

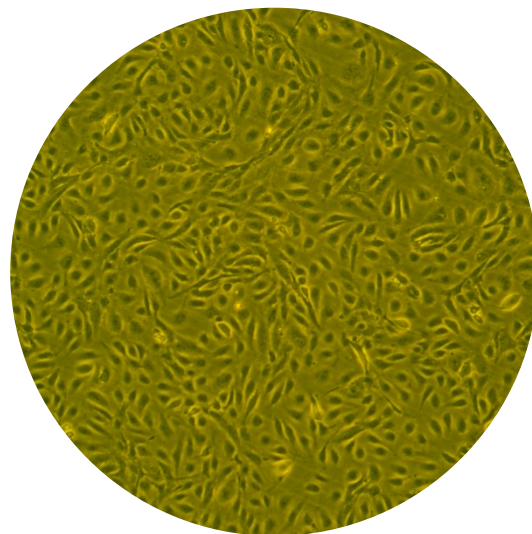
Instruction Sheet

Human Endothelial Cells

FC-0003	LL-0002
FC-0044	LL-0003
FC-0014	LL-0004
FC-0027	LL-0005
FC-0028	
FC-0055	
FC-0032	



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LIFELINE[®]
CELL TECHNOLOGY

Better Solutions for
Breakthrough Results

Human Umbilical Vein Endothelial Cells (HUVEC)	Human Aortic Endothelial Cells (HAoEC)
HUVEC 10-Donor Pool	Human Iliac Artery Endothelial Cells (HIAEC)
Human Coronary Artery Endothelial Cells (HCAEC)	Human Pulmonary Artery Endothelial Cells (HPAEC)

Safety and Use Statement

Lifeline recommends storing cryopreserved vials in liquid nitrogen vapor phase. Handle cryopreserved vials with caution. Always wear eye protection and gloves when working with cell cultures. Aseptically vent any nitrogen from cryopreserved vials by carefully loosening the vial cap in a biosafety cabinet prior to thawing the vials in a water bath. If vials must be stored in liquid phase, the vials should be transferred to vapor phase storage or -80°C for up to 24 hours prior to being thawed.

Quick Steps for Cell Culture:

1. Always wash hands before and after working with cell cultures.
2. Always wear eye protection and gloves when working with cell cultures.
3. Store cryopreserved cells in liquid nitrogen vapor phase.
4. When working with cells or medium, always use a certified biological safety cabinet.
5. Handle cryopreserved vials with caution. Aseptically vent any nitrogen from cryopreserved vials in a biosafety cabinet prior to thawing in a water bath.
6. Feed cells using pre-warmed Lifeline® culture medium according to feeding chart.
7. When cells are 80 to 90% confluent and actively proliferating, passage cells using Lifeline subculture reagents. **When they are ready to passage they will have the density/confluence in the picture on the front page.**
8. Rinse cultures with PBS.
9. Trypsinize cells until rounded, do not over trypsinize.
10. Add TNS to stop trypsinization.
11. Add more PBS to rinse all the cells from the culture surface.
12. Centrifuge at 150 x g for 3 to 5 minutes. Adjust speed and time as appropriate for your centrifuge.
13. Aspirate solution from centrifuge tube, add pre-warmed Lifeline culture medium and gently re-suspend cells.
14. Count cells using a hemacytometer, re-plate at 2,500 to 5,000 cells per cm² (or use a split ratio of 1:3 to 1:8) in vessel containing pre-warmed Lifeline culture medium.
15. Incubate cells using 1 mL of culture medium per 5 cm² surface area at 37°C, 5% CO₂.
16. For any question on cell handling, please contact technical service. We are here to help.

Lifeline Technical Note: There are different and often contradictory terminologies used by cell culture companies to define the passage number of cells. Lifeline's designation of 'primary cells' are cells that have been isolated from tissue, plated onto culture vessels, expanded, harvested and cryopreserved. The



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FC-0003 FC-0028 LL-0002 LM-0002 LS-1029
FC-0044 FC-0055 LL-0003 LS-1019
FC-0014 FC-0032 LL-0004 LS-1020
FC-0027 LL-0005 LS-1035

term 'secondary' indicates that the cells have been isolated, plated and expanded in culture vessels twice before being harvested for cryopreservation.

