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Clonetics™ Conditionally Immortalized Human Brain Microvascular Endothelial Cell System

Cond. Immo. HMVEC-Br – Technical Information & Instructions

Contents:

I. Introduction

Immortalized cell lines offer the possibility of an inexhaustible supply of cells that can be used as models of animal or human tissues. However, a major limitation of current technology is that the available models are almost invariably poor representations of cells found in intact tissues. One major reason for this is that the process that allows the cells to become immortal also has an impact on the characteristics of the cell. Lonza overcomes this limitation using one and/or two patented technologies; human telomerase reverse transcriptase (hTERT) and temperature sensitive Large-T antigen (tsSV40 LTag). The temperature dependent conditional immortalization approach utilizing the large T antigen allows the immortalization to be reserved and the cells to exhibit a range of differentiated functions. hTERT provides chromosomal stability over many cell divisions while maintaining the in vivo nature of primary cells. These technologies allow the development of cell lines from a wide range of human and animal tissues and of novel models relevant to drug discovery.

Conditionally Immortalized HMVEC-Br cells represent a mixed population of human adult brain microvascular endothelial cells isolated from a 60 year old female and were immortalized using tsSV40 LTag and hTERT. These cells become irreversibly growth arrested after differentiation at 37℃.

Similar to primary microvascular endothelial cells, Conditionally Immortalized HMVEC-Br displays a healthy, primary cell-like spindle shape morphology through subsequent passages, expresses a number of endothelial cell markers (CD34+, CD146+, CD105+, CD90-, CD31+, VEGF-RI+, VWF+), maintain contact inhibition and form homogeneous cell monolayers. Upon confluency, the cells display specific submembranous expression of tight junction proteins ZO-1, Cadherin-5, b-Catenin, g-Catenin, and p120 Catenin.

II. General Cell Information

Cat. No.	Description	Recommended Growth Media	Cryopreserved Passage Number	Immortalizing Genes	Seeding Density Upon Thaw*	Time to Subculture
00194607	Brain Microvascular Endothelial	EGM™-2MV BulletKit™ Medium	Passage 12	tsSV40 LTag + hTERT	3,000 – 5,000 cells/cm^2	4-6 days

*Please note that alternative seeding densities may be required for subculture and/or differentiation.

III. Quality Control

Cat. No.	Description	Cells/Vial	Viability	Maximum Productive Population Doublings	Average Doubling Time	Properties
00194607	Brain Microvascular Endothelial	≥500,000 cells	≥70%	>150 PD	45 hrs	CD34+, CD146+, CD105+, CD90-, VEGF-RI +, VWF +, and CD31+; express P-gp; ZO-1ant other TJ proteins

All cells are performance assayed and test negative for HIV-1, mycoplasma, Hepatitis-B, Hepatitis-C, bacteria, yeast and fungi. Cell viability, morphology, cell number, and proliferative capacity are measured after recovery from cryopreservation. Clonetics™ Media are formulated for optimal growth of specific types of human cells. COAs for all media products are available upon request. Please see Section XIV (Product Warranty, Page 7) for more information on Quality Control claims and guarantees.

IV. Cell Growth System Components
(Sold Separately)

- One conditionally immortalized brain microvascular endothelial cell product – (cryopreserved)
- One Microvascular Endothelial Cell Media BulletKit™ Medium - 500 ml

  Clonetics™ EGM™-2MV BulletKit™ (Lonza Catalog No. CC-3202) contains 500 ml of Endothelial Basal Medium-2 (EBM™-2 Medium) and the following growth supplements: human Epidermal Growth Factor (hEGF), 0.5 ml; Vascular Endothelial Growth Factor (VEGF), 0.5 ml; R3-Insulin-like Growth Factor-1 (R3-IGF-1), 0.5 ml; Ascorbic Acid, 0.5 ml; Hydrocortisone, 0.2 ml; human Fibroblast Growth Factor-Beta (hFGF-β), 2.0 ml; Fetal Bovine Serum (FBS), 25.0 ml; Gentamicin/Amphotericin-B (GA), 0.5 ml
- One Trypsin EDTA – 100 ml (Lonza Catalog No. CC-5012)
- One HEPES Buffered Saline Solution (HEPES-BSS) – 100 ml (Lonza Catalog No. CC-5022)
- Rat Tail, Type I Collagen (BD Biosciences Catalog no. 354236 or similar)
- Glacial Acetic Acid (Sigma Catalog No. 27225, or similar)
- Phosphate Buffered Saline Solution (PBS) – 500 ml (Lonza Catalog No. 17-516F, or similar)

NOTE: Additional components are necessary for the cryopreservation of these cells. Please see the corresponding selection below for more information.

