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Protocol for Differentiation of Blood-Brain Barrier Endothelial Cells from Human Pluripotent Stem Cells V.2

BBB Endothelial Cells

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Neurodegeneration Method Development Community
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A Simplified, Fully Defined Differentiation Scheme for Producing Blood-Brain Barrier Endothelial Cells from Human iPSCs. Neal EH, Marinelli NA, Shi Y, McClatchey PM, Balotin KM, Gullett DR, Hagerla KA, Bowman AB, Ess KC, Wikswo JP, Lippmann S. Stem Cell Reports. 2019 Jun 11;12(6):1380-1388.doi: 10.1016/j.stemcr.2019.05.008

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Protocol status: Working
We use this protocol in our group and it is working.

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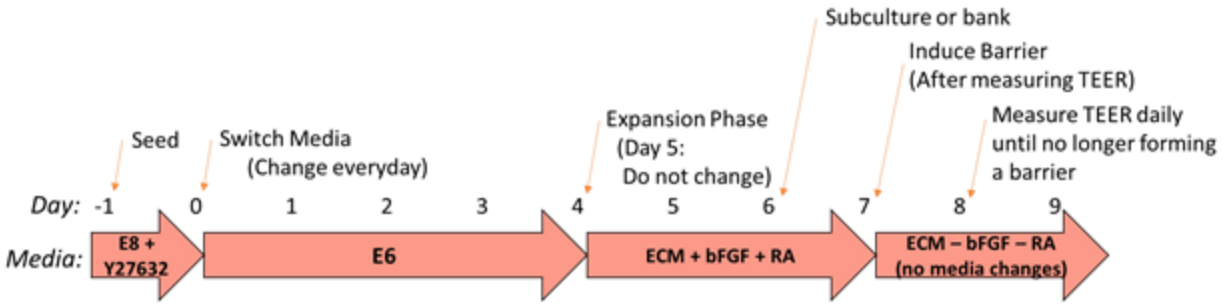
Keywords: human pluripotent stem cells, hPSCs, BBB, endothelial cells, blood-brain barrier, defined differentiation, human induced pluripotent stem cell, iPSC, in vitro model

Abstract

Human induced pluripotent stem cell (iPSC)-derived developmental lineages are key tools for in vitro mechanistic interrogations, drug discovery, and disease modeling. iPSCs have previously been differentiated to endothelial cells with blood-brain barrier (BBB) properties, as defined by high transendothelial electrical resistance (TEER), low passive permeability, and active transporter functions. Typical protocols use undefined components, which impart unacceptable variability on the differentiation process. We demonstrate that replacement of serum with fully defined components, from common medium supplements to a simple mixture of insulin, transferrin, and selenium, yields BBB endothelium with TEER in the range of 2,000-8,000 Ω × cm^2across multiple iPSC lines, with appropriate marker expression and active transporters. The use of a fully defined medium vastly improves the consistency of differentiation, and co-culture of BBB endothelium with iPSC-derived astrocytes produces a robust in vitro neurovascular model. This defined differentiation scheme should broadly enable the use of human BBB endothelium for diverse applications.



Schematic of E6 method for BBB differentiation

Attachments

PDF

BBB_differentiation_...

233KB

Guidelines

We recommend the following antibodies to monitor BMEC differentiation:

Target antigen	Antibody species	Vendor	Clone or product number	Dilution
PECAM-1	Rabbit	Thermo Scientific	RB-10333P	1:10 (FC) 1:25 (ICC)
GLUT-1	Mouse	Thermo Scientific	SPM498	1:50 (ICC&FC)
Occludin	Mouse	Life Technologies	OC-3F10	1:100 (ICC) 1:50 (FC)
Claudin-5	Mouse	Life Technologies	4C3C2	1:100 (ICC) 1:50 (FC)
VE-Cadherin	Mouse	Santa Cruz Biotechnologies	F8	1:25 (ICC) 1:500 (FC)
E-cadherin	Goat	R&D Systems	AF648	1:100 (ICC&FC)
P-glycoprotein	Mouse	Life Technologies	F4	1:25 (ICC) 1:50 (FC)
Breast cancer resistance protein (BCRP)	Mouse	Millipore	5D3	1:25 (ICC) 1:50 (FC)
Multidrug resistance protein 1 (MRP1)	Mouse	Millipore	QCRL-1	1:100 (ICC) 1:50 (FC)
Glial fibrillary acidic protein (GFAP)	Rabbit	Dako	Z0334	1:500 (ICC)
βIII tubulin	Rabbit	Sigma	T2200	1:1000 (ICC)
Nestin	Mouse	Millipore	10C2	1:500 (ICC)
α smooth muscle actin (SMA)	Mouse	American Research Products	1A4	1:100 (ICC)
Platelet-derived growth factor β (PDGFRβ)	Rabbit	Cell Signaling	28E1	1:100 (ICC)