Product	Catalog Number(s)
HUVEC, Human Umbilical Endothelial Cells, Primary – 500,000 cells/vial	FC-0003
HUVEC 10-Donor Pool, Secondary – 500,000 cells/vial	FC-0044
HAoEC, Normal Human Aortic Endothelial Cells, Secondary – 500,000 cells/vial	FC-0014
HAoEC, Normal Human Aortic Endothelial Cells, Tertiary – 500,000 cells/vial	FC-0027
HIAEC, Normal Human Iliac Artery Endothelial Cells, Secondary – 500,000 cells/vial	FC-0028
HPAEC, Normal Human Pulmonary Artery Endothelial Cells, Secondary or Tertiary – 500,000 cells/vial	FC-0055
VascuLife® EnGS Medium Complete Kit (VascuLife Basal Medium-LM-0002, VascuLife EnGS LifeFactors™ Kit-LS-1019)	LL-0002
Kit Components	
• VascuLife® Basal Medium	LM-0002
• VascuLife® EnGS LifeFactors Kit	LS-1019
VascuLife VEGF Medium Complete Kit (VascuLife Basal Medium-LM-0002, VascuLife VEGF LifeFactors Kit-LS-1020)	LL-0003
Kit Components	
• VascuLife® Basal Medium	LM-0002
• VascuLife® VEGF LifeFactors Kit	LS-1020
GA Antimicrobial Supplement, 0.5 mL (Gentamicin 30 mg/mL, Amphotericin B 15 µg/mL); provided with purchase of LL-0002 or LL-0003	LS-1104
HCAEC, Normal Human Coronary Artery Endothelial Cells, Tertiary – 500,000 cells/vial	FC-0032
VascuLife EnGS-Mv Medium Complete Kit (VascuLife Basal Medium-LM-0002, VascuLife EnGS-Mv LifeFactors Kit- LS-1035)	LL-0004
Kit Components	
• VascuLife® Basal Medium	LM-0002
• VascuLife® EnGS-Mv LifeFactors Kit	LS-1035
VascuLife VEGF-Mv Medium Complete Kit (VascuLife Basal Medium-LM-0002, VascuLife VEGF-Mv LifeFactors Kit-LS-1029)	LL-0005
Kit Components	
• VascuLife® Basal Medium	LM-0002
• VascuLife® VEGF-Mv LifeFactors Kit	LS-1029
GA Antimicrobial Supplement, 0.5 mL (Gentamicin 30 mg/mL, Amphotericin B 15 µg/mL); provided with purchase of LL-0004 or LL-0005	LS-1104
Gelatin Solution [0.1%], 100 mL	CM-0004
TrypKit™ Subculture Reagent Kit	LL-0013



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Phenol Red Supplement [33 mM], 1 mL	LS-1009
FrostaLife™ Cryopreservation Solution, 100 mL	LM-0015

Basic Aseptic Technique

Cells, medium and LifeFactors™ should only be used in an aseptic environment, a Class II biological safety cabinet with front access and filtered laminar airflow, or an equivalent device. Always wear gloves and eye protection when working with these materials. Wipe or spray all bottles and vials with 70% ethanol or isopropanol, especially around the area of the cap, before placing them in the biological safety cabinet. Allow these surfaces to dry completely before opening the bottle or vials. Transfer cells, medium or LifeFactors with disposable sterile pipettes. Do not mouth pipette! Take up the volume needed into the pipette, being careful not to touch the sterile tip to the rim of the container or any other surface. Close the container and open the container into which the transfer is being made, again being careful not to touch any surfaces with the sterile tip. Transfer the material and close the container. Wash your hands before and after working with cell cultures. Do not block airflow in a laminar flow hood as this may compromise sterility. Ensure that biological safety cabinets are certified routinely and that the HEPA filters are replaced regularly.

Cryopreserved Vials

Normal Human Endothelial Cells are sold as cryopreserved vials and are shipped in biodegradable insulated packages containing dry ice to ensure the cells remain in a cryopreserved state. To maintain the cells' integrity, unpack the products immediately upon receipt and store at a temperature lower than -150°C or in the vapor phase of a liquid nitrogen dewar. If the cells are to be thawed and plated within 24 hours, they may be stored at -80°C. Do not store the vial for more than 24 hours at -80°C, as the cells will slowly degrade at this temperature.

Medium Preparation

VascuLife® Basal Medium (LM-0002) contains no growth factors, antimicrobials or phenol red. To support cell proliferation, you must add VascuLife EnGS LifeFactors (LS-1019) or VascuLife VEGF LifeFactors (LS-1020) to the basal medium (see VascuLife EnGS Medium instructions or VascuLife VEGF Medium instructions).

