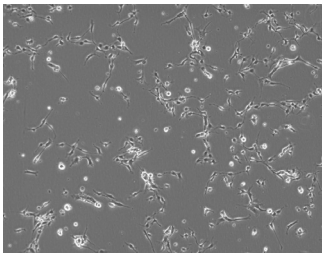
[Day 4]



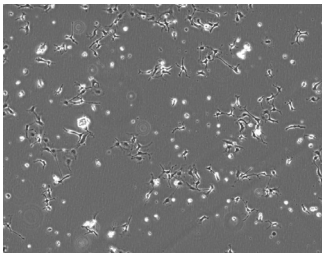
[Day 8]



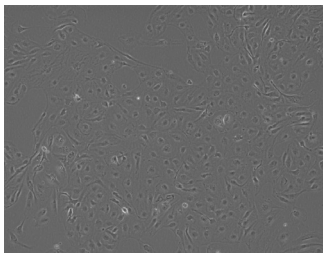
[Day 9]



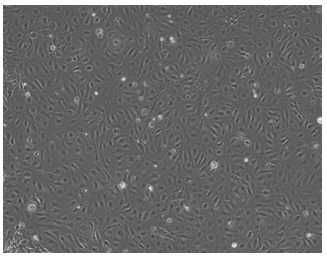
[Day 11]



[Day 14]

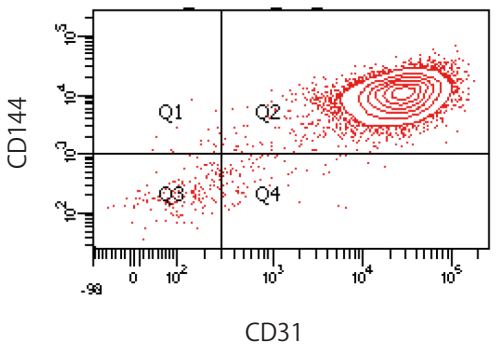


[Day 18]

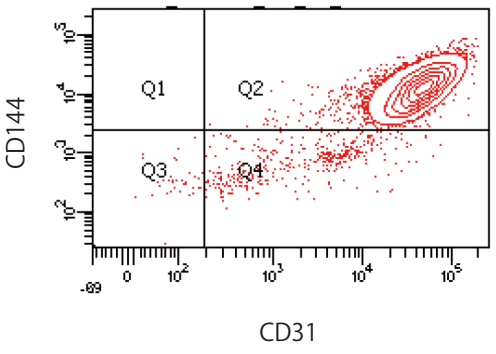


IX-2. Flow Cytometry Analysis

[Day 11]



[Day 18]



X. Related Products

Cellartis® Human iPS Cell Line 12 (ChiPSC12) Kit (Cat. # Y00285)
MiraCell® EC Culture Medium (Cat. # Y50053)*
MiraCell® Endothelial Cells (from ChiPSC12) Kit (Cat. # Y50055)*

* Not available in all geographic locations. Check for availability in your area.