Lippmann, E. S.; Al-Ahmad, A.; Azarin, S. M.; Palecek, S. P.; Shusta, E. V. A retinoic acid-enhanced, multicellular human blood- brain barrier model derived from stem cell sources. *Sci. Rep.* 2014, 4, 4160.

Note

The VE-Cadherin antibody listed above is no longer appropriate.

Materials

MATERIALS

- B-27 Supplement **Gibco - Thermo Fisher Scientific Catalog #**17504044
- Essential 8™ Medium **Gibco - Thermo Fisher Scientific Catalog #**A1517001
- Insulin solution human **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I9278
- Recombinant Human FGF-basic (154 a.a.) **peprotech Catalog #**100-18B
- Human Endothelial-SFM **Thermo Fisher Catalog #**11111044
- Versene Solution **Thermo Fisher Catalog #**15040066
- Essential 6™ Medium **Thermo Fisher Scientific Catalog #**A1516401
- Human Holo-Transferrin Protein CF **R&D Systems Catalog #**2914-HT-001G
- Retinoic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**R2625-50MG

If desired, E8 and E6 may be purchased commercially rather than prepared in-house.
If purchasing E8 and E6 commercially, human holo-transferrin and human insulin solution are not needed.

Plasticware:

FISHER
Corning Tissue Culture Plates (6- or 12-well, 3513 or 3516)
500 ml filter-top bottles (S2GPT05RE)

Safety warnings

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

Before start

REAGENT/MEDIUM PREPARATION:

E4 (prepared according to E4 large scale basal media production protocol)

Large batch previously prepared and stored at -80 °C. If the preparation is not urgent, remove a bottle of E4 from the -80°C and place it in the fridge overnight, which will allow the bottle slowly thaw. However, if preparation is desired for the same day, remove a bottle of E4 from the -80°C, place it on the countertop for ~ 00:20:00, then place it in the 37 °C water bath until it thaws completely (~ 01:00:00 – 02:00:00). Make sure to follow these steps precisely, as premature addition of a frozen bottle of E4 to a water bath can result in rupture of the plastic bottle.

Insulin

Pre-made solution provided by Sigma (catalog #I9278) that needs no additional preparation. Bottles are stored at 4 °C.

Transferrin

Comes as a powder from R&D Systems (Human Holo-Transferrin, CF; catalog #2914-HT-001G). Add 50 mg of transferrin to 5 mL of phosphate-buffered saline (PBS), aliquot at 500 µl/vial, and store at -80 °C. This mixture does not need to be sterile-filtered.

E6 media (prepared according to E6 and E8 media preparation protocol)

Dispense the thawed bottle of E4 into a bottletop filter attached to a 500 ml glass bottle. Add 100 µL of insulin solution and 500 µL of transferrin solution. Vacuum filter and store at 4 °C. E6 media is stable indefinitely.

B27 Supplement

Thaw 10 ml bottle and mix thoroughly. Aliquot into sterile microcentrifuge tubes at 280 µl/tube and store at -20 °C. Upon thawing, unused portions of an aliquot may be stored at 4 °C for up to 1 week for further media preparation.

bFGF, 100 µg/ml (prepared according to E8 media protocol)

Thaw a 500 µl aliquot of bFGF and dilute 1:5000 in EC medium for a final concentration of [M] 20 Mass Percent as described below. Divide the remaining bFGF in 100 µl aliquots and re-freeze at -80 °C. These remaining aliquots can be thawed and used for EC medium but cannot be refrozen a second time.

