TECHNICAL MANUAL

Culture of Human Endothelial Colony-Forming Cells (ECFCs) Using EC-Cult™-XF Culture Kit

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1.0 Introduction

Human endothelial colony-forming cells (ECFCs), or late outgrowth endothelial cells (OECs), form a distinct subset of putative human endothelial progenitor cells that circulate in blood. ECFCs form a hierarchy composed of cells with a high-proliferative potential (HPP-ECFCs), giving rise to low-proliferative potential ECFCs (LPP-ECFCs), which generate endothelial clusters and finally, mature non-dividing endothelial cells (ECs).[1,2,3]

EC-Cult™-XF Medium is a xeno-free medium for the culture of human ECFCs, mature ECs, and placental pericytes. For expansion and passaging of ECs and pericytes, refer to the Product Information Sheet (PIS) for EC-Cult™-XF Culture Kit (Document #DX20709), available at www.stemcell.com or contact us to request a copy. EC-Cult™-XF Medium can also be used for the derivation and expansion of ECFCs from primary human umbilical cord blood and adult peripheral blood in a standardized 7- to 14-day assay. During the ECFC assay, colonies can be allowed to grow as a polyclonal cell line (section 5.3), or single colonies can be isolated through the use of cloning cylinders for further subculture (section 5.2). ECFCs previously derived or cultured in serum-containing or serum-free conditions can also be expanded using EC-Cult™-XF Medium. EC-Cult™-XF Medium requires supplementation with heparin; since Heparin Solution contains non-human animal-derived components, the complete medium will not be xeno-free. Cultureware must be coated with Animal Component-Free Cell Attachment Substrate (Component #07130; included in EC-Cult™-XF Culture Kit).

EC-Cult™-XF Medium specifically enhances the survival and growth of HPP-ECFCs compared to serum-containing medium.

Human ECFCs derived and maintained in complete EC-Cult™ Medium have been shown to:

- Display a cobblestone appearance with variations in colony size
- Possess clonal ability that covers the entire ECFC hierarchy (high-proliferative, low-proliferative, endothelial clusters, and mature non-dividing cells)
- Expand equally compared to ECFCs cultured in serum-containing medium
- Uniformly express high levels of multiple endothelial cell antigens (e.g. CD31, CD105, CD144) and do not express the hematopoietic marker CD45
- Form capillary tube-like structures on Corning® Matrigel®
- Uptake acetylated low-density lipoproteins
- Enhance revascularization when injected in animal models of ischemia[4]

2.0 Materials, Reagents, and Equipment

2.1 EC-Cult™-XF Culture Kit (Catalog #08000)

For component storage and stability information, refer to the Product Information Sheet (PIS) for EC-Cult™-XF Culture Kit (Document #DX20709); the PIS is also available at www.stemcell.com, or contact us to request a copy.

The following components are sold as a complete kit and are not available for individual sale.

COMPONENT NAME	COMPONENT #	SIZE
EC-Cult™-XF Basal Medium	08003	150 mL
EC-Cult™-XF 2.5X Supplement*	08004	100 mL
Animal Component-Free Cell Attachment Substrate	07130	1 mL

*This component contains material derived from human plasma. Donors have been tested and found negative for HIV-1 and -2, hepatitis B, and hepatitis C prior to donation. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.

2.2 Additional Required Materials

PRODUCT	CATALOG #
Heparin Solution	07980
Polyethersulfone (PES) filter unit, 0.2 - 0.22 µm	e.g. Fisher 09-741-04 (0.2 µm, 250 mL) or Fisher SCGP00525 (0.22 µm, 50 mL)
D-PBS (Without Ca++ and Mg++)	37350
Animal Component-Free Cell Dissociation Kit • ACF Enzymatic Dissociation Solution • ACF Enzyme Inhibition Solution	05426
Costar® 6-Well Flat-Bottom Plate, Tissue Culture-Treated	38015
3% Acetic Acid with Methylene Blue	07060
Trypan Blue	07050
Lymphoprep™	07801
CryoStor® CS10	07930
Falcon® Conical Tubes	38009 (15 mL) or 38010 (50 mL)