V. Unpacking and Storage Instructions

1. Check all containers for leakage or breakage.
2. For cryopreserved cells: Remove cryovials from the dry ice packaging and immediately place into liquid nitrogen storage. Alternatively, thaw and use the cells immediately. If no dry ice remains, please contact Customer Service.
3. BulletKit™ Medium instructions: store basal medium at 2°-8℃ and SingleQuots ™ Kit at ≤-20℃ in a freezer that is not self-defrosting. Once thawed, SingleQuots™ Kit should be stored at 2°-8℃ and added to basal medium within 72 hours. After SingleQuots™ Kit is added to basal medium, use within 1 month. Do not re-freeze.
4. The Trypsin/EDTA Solution and HEPES-BSS are sterile-filtered and then stored at –20℃ until shipment. Store Trypsin/EDTA

and HEPES-BSS at ≤-20℃ in a freezer that is not self-defrosting. Trypsin/EDTA Solution has a limited shelf life or activation at 4℃. Trypsin/EDTA and HEPES-BSS may thaw during transport .If, upon arrival, Trypsin/EDTA is thawed, store at 4℃ and use within 3 days or immediately aliquot and refreeze at –20℃. If, upon arrival, HEPES-BSS is thawed, store at 4℃ and use within one month or immediately aliquot and refreeze at –20℃. Trypsin/EDTA and HEPES-BSS may be thawed and refrozen once.

5. Rat Tail, Type I Collagen can be stored at 2°-8℃. Do not freeze.
6. Phosphate Buffered Saline can be stored at ambient temperature.

NOTE: To keep Trypsin/EDTA fresh and active after thawing, you may aliquot it into sterile centrifuge tubes and re-freeze at –20°C.

Using media or reagents other than what is recommended will void the cell warranty. Please contact Scientific Support if you need help selecting media and/or reagents.

VI. Preparation of Culture Media

1. Decontaminate external surfaces of all vials, including the medium bottle, with ethanol or isopropanol.
2. To formulate Microvascular Endothelial Growth Media-2 (EGM™-2MV Medium), transfer the contents of the EGM™-2MV SingleQuots™ Kit (Catalog No. CC-4147 containing human Epidermal Growth Factor [hEGF], Vascular Endothelial Growth Factor [VEGF], R3-Insulin-like Growth Factor-1 [R3-IGF-1], Ascorbic Acid, Hydrocortisone, human Fibroblast Growth Factor-Beta [hFGF-β], Fetal Bovine Serum [FBS], and Gentamicin/Amphotericin-B [GA]) to EBM™-2 Basal Medium with a pipette, and rinse each vial with medium.
3. When preparing these BulletKit™ Media, it may not be possible to recover the entire volume listed for each vial. Small losses (up to 10%) should not affect the cell growth characteristics of the supplemented medium.
4. Transfer the label provided with each kit to the basal medium bottle(s) being supplemented (avoid covering the basal medium lot # and expiration date). Use it to record the date and amount of each supplement added. After SingleQuots™ Kit is added to basal medium, store at 2°-8℃ and use within 1 month. Do not freeze medium.

NOTE: If there is concern that sterility was compromised during the supplementation process, the entire newly prepared growth medium may be re-filtered with a 0.2 µm filter to assure sterility. Routine re-filtration is not recommended.

VII. Collagen Plate Coating

Collagen Plate Coating Components
(Sold Separately)

- Rat Tail, Type I Collagen (BD Biosciences Catalog no. 354236 or similar)
- Glacial Acetic Acid (Sigma Catalog No. 27225, or similar)
- Phosphate Buffered Saline Solution (PBS) – 500 ml (Lonza Catalog No. 17-516F, or similar)

