



Human Endothelial-SFM

Cat. No.: 11111

500 mL

Storage Conditions: 2 to 8°C, protect from light.

CAUTION: Human origin materials are non-reactive (donor level) for anti-HIV 1 & 2, anti-HCV, and HB_sAg. Handle in accordance with established bio-safety practices.

Background

The ability to cultivate vascular endothelial cells *in vitro* has led to the development of many useful models for the study of endothelial cell physiology. The use of such models has greatly advanced our understanding of vascular endothelial cell physiology under both normal and pathological states. However, much of our knowledge of endothelial cell function has resulted from *in vitro* experimentation utilizing serum-supplemented media¹. The use of high concentrations of animal sera creates many obstacles, such as lot-to-lot performance variability, presence of adventitious agents, and fluctuations in price and availability. The presence of serum can mask a desired effect or detection of a mediator present in low quantities. Additionally, the undefined character of serum makes it undesirable for media supplementation as endothelial cell research moves towards therapeutic applications^{2,3,4}.

Human Endothelial Serum Free Medium (SFM) was initially developed to support the long-term propagation of human umbilical vein endothelial cells (HUVEC)^{5,6}. This formulation has been demonstrated to successfully support primary isolation and subsequent secondary growth of HUVEC for up to 15 passages. HUVEC cultured in Human Endothelial-SFM exhibit the histotypic "cobblestone" morphology and retain endothelial specific markers including: expression of Factor VIII-related antigen, UEA-1 lectin binding, uptake of DiI-acetylated LDL, vimentin and IL-1 α -induced ICAM-1 expression. Additionally, HUVEC cultured in SFM have been shown to maintain cAMP and prostacyclin signal transduction systems. When supplemented with human recombinant basic fibroblast growth factor (hrbFGF) and human recombinant epidermal growth factor (hrEGF) the growth rate of HUVEC is similar to that obtained for cells cultured in Medium 199 supplemented with 20% FBS, acidic FGF and heparin.

Human Endothelial-SFM has also been shown to support the growth and retention of physiological markers for human umbilical arterial and dermal microvascular endothelial cells.⁷ Outlined below are basic instructions and procedures which should be followed when culturing human endothelial cells in SFM.

Preparation and Use of Growth and Attachment Factors

***Attachment Factor:** Human plasma fibronectin is recommended (GIBCO Cat. No. 33016).

Preparation: Reconstitute in sterile distilled water to yield a 1 mg/mL stock solution. Complete solubilization will take approximately 30 minutes at room temperature with periodic gentle swirling (**do not vortex, heat or filter sterilize**).

Storage: Aliquot at appropriate volumes into sterile polypropylene vials and store at -70°C.

Avoid subjecting to repeated freeze-thaw cycles.

Usage: For secondary cultures, human plasma fibronectin can be added directly to the culture vessel containing growth medium prior to the addition of cells. Fibronectin is required when passaging cells, but it is not required for fluid changes. When establishing primary cultures, better cell attachment and spreading is achieved when tissue flasks are pretreated with fibronectin (see isolation procedures for further details). We recommend using fibronectin at a concentration of 20 μ g/mL for the establishment of primary cultures and at a concentration of 10 μ g/mL for secondary cultures. If an attachment factor other than human plasma fibronectin is desired, it should be titrated to determine the optimal concentration required for cell attachment and spreading. **Do not add fibronectin to the bottle of culture medium.** There is a sufficient amount of fibronectin to supplement a total of 200 mL of media. The remaining 300 mL of media is used to feed the cell cultures. For additional information please consult manufacturers' product insert.

***We have found that human plasma fibronectin was not optimal for arterial cell growth past 48 hours or for extended subculturing (>3 passages). We recommend plating cells in SFM supplementing with 5% FBS for 2 hours at 37°C after which time the serum-supplemented medium can be removed and fresh SFM and growth factors added. For use of additional attachment factors please see reference #7.**

***Growth Factor:** Human recombinant basic FGF (GIBCO Cat. No. 13256).

Preparation: Reconstitute in Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO Cat. No. 14190) containing a minimum of 0.1% human serum albumin to yield a stock solution of 2 μ g/mL. **Do not filter sterilize.**

Storage: Aliquot at appropriate volumes into sterile polypropylene vials and store at -70°C.

Avoid subjecting to repeated freeze-thaw cycles.

Usage: hrbFGF should be added directly to the culture medium prior to the addition of cells and should **not** be added to the bottle of culture medium. We recommend using hrbFGF at a final concentration of 20 ng/mL. For additional information please consult manufacturers' product insert.