For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

2.3 Equipment Required

- Biosafety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and > 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with swinging bucket rotor
- Pipette-aid
- Hemocytometer
- Inverted microscope with 2X, 4X, 10X, and 20X phase objectives
- Cryovials for cell preservation
- Isopropanol freezing container (e.g. Fisher Catalog #1535050)
- -135°C Freezer or liquid nitrogen vapor tank

3.0 Preparation of Materials and Reagents

3.1 Complete EC-Cult™ Medium for ECFCs

Use sterile techniques to prepare complete EC-Cult™ Medium (Basal Medium + 2.5X Supplement + heparin). The following example is for preparing 250 mL of complete medium. If preparing other volumes, adjust accordingly.

NOTE: For complete EC-Cult™ Medium for various types of mature endothelial cells, refer to the PIS for EC-Cult™-XF Culture Kit (Document #DX20709), available at www.stemcell.com or contact us to request a copy. A lower concentration of heparin is required when culturing mature endothelial cells.

1. Thaw the 2.5X Supplement either at 2 - 8°C overnight or at 37°C until fully thawed. Mix thoroughly but do not vortex.

   Note: Some precipitate may form. This will not affect product performance and will be removed when the complete medium is filtered (step 5).

   Note: Once thawed, use immediately or aliquot and store at -20°C for up to 10 months. Do not exceed the shelf life of the supplement. After thawing the aliquots, use immediately. Do not re-freeze.
2. Warm EC-Cult™-XF Basal Medium and Heparin Solution to room temperature (15 - 25°C).
3. Add 100 mL of 2.5X Supplement to 143.75 mL of Basal Medium.
4. Add 6.2 mL of Heparin Solution (final concentration 50 µg/mL). Mix thoroughly.

   Note: Since Heparin Solution contains non-human animal-derived components, the complete medium will not be xeno-free.
5. Filter the complete medium through a 0.2 - 0.22 µm PES filter unit.

   Note: If not used immediately, store complete EC-Cult™ Medium at 2 - 8°C for up to 15 days. If a precipitate forms, filter again as described. This will not affect performance of the medium.

3.2 Coating Cultureware with Animal Component-Free (ACF) Cell Attachment Substrate

Use sterile techniques when coating cultureware with ACF Cell Attachment Substrate.

Note: Only use tissue culture-treated cultureware.

1. Dilute ACF Cell Attachment Substrate 1 in 100 in D-PBS (Without Ca++ and Mg++) (PBS).

   For example, add 100 µL of ACF Cell Attachment Substrate to 9.9 mL of PBS.
2. Gently mix diluted ACF Cell Attachment Substrate. Do not vortex.
3. Immediately use diluted ACF Cell Attachment Substrate to coat cultureware. Refer to Table 1 for recommended coating volumes for various cultureware.

Table 1. Recommended Volumes for Coating Cultureware with Diluted ACF Cell Attachment Substrate

CULTUREWARE	VOLUME OF DILUTED ACF CELL ATTACHMENT SUBSTRATE
24-well plate	0.4 mL/well
6-well plate	1.0 mL/well
T-25 cm^2 flask	4 - 5 mL/flask
T-75 cm^2 flask	8 - 9 mL/flask

4. Gently rock cultureware back and forth to spread ACF Cell Attachment Substrate evenly across the surface. Seal with Parafilm®.
5. Incubate sealed cultureware at room temperature (15 - 25°C) for at least 2 hours before use. Do not let ACF Cell Attachment Substrate evaporate.
   - *For ECFC derivation, plates must be coated and used on the same day as cord blood processing. Proceed to step 6.*
   - *For established ECFC cultures, sealed coated cultureware can be stored at 2 - 8°C for up to 3 days after coating. Allow stored coated cultureware to warm to room temperature (15 - 25°C) for 30 minutes before proceeding to step 6.*
6. Gently tilt cultureware onto one side and allow excess ACF Cell Attachment Substrate to collect at the edge. Remove excess solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
7. Wash cultureware twice using PBS (e.g. use 2 x 2 mL/well if using a 6-well plate).
8. Aspirate PBS. The coated cultureware is now ready for use.
   - Proceed to section 4.0 for isolation of mononuclear cells
     OR
   - Proceed to section 6.0 for passaging or expansion of established ECFCs

3.3 Preparation of Cloning Cylinders: For First Passage/Subculture of ECFCs Using Single Clones

Cloning cylinders create a barrier between individual colonies growing in the ECFC assay. Prepare cloning cylinders just prior to use.

