BBB Endothelial Cells

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

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1 Works for me dx.doi.org/10.17504/protocols.io.8g5hty6

Neurodegeneration Method Development Community

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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

ATTACHMENTS

BBB_subculturing_protocol_-_B27_update.pdf

GUIDELINES

Additional notes:

- Cells should reach confluence 24 – 48 hours post-split.
- If not treated with RA, cells should reach baseline TEER reading of 200 – 250 ohms (empty filter not subtracted) when confluent. If retinoic acid is used, initial TEER at 24 hours post-split can range anywhere from 200 – 2000 ohms depending on the cell fidelity. If using RA, TEER can reach 2000 – 4000 ohms, depending on the cell line used.
- If using filters for permeability screens, 48 hours post-split is usually the optimal time (maximum TEER).

TROUBLESHOOTING (subculture onto plates and filters)

Problem: Large clumps of cells are accumulating on top of monolayer

Solutions:

1. Increase trituration, particularly if you are not triturating vigorously. Cell clumps can accumulate on the top of the monolayer and decrease viability, while dispersed cells are more likely to remain in solution and can be removed the following day during the medium change.
2. Try increasing length of enzyme treatment (i.e., if using 15 minutes accutase try 20 minutes). Singularized cells (as opposed to clumps of cells) are more likely to form an even monolayer.
3. Try adjusting initial cell density at start of differentiation (day 0). Clumps can indicate that initial cell density was too high.

Problem: Cells have not filled in (resulting in low- to zero TEER if using filters)

Solutions:

1. Treat cells more gently, too much trituration can hurt the cells (particularly if not using RA treatment, these cells tend to be more fragile).
2. Decrease enzyme treatment length (i.e., if using 15 minutes, try 10 minutes). If using accutase, try versene instead.
3. Try adding more cells to the filter. If using accutase, increase number of cells added to filter (i.e., if using 1 million cells try using 1.2 million cells).
4. Try adjusting initial cell density at start of differentiation (day 0). Low attachment to plates/filters can indicate that initial cell density was too low.

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Problem: Monolayer is intact but TEER is not spiking, even with RA addition

Solution:

Ensure that media is changed after 1 day post-split, and does not contain RA or bFGF. Addition of either RA or bFGF eliminates any potential spike in TEER.

MATERIALS

NAME	CATALOG #	VENDOR
B-27 Supplement	17504044	Gibco - Thermo Fischer
Gibco™ DPBS no calcium no magnesium	14190144	Thermo Fisher Scientific
Recombinant Human FGF-basic (154 a.a.)	100-18B	peprotech
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501	Thermo Fisher Scientific
Human Endothelial-SFM	11111044	Thermo Fisher
Retinoic acid	R2625-50MG	Sigma Aldrich
Fibronectin bovine plasma	F1141-5MG	Sigma Aldrich
Collagen from human placenta	C5533-5MG	Sigma Aldrich

MATERIALS TEXT

Plasticware

FISHER

Corning Tissue Culture Plates (6- or 12-well, 3513 or 3516)
500 ml filter-top bottles (S2GPT05RE)
Corning Transwell Polyester Filters (07-200-161)

Equipment

EVOM2 located in tissue culture room
Chopstick electrodes or EndOhm chamber located in tissue culture room

SAFETY WARNINGS

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

BEFORE STARTING

REAGENT/MEDIUM PREPARATION:

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 **20 ng/ml** 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.

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Retinoic acid (RA)

Dilute **50 mg** RA in **16.6 ml** DMSO to create a stock solution of **10 Milimolar (mM)** 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** .

Note that after subculture, purified BMECs are changed to EC medium containing 200X B27, but without bFGF.

Collagen IV, 1 mg/ml

Dissolve **5 mg** of collagen IV in **5 ml** of sterile-filtered **0.5 mg/ml** acetic acid.

ECM solution for plate and filters

Mix 5 parts sterile ddH_2O with 4 parts **1 mg/ml** collagen IV and 1 part fibronectin. Exact volume depends on the number of filters being coated (200 µl/filter) and number of plates being coated.

Subculturing (Day 6)

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

step case

Plates

Subculturing onto Plates using Accutase.

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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

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.

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).

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

4

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

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

5

Wash each well once with **2 ml** PBS.

6

Add **1 ml** accutase (warmed to **Room temperature**) to each well.

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7 

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

step case

If cells have not been treated with RA

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).

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

10 

Add cells to 15 ml conical containing spent media.

11 

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

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.

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

14 

Add appropriate volume of cells to each well.

15 

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

16 

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

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step case

Filters

Subculturing onto Filters using Accutase.

2

Note: owing to the complexity of filter washing and separate addition of media to the top and bottom chambers of filters, make sure you calculate the necessary volumes of EC media before starting the passaging step.

Coat Transwell filters with **200 µl** of ECM filter solution.

3

Incubate at least **04:00:00** in **37 °C** incubator, up to overnight.