***Growth Factor:** Human recombinant EGF (GIBCO Cat. No. 13247). **Preparation:** Reconstitute in D-PBS containing a minimum of 0.1% human serum albumin to yield a stock solution of 2 μ g/mL. **Do not filter sterilize.**

Storage: Aliquot at appropriate volumes into sterile polypropylene vials and store at -70°C.

Avoid subjecting to repeated freeze-thaw cycles.

Usage: hrEGF should be added directly to the culture medium prior to the addition of cells and should **not** be added to the bottle of culture medium. We recommend using hrEGF at a final concentration of 10 ng/mL. For additional information please consult manufactures' product insert.

***We have found that Endothelial Cell Growth Supplement used at a concentration of 25-50 μ g/mL is toxic to HUVEC cultured in SFM. If growth factors other than hrbFGF and hrEGF are used, they should be titrated to determine the optimal concentration required to support endothelial cell growth.**

Isolation and Establishment of Primary Cultures

For isolation of HUVEC we use a modification of the procedure described by Jaffe et al.⁸. Briefly, untraumatized umbilical cord segments are cannulated and flushed with 50 mL of Medium 199 supplemented with penicillin (10 U/mL) and streptomycin (10 μ g/mL) (please see Additional Considerations for the use of antibiotics). Endothelial cells are isolated by incubating umbilical veins with 0.1% collagenase (GIBCO Cat. No. 17101) in Medium 199 for 25 minutes at 22°C. Cords are then flushed with 50 mL of Medium 199, the cell suspension centrifuged (100 X g, 22°C for 5 minutes) and the cell pellet washed two times in Medium 199. The cell pellet should be resuspended in 2 mL of Human Endothelial-SFM. Primary cultures are established in 25 cm² tissue culture flasks (please see Additional Considerations) preincubated with human plasma fibronectin (20 μ g/mL, 100 μ g/flask) in 3 mL of Human Endothelial-SFM. The cell suspension

should be added directly to each flask resulting in a final volume of 5 mL. Each flask should then be supplemented with hrbFGF (20 ng/mL, 100 ng/flask), hrEGF (10 ng/mL, 50 ng/flask), penicillin (10 U/mL) and streptomycin (10 μ g/mL). Cultures are maintained with a loosened cap at 37°C in humidified air containing 5% CO₂. Primary cultures should be fluid changed 24 hours postseeding and the cell sheet washed with D-PBS to remove adherent blood cells. Following 48-72 hours in culture, HUVEC should be subcultured (see details on subculturing below) or fluid changed with fresh growth factors added.

Antibiotics should be removed from the cultures at this time.

Additional Considerations

- Due to the presence of contaminating blood cells which occurs during endothelial isolation, we recommend that flasks be pretreated with fibronectin at 20 μ g/mL for 1-1½ hours at 37°C in SFM. The cell suspension should be added directly to the flask to result in a final volume of 5 mL of Human Endothelial-SFM. Please note that for secondary cultures, fibronectin can be added directly to the serum-free culture medium at a concentration of 10 μ g/mL.
- We have found primary HUVEC cultures established in SFM to be very sensitive to antibiotics. We recommend using penicillin at a concentration of 10 U/mL and streptomycin at a concentration of 10 μ g/mL. Additionally, antibiotics should be removed from the culture medium 48-72 hours after the establishment of primary cultures.
- We have found the serum-free (as well as serum-supplemented) growth of HUVEC can be influenced by the type of tissue culture flasks used. We recommend that flasks be rinsed with DPBS prior to use.

Establishment of Secondary Cultures

Upon reaching confluency, endothelial cultures should be subcultured using 0.05% Trypsin-EDTA (GIBCO Cat. No. 25300). Following removal of spent culture medium, an appropriate amount of trypsin should be added to ensure complete coverage of the flask surface. The cell sheet should be coated with trypsin for approximately 10-20 seconds, the flask then stood upright and the trypsin removed prior to cell detachment. **Please note that HUVEC cultured in SFM detach very readily from plastic surfaces.** Following removal of trypsin, the flask should be observed using a phase contrast microscope to confirm complete cell detachment. Following detachment, flasks should be rinsed with an appropriate volume of SFM and the resulting cell suspension collected and transferred to a sterile polypropylene centrifugation tube and centrifuged for 3-5 minutes at 100 X g (22°C). Following centrifugation, the culture medium is removed and the cell pellet resuspended in 5-10 mL of fresh SFM and recentrifuged as described above. Following the second wash step the cell pellet is resuspended in an appropriate volume of SFM and transferred to a new flask supplemented with fibronectin (10 μ g/mL), hrbFGF (20 ng/mL) and hrEGF (10 ng/mL). Cultures are maintained with a loosened cap at 37°C in a humidified atmosphere containing 5% CO₂ in air.