1. Spread a dime-sized amount of vacuum grease (e.g. Sigma Catalog #Z273554) into a thin layer in a glass dish.
2. Sterilize the grease-containing glass dish in an autoclave and cool completely.
3. Using sterile forceps, remove a cloning cylinder (e.g. Fisher Catalog #14-512-79) from its packaging. Dip the bottom surface into the vacuum grease in the sterilized glass dish to coat.

   Note: Apply the minimum amount of grease necessary to coat the bottom surface and to form a good seal with a culture plate. Excess grease will interfere with the collection of cells.
4. Lightly set the prepared cylinder, greased side down, in a sterile 100 mm culture dish (e.g. Catalog #27110) until use.

4.0 Isolation of Mononuclear Cells (MNCs)

EC-Cult™-XF Medium can be used for the derivation and expansion of ECFCs from primary human umbilical cord blood and adult peripheral blood. The frequency of ECFCs is much higher in cord blood compared to adult peripheral blood. ECFCs in cord blood have a greater replicative capacity, and can therefore be maintained in culture for a longer period of time (i.e. more passages) before reaching senescence.[1]

Umbilical cord blood and adult peripheral blood should be processed as soon as possible after collection. The number of colonies will decrease as the time between blood collection and processing increases. ECFC recovery is most successful when **fresh cord blood** is used. EC-Cult™-XF Medium can be used to isolate ECFCs from cord blood samples up to 36 hours post-collection; however, the success rate will be lower with samples older than 6 hours post-collection.

Note: The number of ECFC colonies, time of appearance, growth kinetics, and the potential growth of contaminating mesenchymal stromal cells are largely donor-dependent. Results can vary widely depending on age/gestational age of the donor, as well as disease state and genetic factors.[5]

The protocol described below is for isolating mononuclear cells (MNCs) from cord blood or adult peripheral blood in preparation for the ECFC assay. Recommended starting volumes are as follows:

- For **cord blood**: Process a minimum of 35 - 50 mL of fresh whole cord blood to ensure a sufficient yield of MNCs.
- For **adult peripheral blood**: Process a minimum of 100 mL.

The following example is for processing 50 mL of cord blood. For other volumes, adjust accordingly.

1. Coat two 6-well plates with ACF Cell Attachment Substrate (see section 3.2).

   Note: Plates must be coated on the day of MNC isolation. Do not use older plates.
2. Add 15 mL of Lymphoprep™ to each of 6 x 50 mL conical tubes.
3. Dilute 50 mL of cord blood with 100 - 150 mL of PBS.

   Note: A higher dilution generally results in better isolation of ECFCs.
4. Using a 10 or 25 mL pipette, slowly add up to 35 mL of diluted cord blood on top of Lymphoprep™, ensuring that the layers do not mix. Repeat for each tube.
5. Balance the tubes in the centrifuge.

   Note: It is recommended to weigh the tubes prior to centrifuging.
6. Centrifuge the 6 tubes at 560 x *g* for 30 minutes with the **brake off**.

   *Note: During this step, the **brake must be off**, otherwise the layers will be disturbed during the sudden deceleration.*
7. From one tube, collect the hazy MNC layer at the plasma:Lymphoprep™ interface using a transfer pipette or a 5 - 10 mL serological pipette and place in a new 50 mL conical tube. Top up with PBS. Repeat for remaining tubes.

   Note: If it is difficult to see the cells at the interface, remove some of the Lymphoprep™ and/or plasma along with the cells in order to maximize cell recovery.

   Note: Cord blood often contains nucleated red blood cells (nRBCs)—red blood cells that have not completely lost their nucleus. They may be visible in the interface along with the other MNCs. Their presence and abundance are donor-dependent, completely normal, and will not affect the subsequent ECFC assay.