To support optimal proliferation of HCAEC, you must add VascuLife EnGS-Mv LifeFactors (LS-1035) or VascuLife VEGF-Mv LifeFactors (LS-1029) to the basal medium (see VascuLife EnGS-Mv Medium instructions or VascuLife VEGF-Mv Medium instructions).

A vial of Gentamicin and Amphotericin B (GA; LS-1104) is provided with the purchase of VascuLife EnGS (LL-0002), VascuLife VEGF (LL-0003), VascuLife EnGS-Mv (LL-0004), or VascuLife VEGF-Mv (LL-0005) Medium Complete Kits for your convenience. The use of GA is recommended to inhibit potential fungal or bacterial contamination of eukaryotic cell cultures. Phenol Red (LS-1009) may be purchased, but is not required.



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Pre-warming Medium

If using less than 100 mL of medium, Lifeline® recommends warming only the volume needed in a sterile conical tube. Repeated warming of the entire bottle over extended periods will cause degradation and reduced shelf life of the medium. Medium will warm to 37°C in 10 to 30 minutes, depending on the volume. Do not leave medium in water bath for extended periods.

Thawing and Plating Cryopreserved Cells

Remove vial from dewar and check the cap to be sure that the vial is securely sealed. Spray the vial with 70% ethanol or isopropanol and transfer it to a biosafety cabinet. Allow it to dry thoroughly and carefully loosen the cap to vent any liquid nitrogen that may have entered the vial. Recap the vial and hold only the bottom half of the vial in a 37°C water bath for approximately one minute or until vial is almost completely thawed—a small amount of ice should still be visible. To avoid potential contamination, do not allow the vial cap to make contact with the water. Do not over thaw as this may damage the cells. Dry the vial, spray the exterior of the vial with 70% ethanol or isopropanol and place the vial in a biological safety cabinet and allow it to dry. Carefully remove the cap to avoid contamination or splatter. Gently resuspend the cells in the vial using a 1- or 2-mL sterile pipette. Do not centrifuge; the cells may be directly plated from the vial. Plate cells into the respective pre-warmed fully supplemented Vasculife® Medium (respective to cell type) in the desired culture vessel at a density of 2,500 to 5,000 cells per cm². Flasks with vented caps, commonly referred to as filter caps, are strongly recommended. Gently rock the culture vessel from side to side and front to back to evenly distribute cells within the vessel. Place seeded culture vessel in a 37°C, 5% CO₂ incubator. Refeed the cells after they have attached (approximately 4 to 36 hours after inoculation) to remove cryopreservation reagents.

Standard Calculation for Plating of Cells

Gently re-suspend the cells evenly in the medium. Using a clean hemacytometer, and aseptic technique, remove 20 µL of the cell suspension to a separate tube, such as a microcentrifuge tube. Add an equal volume of 0.4% Trypan Blue solution to the cell suspension in the microcentrifuge tube. Perform cell counts between 1 to 5 minutes after the addition of Trypan Blue to the cell suspension. Place 10 µL of the cell suspension into at least one chamber of the hemacytometer. Count a minimum of 4 quadrants on the hemacytometer (see diagram below). Dead and dying cells are permeable to Trypan Blue and will look completely blue. For accurate cell counts, optimal number of cells per quadrant should be 25 to 75 cells. After counting the cells, calculate the average of the 4 quadrants. Take the cell count average and multiply by the dilution factor and by 10⁴ to get the number of cells per mL. Multiply the desired seeding density (2,500 to 5,000 viable cells per cm²) by the surface area of the vessel(s) to be inoculated. This will give you the total number of cells to inoculate one vessel. Divide the number of cells needed to inoculate one vessel by the total number of cells in the cell suspension. This will give you the volume of cell suspension with which to inoculate each vessel. Inoculate the cells into the culture vessels prepared with pre-warmed culture medium. Mix gently to evenly distribute the cells and place culture vessels into the incubator at 37°C, 5% CO₂.



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Sample calculation:

Average viable cells per quadrant = 31

$31 \text{ cells/quadrant} \times 10,000 \text{ quadrants/mL} \times 2 \text{ (dilution factor*)} = 620,000 \text{ cells/mL}$

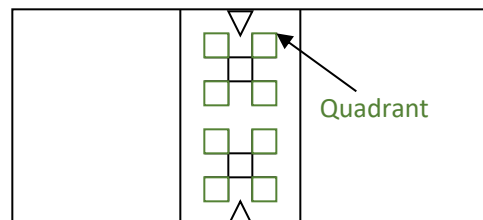
*if using equal volumes of Trypan Blue and cell suspension.