1. Prepare a 0.02N Acetic Acid stock solution by adding 1.0 ml of glacial acetic acid to 870 ml of sterile tissue grade water. Gently swirl to mix.
2. Pour 1.0 ml of 0.02N Acetic Acid stock solution into a sterile beaker for every 10 cm^2 of surface area of the vessels intended to be coated (i.e. four T-25 would require 10 ml of 0.02N Acetic Acid stock solution or two T-75 flasks would require 15 ml of 0.02N Acetic Acid stock solution).
3. Create a 30 µg/ml collagen stock solution by adding rat tail, type I collagen to the 0.02N Acetic Acid stock solution (i.e. add 300 µg rat tail, type I collagen to 10 ml of 0.02N Acetic Acid stock solution or add 450 µg rat tail, type I collagen to 15 ml of 0.02N Acetic Acid stock solution).
4. Using pipette tips, add 1.0 ml of 30 µg/ml collagen stock solution per every ten square centimeter of culture surface area (i.e. 2.5 ml of 30 µg/ml collagen stock solution per T-25 flask or 7.5 ml of 30 µg/ml collagen stock solution per T-75 flask).
5. Incubate the vessels at room temperature for one hour, then aspirate the collagen stock solution using a sterile pipette.
6. After incubation, thoroughly wash vessels with PBS.

7. Collagen coated vessels may be used immediately or may be allowed to air-dry in the hood for two hours or until completely dry, then stored at 2°-8°C for up to three days.

VIII. Thawing of Cells / Initiation of Culture Process

NOTE: For proliferation of these cells, cells must be cultured at 33°C±1°C, 5% CO_2, 90%±2% humidity. Culturing these cells at 37°C will cause permanent and irreversible differentiation.

1. The recommended seeding density when initially thawing Conditionally Immortalized HMVEC-Br from cryopreservation is 3,000-5,000 cells/cm^2. One ampoule of Conditionally Immortalized HMVEC-Br containing ≥500,000 cells contains enough cells to plate two T-75 flasks.
2. To set up culture vessels, calculate the number of vessels needed based on the recommended seeding density and the surface area of the vessels being used and coat with collagen as described in Section VII (Collagen Plate Coating, Page 3).
3. Add the appropriate amount of medium to the vessels (1 ml/5 cm^2) and allow the vessels to equilibrate in a 33°C±1°C, 5% CO_2, 90%±2% humidity incubator for at least 30 minutes.
4. Prior to thawing cells, add 9 ml of culture medium to a 15 ml sterile centrifuge tube and allow the vessels to equilibrate to room temperature.
5. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure and then retighten. Quickly thaw the cryovial in a 37°C water bath being careful not to submerge the entire vial. Watch your cryovial closely; when the last sliver of ice melts, remove it. Do not submerge it completely. Thawing the cells for longer than 2 minutes results in less than optimal results.
6. Using a micropipette, gently add the thawed cell suspension to the previously prepared 15 ml sterile centrifuge tube containing 9 ml of medium equilibrated to room temperature.
7. Centrifuge at 150 x g for 5 minutes at room temperature.
8. Carefully discard the supernatant and resuspend the pellet in 1 ml of medium using a micropipette. Dispense cells into the culture vessels set up earlier. Gently rock the culture vessel to evenly distribute the cells and return to the 33°C±1°C, 5% CO_2, 90%±2% humidity incubator.
9. Change the growth medium the day after seeding.

IX. Maintenance

1. Change the growth medium the day after seeding and every 2 days thereafter.
2. Increase the media volume by 2 ml/75 cm^2 of culture area with each successive feeding.
3. Warm an appropriate amount of medium to 33°C in a sterile container. Remove the medium and replace it with the warmed, fresh medium and return the flask to the incubator.
4. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer and warm only the required volume to a sterile secondary container.

X. Subculturing

NOTE: Lonza warrants its Clonetics™ Cells only if Lonza Subculturing Reagents are used. The recommended subculturing reagents for these cells are Trypsin/EDTA (CC-5012) and HEPES-BSS (CC-5022).

The following instructions are for a 25 cm^2 flask. Adjust all volumes accordingly for other size flasks.

1. Subculture the cells when they are 70%-90% confluent.
2. For each 25 cm^2 of cells to be subcultured:
   a. Thaw 2 ml of Trypsin/EDTA and allow to come to room temperature.
   b. Allow 5 ml of HEPES Buffered Saline Solution (HEPES-BSS) to come to room temperature.
   c. Remove growth medium from 4°C storage and allow warming to room temperature.
   d. Prepare new culture vessels.
3. Subculture one flask at a time. All flasks following the first flask will be subcultured following an optimization of this protocol (explained later in this procedure), based on calculated cell count, cell viability, and seeding density.