8. Centrifuge the 6 tubes at 300 x *g* for 10 minutes with the **brake on**.
9. Remove and discard the supernatants. Resuspend each cell pellet in 2 mL of PBS. Combine the cell suspensions from all 6 tubes into 4 tubes and top up each tube with PBS.
10. Centrifuge the 4 tubes at 300 x *g* for 10 minutes with the **brake on**.
11. Remove and discard the supernatants. Resuspend each cell pellet in 2 mL of PBS. Combine the cell suspensions from all 4 tubes into 2 tubes and top up each tube with PBS.
12. Centrifuge the 2 tubes at 200 x *g* for 10 minutes with the **brake on**.

    Note: The lower speed improves platelet removal from the sample.
13. Carefully remove and discard the supernatants. Take extra precaution at this step, as the cells may become dislodged.
14. Resuspend one of the cell pellets in 5 - 10 mL of PBS. Add this cell suspension to the remaining cell pellet to resuspend. Transfer the combined cell suspension into one 15 mL conical tube for counting.
15. Count the nucleated cells using 3% Acetic Acid with Methylene Blue at a 1 in 50 to 1 in 100 dilution. Trypan Blue can be used to assess cell viability and to confirm the cell count.

    Note: If the cell suspension is very red due to contaminating nRBCs, the nucleated cell count may be artificially high. Exclude nRBCs if possible; at 20X magnification, nRBCs appear perfectly round, whereas MNCs tend to have wavy edges.

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5.0 Endothelial Colony-Forming Cell (ECFC) Assay

5.1 ECFC Assay Setup

The following protocol is for setting up an ECFC assay in a 6-well plate.

Day 0: Plating

1. Prepare complete EC-Cult™ Medium (see section 3.1).
2. Retrieve the coated 6-well plates prepared in section 4.0 step 1.
3. Calculate the total number of wells that can be plated (based on the MNC count in section 4.0 step 15). The recommended plating density is 35 - 50 x 10^6 cells/well (cord blood) or ≥ 50 x 10^6 cells/well (adult peripheral blood).
4. Centrifuge the cells at 300 x *g* for 5 minutes.
5. Resuspend the cells in complete EC-Cult™ Medium (2 mL/well to be plated).
6. Add 2 mL of cell suspension to each well by touching the tip of the pipette to the edge of the well and slowly dispensing.
7. Gently swirl the plate in a “figure 8” pattern to ensure the cells are evenly distributed.
8. Incubate at 37°C and 5% CO_2 for 24 hours.

Day 1 - 7: Daily medium changes

Note: Careful and slow medium changes during the first week of culture are critical for successful ECFC outgrowth. Ensure that medium is not added or removed too quickly, as this can dislodge the loosely adherent cells and lead to diminished colony numbers.

Note: From Day 1 - 7, do not exceed 24 hours between medium changes.

9. Perform a daily medium change as follows:
   a) Warm complete EC-Cult™ Medium to room temperature (15 - 25°C).
   b) Using a pipette, **slowly** remove the medium from the well, being cautious not to dislodge the cells that have adhered to the bottom. Plates can be gently tilted to facilitate removal of cells in suspension (usually nRBCs at first). Leave some liquid in the well to prevent drying of the plate surface. Discard the medium.

      Note: Remove medium from one well at a time and at a rate of 1 mL every 5 - 10 seconds.
   c) **Slowly** add 2 mL of warm complete EC-Cult™ Medium to each well by touching the tip of the pipette to one edge of the well and slowly dispensing (1 mL every 5 - 10 seconds).

d) Observe the wells under a microscope. Refer to Figure 1 for an image of an ECFC colony at Day 11.

- From **fresh cord blood** (isolated within 6 hours of delivery), ECFC colonies can appear as early as Day 5 (visible at 10X magnification).
- From **older cord blood** samples, ECFC colonies typically arise at Day 7 - 14 (visible at 4X magnification).
- From **adult peripheral blood**, ECFC colonies can be expected to arise at Day 14 - 28.

Note: Circle colonies with a marker on the underside of the well to monitor their appearance and growth. Count individual colonies early before their borders merge.

e) Incubate at 37°C and 5% CO_2.