4 Aspirate filters and allow to dry in sterile hood, similar to the description for plates. Filters should dry for at least **00:20:00** but no longer than **00:30:00** . If cells are still dissociating after **00:30:00** , move to step 10.1 before performing steps 5 – 9.

5

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

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

6

Wash each well once with **2 ml** PBS.

7

Add **1 ml** accutase (warmed to **Room temperature**) to each well.

8

Incubate at **37 °C** . The length of time depends on cell treatment:

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step case

If cells have been treated with RA

8 

If cells have been treated with RA, incubate at **37 °C** for **00:20:00** to **00:25:00** typically suffices, though up to **00:45:00** may be necessary for some differentiations.

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

10 

Add cells to 15 ml conical containing spent media.

11 

Spin down cells at **1000 rpm 00:04:00** .

12 

Aspirate media, and resuspend cells in appropriate volume of EC media (with or without **10 Micromolar (µM)** RA). 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 (with or without **10 Micromolar (µM)** RA) in which to resuspend cells.

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

14 

Add appropriate volume of cells to each well.

15 

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

16 

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

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step case

If cells have not been treated with RA

9

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). Do not go over **00:20:00** .

10 During accutase incubation, prepare filters:

1. Add **0.5 ml** hESFM to the top of each filter for approximately **00:05:00** to wet filters.
2. After **00:05:00** , aspirate hESFM from the top of each filter
3. Add **1.5 ml** EC medium (with or without **10 Micromolar (µM)** RA) to the basolateral chamber of each filter.

11

Add **0.5 ml** hESFM to the top of each filter for approximately **00:05:00** to wet filters.

12

After **00:05:00** , aspirate hESFM from the top of each filter.

13

Add **1.5 ml** EC medium (with or without **10 Micromolar (µM)** RA) to the basolateral chamber of each filter.

14 Following accutase incubation, use p1000 to collect cells and spray gently over surface 2–3x to dislodge any remaining cells. Triturate briefly to break up cell clumps.

15

Add accutased cells to the conical containing spent medium.

16

Spin down for **00:04:00** at **1000 rpm** .

17

Aspirate media, resuspend cells in appropriate volume of EC media (with or without **10 Micromolar (µM)** RA). Cells are seeded based on a 1:3 ratio (i.e. 1 well of a six well plate seeds three Transwell filters).

- Volume of EC media containing cells = # of wells of a 6 well plate being subcultured * 0.5 ml
- For 3 filters, resuspend cells in **1.5 ml** EC medium, for 6 use **3 ml** , etc.

18

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

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19 24 hours later (i.e., day 7), aspirate and change to EC medium **lacking bFGF and RA**.
1. Aspirate EC media from both chambers (do not touch monolayer with pipet tip!).
2. Gently add EC medium **lacking bFGF and RA**, **0.5 ml** in top chamber and **1.5 ml** in bottom chamber. **DO NOT include bFGF and RA in the medium** – these factors prevent the monolayer from tightening.

20 

Aspirate EC media from both chambers (do not touch monolayer with pipet tip!).

21 

Gently add EC medium **lacking bFGF and RA**, **0.5 ml** in top chamber and **1.5 ml** in bottom chamber. **DO NOT include bFGF and RA in the medium** – these factors prevent the monolayer from tightening.

step case

If cells have been treated with RA

9 

If cells have been treated with RA, incubate at **37 °C** for **00:20:00** – **00:45:00** , or until cells are dissociated from plate.

10 During accutase incubation, prepare filters:
1. Add **0.5 ml** hESFM to the top of each filter for approximately **00:05:00** to wet filters.
2. After **00:05:00** , aspirate hESFM from the top of each filter.
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16 

Spin down for **00:04:00** at **1000 rpm** .

17 

Aspirate media, resuspend cells in appropriate volume of EC media (with or without **10 Micromolar (µM)** RA). Cells are seeded based on a 1:3 ratio (i.e. 1 well of a six well plate seeds three Transwell filters).

- Volume of EC media containing cells = # of wells of a 6 well plate being subcultured * 0.5 ml
- For 3 filters, resuspend cells in **1.5 ml** EC medium, for 6 use **3 ml** , etc.

18 

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

19 24 hours later (i.e., day 7), aspirate and change to EC medium **lacking bFGF or RA**.

1. Aspirate EC media from both chambers (do not touch monolayer with pipet tip!).
2. Gently add EC medium **lacking bFGF or RA**, **0.5 ml** in top chamber and **1.5 ml** in bottom chamber. **DO NOT include bFGF or RA in the medium** – these factors prevent the monolayer from tightening.

20 

Aspirate EC media from both chambers (do not touch monolayer with pipet tip!).

21 

Gently add EC medium **lacking bFGF or RA**, **0.5 ml** in top chamber and **1.5 ml** in bottom chamber. **DO NOT include bFGF or RA in the medium** – these factors prevent the monolayer from tightening.

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