NOTE: The following steps must be performed in a sterile field.

4. Aspirate the medium from one culture vessel.
5. Rinse the cells with 5 ml of room temperature HEPES-BSS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
6. Aspirate the HEPES-BSS from the flask.
7. Cover the cells with 2 ml of Trypsin/EDTA solution.
8. Place the culture vessels into a 33°C humidified incubator for 3-5 minutes. Periodically examine the cell layer microscopically and check for cell detachment.
9. Allow the trypsinization to continue until approximately 90% of the cells are rounded up.
10. At this point, tap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and tap again. If cells still do not detach, wait and tap every 30 seconds thereafter. This entire process should take no more than 5 minutes.
11. After cells are released, quickly transfer the detached cells to a sterile 15 ml centrifuge tube containing 4 ml of growth medium.
12. Rinse the flask with a final 1-2 ml of growth medium to collect residual cells, and add this rinse to the centrifuge tube.
13. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%.
14. Centrifuge the harvested cells at 150 x g for five minutes to pellet the cells.
    a. Aspirate most of the supernatant, except for 100-200 µl.
    b. Flick the cryovial with your finger to loosen the pellet.
15. Dilute the cells to a final volume of 2 to 3 ml of growth medium and note the total volume of the diluted cell suspension.
16. Determine cell count and viability using a hemacytometer and Trypan Blue or cell counter. Make a note of your cell yield for later use.
17. If necessary, dilute the suspension with growth medium to achieve the desired “cells/ml” and re-count the cells.
18. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

19. The number of flasks needed depends upon cell yield and seeding density. If seeding into flasks for further proliferation or differentiation, the recommended density is 3,000 -5,000 cells/cm^2. Determine the total number of flasks to inoculate by using the following equation.

$$\text{Total \# of Flasks to innoculate} = \frac{\text{Total \# of viable cells}}{\text{Growth area} \times \text{Rec. Seeding Density}}$$

20. Use the following equation to calculate the volume of cell suspension to seed into your flasks.

$$\text{Seeding Volume} = \frac{\text{Total volume of diluted cell suspension}}{\text{\# of flasks as determined in step 18}}$$

21. Prepare flasks by labeling each flask with the passage number, cell type, and date.
22. Coat each vessel with collagen as described in Section VII (Collagen Plate Coating, Page 3).
23. Carefully transfer growth medium to new collagen coated culture vessels by adding 1 ml growth medium for every 5 cm^2 surface area of the flask (1 ml/5 cm^2) for further culturing of the cells or for differentiation of the cells.
24. After mixing the diluted cells with a 5 ml pipet to ensure a uniform suspension, dispense the calculated volume into the prepared subculture flasks.
25. If not using vented caps, loosen caps of flasks. Place the new culture vessels into a 33℃±1℃, 5% CO_2, 90%±2% humidity incubator.

XI. Cryopreservation

NOTE: Cryopreservation may compromise cell quality and performance.

Cryopreservation Media:

Description	Base Media	DMSO	FBS
Cond. Immo. HMVEC-Br	70% EGM™-2MV	10% DMSO	20% FBS

1. Prepare cryopreservation media according to the chart listed above and chill to 4℃.

2. Prepare freezing vials or ampoules by labeling each with the passage number, cell type and date.
3. Sterile filter cryopreservation media using a 0.2 micron filter
4. Harvest and centrifuge cells according to steps 1 to 14 of Section X (Subculturing, Page 4).
5. Resuspend cells in cold cryopreservation media at ≥700,000 cells per ml.

NOTE: Work Quickly! Once exposed to the DMSO, cells become very fragile.

6. Pipet aliquots (1 ml each) into freezing vials or ampoules and seal.
7. Insulate aliquots with Styrofoam or propanol freezing canister.
8. Store cells at -80°C overnight.
9. Within 12 to 24 hours, place cells in liquid nitrogen (-200°C) for long-term storage. Cells will be compromised by storage in -80°C.

XII. Differentiation

Differentiation Components (Sold Separately)

- One conditionally immortalized brain microvascular endothelial cell product – (cryopreserved)
- One Microvascular Endothelial Cell Media BulletKit™ Medium - 500 ml (prepared as described in Section VI [Preparation of Culture Media, Page 3)

NOTE: For differentiation of these cells, cells must be cultured at 37°C±1°C, 5% CO_2, 90%±2% humidity. Culturing these cells at 37°C will cause permanent and irreversible differen tiation.