Additional Considerations

- For HUVEC cultured in SFM we recommend using a 1:2 subculturing ratio every 96-120 hours with fluid changing after 72 hours of culture. To fluid change, 50% of the spent culture medium should be removed and replaced with fresh SFM supplemented with hrbFGF and hrEGF (both added based on total volume, addition of fibronectin is not necessary).
- Endothelial cells cultured in SFM are very sensitive to proteolytic enzymes such as trypsin. It is very important that all residual trypsin be removed prior to cell detachment and that the cells be washed in SFM at least two times prior to replating.
- Care should be exercised in the handling of endothelial cells. Avoid centrifugation forces in excess of 100 X g as well as vigorous pipeting to resuspend cell pellets following centrifugation.
- As stated previously, endothelial cells cultured in SFM have been found to be sensitive to antibiotics. The use of antibiotics for secondary cultures should be avoided. However, if antibiotics must be used for secondary cultures we recommend using penicillin at a concentration of 10 U/mL and streptomycin at a concentration of 10 μ g/mL.

Cryopreservation and Recovery

- For cryopreservation of endothelial cells in SFM the following cryopreservation medium should be used: 92.5% Human Endothelial-SFM (50% fresh medium; 50% conditioned medium) and 7.5% DMSO. Alternatively, cells may be frozen in serum-supplemented medium consisting of 92.5% Medium 199 supplemented with 20% FBS and 7.5% DMSO.
- Confluent cultures should be harvested as described above and washed twice in SFM prior to resuspension in fresh SFM. An aliquot is then removed and the viable cell density determined. The cell suspension is recentrifuged, the supernatant removed and the cell pellet resuspended in an appropriate volume of cryopreservation medium to yield a final cell density of 2-3 X 10⁶ cells/mL.
- Aliquot 1 mL of the cell suspension into sterile freezing vials and cryopreserve using a Cryo-Med[®] Cryopreservation Unit, Nalgene Freezing Container or similar device to achieve a freezing rate of -1°C/minute. Following cryopreservation, vials should be stored in liquid nitrogen. In the absence of such a unit, perform the following time and temperature sequence: 45 minutes at 4°C, 1 hour at -20°C, overnight at -70°C, store in liquid nitrogen.
- Recovery of frozen stocks should be done in a 37°C water bath and after rapid thawing, cells should be transferred to a culture flask containing SFM supplemented with fibronectin, hrbFGF and hrEGF, and incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. After 24 hours, cultures should be fluid changed by replacing 50% of the spent culture medium with fresh SFM and growth factors.

References:

- Gimbrone, M.A. Culture of Vascular Endothelium. *Progress in Hemostasis and Thrombosis* 3:1-28. (1976).
- Jayme, D.W., Epstein, D.A. and Conrad, D.R. Fetal Bovine Serum Alternatives. *Nature* 334:547-548. (1988).
- Rosa, M.D. Serum Substitutes: Is There a Solution? *BioPharm* 2:16-17. (1989).
- Gorfin, S., Spector, A., DeLuca, D. and Weiss, S. Growth and Physiological Functions of Vascular Endothelial Cells in a New Serum-Free Medium (SFM). *Experimental Cell Research* 206:291-301. (1993).
- Battista, P.J., Bowen, H.J. and Gorfin, S.F. A Serum-Free Medium for the Culture of Human Umbilical Vein Endothelial Cells. *Focus* 17:10-13. (1995).
- Soderland, C., Veres, J.S. and Battista, P.J. Serum-Free Culture of Human Endothelial Cells for Leukocyte Adhesion Applications. *Focus* 17:14-17. (1995).
- Battista, P.J. and Soderland, C. Serum-Free Culture of Human Arterial and Microvascular Endothelial Cells. *Focus* 17:106-108. (1995).
- Jaffe, E.A., Nachman, R.L., Becker, C.G. and Minick, C.R. Culture of Human Endothelial Cells Derived From Umbilical Veins: Identification by Morphological and Immunological Criteria. *Journal of Clinical Investigation* 52:2745-2756. (1973).

Cryo-Med[®] is a registered trademark of Forma Scientific Inc.

For further information on this or other GIBCO[™] products, contact Technical Services at the following:

United States TECH-LINESM: 1 800 955 6288

Canada TECH-LINE: 1 800 757 8257

Outside the U.S. and Canada, refer to the GIBCO products catalogue for the TECH-LINE in your region.

You may also contact your Invitrogen Sales Representative or our World Wide Web site at www.invitrogen.com.

**For research use only.
CAUTION: Not intended for human or animal
diagnostic or therapeutic uses.**

April 2002

Form No. 3759