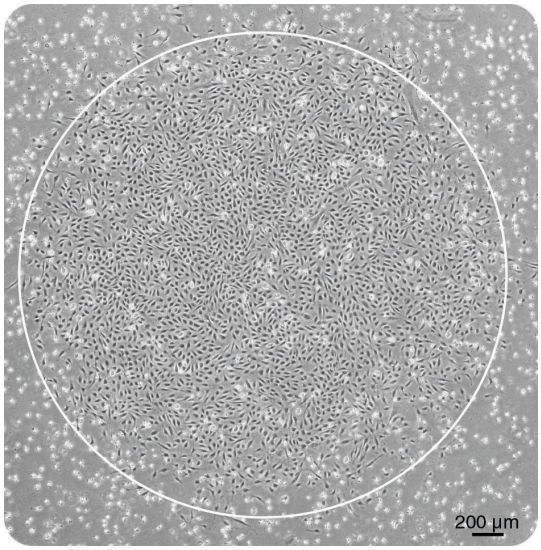

Figure 1. ECFC Colony at Day 11 Obtained Using Complete EC-Cult™ Medium
Round colonies with cells showing cobblestone-like morphology.

10. Periodically monitor the cultures for mesenchymal stem and progenitor cell (MSC) contamination. Cells within MSC clusters are thin, elongated, spiky, and highly migratory. Initially, the MSCs may appear loosely packed, but they can quickly overtake the ECFCs. If MSC contamination occurs, ECFCs should be subcultured into new culture vessels (using cloning cylinders; see section 5.2). Refer to Figure 2 for images of MSC contamination.

    Note: If cells start to ball up or peel during the ECFC assay, they should be passaged immediately to prevent further loss of cells.

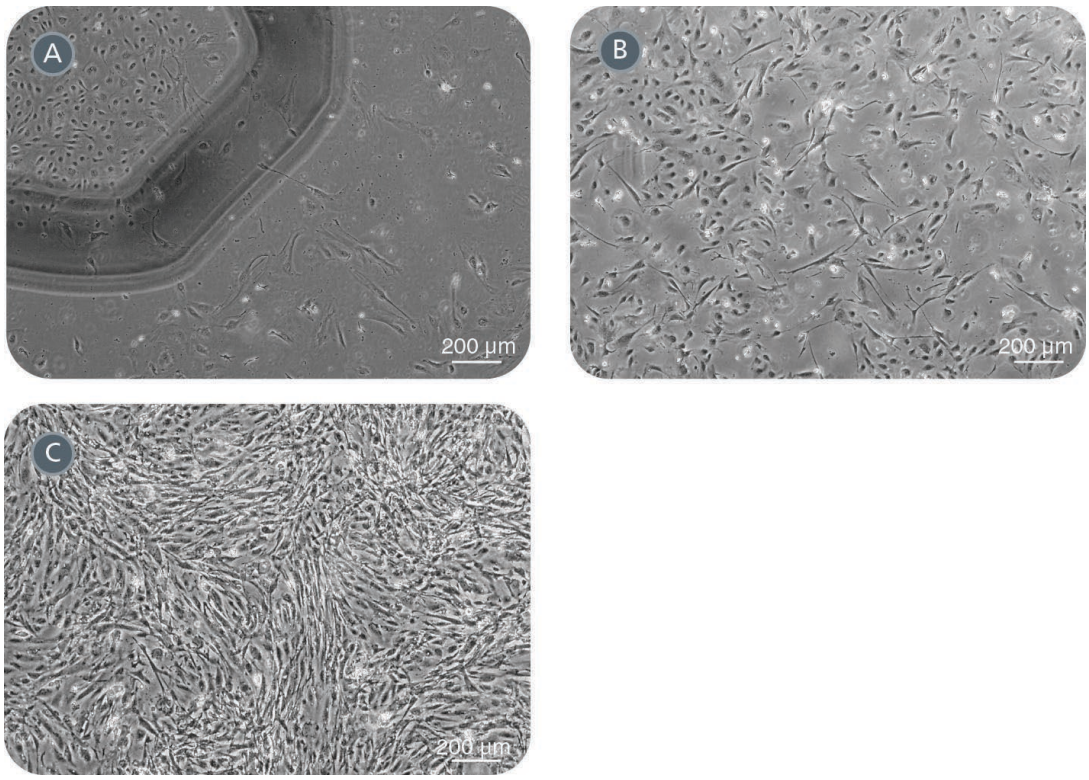

Figure 2. Progression of MSC Contamination

A) Upper left corner: Endothelial colony with tight, round, cobblestone-like cells. Bottom right corner: Invading MSCs.
B) Mixed culture of ECFCs and MSCs: MSCs starting to take over the ECFC culture.
C) MSCs covering the entire well.