1. The recommended seeding density when plating Conditionally Immortalized HMVEC-Br for differentiation is 3,000-5,000 cells/cm^2.
2. To set up culture vessels, calculate the number of vessels needed based on the recommended seeding density and the surface area of the vessels being used.
3. Coat each vessel with collagen as described in Section VII (Collagen Plate Coating, Page 3).
4. Add the appropriate amount of growth medium (EGM™-2MV Medium) to the vessels (1 ml/5 cm^2) and allow the vessels to equilibrate in a 33°C±1°C, 5% CO_2, 90%±2% humidity incubator for at least 30 minutes
5. Subculture cells according to Section X (Subculturing, Page 4).
6. Culture cells under standard culturing conditions in a 33°C±1°C, 5% CO_2, 90%±2% humidity incubator in growth medium (EGM™-2MV Medium).
7. When culture has achieved desired confluence, transfer flasks to 37°C±1°C, 5% CO_2, 90%±2% humidity incubator.
8. After 24-48 hours in 37°C incubator, differentiation will have occurred.
9. If the microvascular endothelial cells are to be used in assays that require an extended period in culture following differentiation, replace the growth medium every other day to maintain the cultures for ~2 to 3 weeks post differentiation.

XIII. Ordering Information

Cryopreserved Brain Microvascular Endothelial Cells:

Cat. No.	Product	Description
00194607	Cond. Immo. HMVEC-Br	≥500,000 cells

Endothelial Growth Media (Sold Separately):

Cat. No.	Product	Description
CC-3202	EGM™-2MV BulletKit™ Medium	500 ml EBM™-2 Basal Medium plus CC-4147 SingleQuots™ Kit to formulate EGM™-2MV Medium (growth medium)
CC-3156	EBM™-2 Basal Medium	Endothelial basal medium-2 (500 ml)
CC-4147	EGM™-2MV SingleQuots™ Kit	Formulates 500 ml of EBM™-2 Basal Medium to EGM™-2MV Growth Medium; contains hEGF, 0.5 ml; VEGF, 0.5 ml; R3-IGF-1, 0.5 ml; Ascorbic Acid, 0.5 ml; Hydrocortisone, 0.2 ml; hFGF-β, 2.0 ml; FBS, 25.0 ml; GA, 0.5 ml

Subculturing Reagents (Sold Separately):

Cat. No.	Product	Description
CC-5012	Trypsin/EDTA Solution	100 ml
CC-5022	HEPES-BSS (1X)	HEPES Buffered Saline Solution (1X) (100 ml)

Collagen Plate Coating Reagents
(Sold Separately):

Cat. No.	Product	Description
17-516F	PBS (1X)	Phosphate Buffered Saline Solution (1X) (500 ml)

Additional components are required for collagen plate coating please see Section VIII (Collagen Plate Coating, Page 3) for a complete listing of required components.

XIV. Product Warranty

Cultures have a finite lifespan *in vitro.*

Lonza guarantees the performance of its cells in the following manner only if Clonetics™ Media and Reagents are used exclusively, and the recommend protocols are followed. The performance of cells is not guaranteed if any modifications are made to the complete cell system.

1. Conditionally Immortalized HMVEC-Br will become irreversibly differentiated if cultured at 37℃. To avoid the loss of your cells and forfeiture of your warranty, proliferation of these cells must occur at 33℃.

When placing an order or for Scientific Support, please refer to the product numbers and descriptions listed above. For a complete listing of all Clonetics™ Products, refer to the Lonza website or the current Lonza catalog. To obtain a catalog, additional information or want to speak with Scientific Support, you may contact Lonza by web, e-mail, telephone, fax or mail
(See page 1 for details).

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* procedures.

WARNING: CLONETICS™ AND POIETICS™ PRODUCTS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS. Each donor is tested and found non-reactive by an FDA-approved method for the presence of HIV-I, hepatitis B virus and hepatitis C virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV, hepatitis B virus, and hepatitis C virus. Testing cannot offer complete assurance that HIV-1, hepatitis B virus, and hepatitis C virus are absent. All human-sourced products should be handled at the biological safety level 2 to minimize exposure of potentially infectious products, as recommended in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 5th ed. If you require further information, please contact your site safety officer or Scientific Support.