Retinoic acid (RA)

Dilute 50 mg RA in 16.6 mL DMSO to create a stock solution of 10 Mass Percent and store 1 mL aliquots at -80 °C. To prepare working stocks, divide a 1 ml stock tube into 50 µl aliquots and store at -20 °C. Dilute working stocks 1:1000 in EC medium for a final concentration of 10 micromolar (µM).

EC medium w/ 200X B27 + 20 ng/ml bFGF

For 50 ml: add 250 µL of B27 and 10 µL bFGF to 50 mL of hESFM.

Good for up to two weeks at 4 °C.

EC medium w/ 200X B27

For 50 ml: add 250 µL of B27 to 50 mL of hESFM.

BBB differentiation (Day 0–4)

1

Note

Note: Cells are seeded for differentiation in E8 medium according to the standardized single cell seeding protocol

On **day 0**, aspirate E8 medium and add 2 mL of E6 per well.

2 Change medium every day using 2 mL of E6 per well.

BBB expansion (Day 4–6)

3 At **day 4** of E6 treatment, aspirate and add 2 mL of EC medium with bFGF (basic fibroblast growth factor) and [M] 10 micromolar (µM) RA to each well.

Note

Medium is NOT changed during expansion phase.

4 BBB subculturing:
On **day 6**, subculture BBB onto plates and Transwell filters according to the following protocol:

Protocol

BBB Endothelial Cells

NAME

Protocol for Subculture of Differentiated Blood-Brain Barrier Endothelial Cells onto Plates and Filters

CREATED BY
Emma Neal

PREVIEW

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4.1 Please select between subculturing onto **plates** or **filters**.

STEP CASE

Plates 15 steps

Subculturing onto Plates using Accutase.

4.2 Coat plates with ECM plate solution for at least 01:00:00 at 37 °C. Volume depends on plate type (see Table):

	Plate type for subculture phase	Volume of ECM solution for coating	Working volume of EC media for cell culture
	6-well	800 µl	2 ml
	12-well	250 µl	1 ml
	24-well	200 µl	500 µl
	48-well	100 µl	400 µl

96-well	50 µl	200 µl

Note

If desired, plates may be coated Overnight. If coating overnight, add necessary volume of ECM and an equal volume of ddH_2O to each well to prevent excessive evaporation. If using glass plates, overnight incubation is needed to achieve adequate protein adsorption.

4.3 Aspirate plates and allow to dry in sterile hood (place the plate in the back of the hood and leave the lid slightly ajar).

Note

Plates only need to dry for 00:05:00 (can be aspirated during accutase incubation). Do not over dry!

4.4 Retrieve cells from incubator and transfer equal volume of spent media to 15 ml conical corresponding to the number of wells being accutased.

Note

For example, if accutasing 4 wells, save 4 ml of spent media and discard the rest.

4.5 Wash each well once with 2 mL PBS.

4.6 Add 1 mL accutase (warmed to Room temperature) to each well.

4.7 Incubate at 37 °C, length of time depends on cell treatment:

STEP CASE

If cells have not been treated with RA 9 steps

4.8 If cells have not been treated with RA, incubate at 37 °C for 00:20:00, or until cells are dissociated from plate (whichever comes first).

4.9 Using p1000, collect cells, and spray gently over surface 2–3x to dislodge any remaining cells. Triturate briefly to break up cell clumps.

4.10 Add cells to 15 ml conical containing spent media.

4.11 Spin down cells at 1000 rpm, 00:04:00.

4.12 Aspirate media, and resuspend cells in appropriate volume of EC media. For 6- and 12-wells, cells are seeded based on a split ratio:

- 1 well of a 6-well plate is split to 1 well of a 6-well plate [1:1]
- 1 well of a 6-well plate is split to 3 wells of a 12-well plate [1:3]
- For smaller plates (24-, 48-, or 96-wells), seed 1 million cells/cm^2.
- Multiply split ratio by the working volume found in the table to arrive at total volume of EC media in which to resuspend cells.

4.13 Thoroughly triturate 3 – 4 times to yield single cell suspension.

4.14 Add appropriate volume of cells to each well.

4.15 Place plate in incubator, shaking plate back and forth to distribute cells evenly (do not swirl).

4.16 24 hours later (i.e., day 7), aspirate spent media and add appropriate volume of EC medium (**without bFGF or RA**).