Inoculating a T-75 flask at 2,500 cells/cm²:

$2,500 \text{ cells/cm}^2 \times 75 \text{ cm}^2 = 187,500 \text{ cells/flask}$

Calculate volume of cell suspension to inoculate each flask with:

$187,500 \text{ cells/flask divided by } 620,000 \text{ cells/mL} = 0.302 \text{ mL/flask}$



Passaging Cells

Normal Human Endothelial Cells may be passaged once the culture is 80 to 90% confluent and actively proliferating. Human Coronary Artery Endothelial Cells (HCAEC) may be passaged once the culture is greater than 90% confluent. **Refer to the picture on the front page for appropriate density/confluence.** Human Endothelial Cells are not contact inhibited; however Lifeline® recommends that endothelial cells be passaged before reaching confluence since post-confluent cells exhibit slower proliferation after passaging.

Lifeline recommends using the TrypKit™ Subculture Reagent Kit (LL-0013). Aspirate the medium from the culture vessel. Rinse the flasks with Lifeline's Phosphate Buffered Saline (PBS; CM-0001) by adding at least 1 mL of PBS per each 5 cm² and gently tilting the flask to cover the surface with PBS. Aspirate the PBS from the culture vessel, repeat the rinse if desired. Add at least 2 mL of Lifeline's 0.05% Trypsin/0.02% EDTA (CM-0017) per each 25 cm² to the vessel. Swirl gently to ensure all cells are coated with the Trypsin/EDTA. Observe the cells carefully under the microscope. When the cells round up they are ready to be released. This normally takes from 2 to 3 minutes depending on the confluence of the cells. Do not over trypsinize as this may damage the cells. Detach the cells by gently striking the culture vessel against your hand several times. Observe the cells under the microscope to be sure they have become detached. Once the cells are fully detached, add Lifeline's Trypsin Neutralizing Solution (TNS; CM-0018) using a volume equal to the amount of Trypsin/EDTA that was originally used. Gently swirl to ensure all of the trypsin solution is neutralized. Using aseptic laboratory techniques, pipette the cells into a sterile centrifuge tube. Collect the remaining cells by rinsing the culture vessel with at least 1 mL of PBS per each 5 cm² and pipetting the cells into the sterile centrifuge tube. Check culture vessel under the microscope for cells still attached and repeat steps if necessary to retrieve all the cells from the vessel. All steps must be completed under aseptic conditions in a biological safety cabinet.

Centrifuge the cells at $150 \times g$ for 3 to 5 minutes. For best results, calculate speed for individual centrifuge type. Time may also be centrifuge dependent. Do not over centrifuge cells as this will cause cell damage. After centrifugation, the cells should form a clean loose pellet. Please consult Lifeline's technical service department if issues arise from trypsinization or centrifugation.

Aspirate the neutralized trypsin solution from the centrifuge tube and resuspend the cell pellet in pre-warmed Vasculife® Medium by gently pipetting up and down with a 2- or 5-mL pipette. Count cells using a hemacytometer, and re-plate at 2,500 to 5,000 cells per cm² (**or use a split ratio of 1:3 to 1:8**) in vessel containing the respective pre-warmed culture medium.



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FC-0027		LL-0005	LS-1035	

*To calculate RCF (x g)

$$RCF = 0.00001118 \times (\text{rpm})^2 \times r$$

r = rotational radius in centimeters

rpm = rotations or revolutions per minute

Recommended Feeding Guidelines

Guidelines for a T-25 Flask. Adjust volumes according to culture surface area.
Every other day, remove medium and feed with 5 mL of fresh supplemented medium.
Most cultures which are 50% confluent will be ready for passage within two days and should be fed with 7 to 8 mL of supplemented medium.
<u>Do not use more than 10 mL of medium per 25 cm² of culture surface</u> to ensure that the media depth is at a level where gas diffusion will be sufficient to support the cells' requirements for oxygen.

The depth of the medium affects gas diffusion gradients through the culture medium to the cells. The volumes of medium recommended in this table result in a range of depths between 2 mm and 5 mm, which is compatible with general recommendations, 10 mL being at the maximum depth allowable (5 mm).



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