Day 8 - 21: Perform medium changes every other day and monitor colony growth

11. Continue performing slow medium changes (see step 9) every other day until cells are ready to be passaged.

    ECFCs can be grown as single clones/colonies or allowed to merge as a polyclonal mix:

    - **Single clones**: Individual colonies (clones) can be isolated and further expanded. Visualize the colonies and outline the colony borders with a fine-tipped marker on the underside of the well. Monitor their growth and passage as described in **section 5.2** before they merge with other colonies in the well.
    - **Polyclonal growth**: Colonies are allowed to merge with one another in the wells as they grow. Once the wells reach 80 - 90% confluency, the cells can be passaged into larger culture vessels, as described in **section 5.3**.

    Note: Do not leave colonies for longer than 21 days in the same culture vessel.

12. When appropriate, proceed to **section 5.2** for passaging as single clones or **section 5.3** for passaging a polyclonal mix.

5.2 First Passage of ECFCs – Subculturing Single Clones

The protocol below describes how to perform the first passage of ECFCs using cloning cylinders to separate individual clones. Each individual colony on the 6-well plate (from section 5.1) is isolated and placed in one well of a 24-well plate coated with ACF Cell Attachment Substrate.

The timing for the first passage varies depending on the growth kinetics of each sample; colonies should be subcultured before adjacent colonies start to merge. If a culture has > 20 colonies by Day 10, it should be carefully monitored, as colonies can expand rapidly.

Note: ECFCs should be subcultured immediately if MSC contamination is observed.

1. Coat a 24-well plate with ACF Cell Attachment Substrate (see section 3.2).
2. Prepare the required number of cloning cylinders (see section 3.3).
3. Passage cells using Animal Component-Free Cell Dissociation Kit as follows:
   a) Warm ACF Enzymatic Dissociation Solution and ACF Enzyme Inhibition Solution to room temperature (15 - 25°C). Do not incubate at 37°C.
   b) Visualize the ECFC colony under an inverted microscope. Circle the colony using a marker on the underside of the well. If MSC contamination occurs, circle or color the area of the MSC colony, including generous borders.
   c) Wash each well twice with 2 mL of PBS.
   d) Use sterile forceps to place a sterile, greased cloning cylinder (from step 2) around each colony and firmly press against the plate.
   e) Add 0.2 - 0.3 mL of ACF Enzymatic Dissociation Solution to the center of each cylinder and incubate at 37°C for 4 - 5 minutes.
   f) Gently tap the plate to detach cells. If less than 90% of the cells have detached, incubate at 37°C for an additional 1 - 2 minutes and tap again.
   g) Add 0.2 - 0.3 mL of ACF Enzyme Inhibition Solution to each isolated colony. Pipette up and down to create a single-cell suspension and to lift any remaining cells. Add each cell suspension to individual microfuge tubes.
   h) Wash each colony with PBS and add the wash to the corresponding tube from step g).
   i) Centrifuge microfuge tubes at 300 x *g* for 5 minutes with the **brake on**.
   j) Remove and discard the supernatants. Resuspend each cell pellet in 0.5 mL of complete EC-Cult™ Medium.
   k) Transfer one cell suspension into one well of a coated 24-well plate (prepared in step 1). Repeat for each cell suspension.
   l) Incubate at 37°C and 5% CO_2. Proceed to section 6.0 when cells are ready to be expanded. If cells have not reached confluency in 3 days, a full medium change is recommended.

Note: Cells from the first passage may contain contaminating monocytes, macrophages, or hematopoietic stem cells. With further passaging, these other cell types will be selected out.

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5.3 First Passage of ECFCs – Polyclonal Growth

The following protocol describes how to perform the first passage of ECFCs using a polyclonal mix. ECFCs from one well of the 6-well plate (from section 5.1) should be passaged into one T-25 cm^2 flask when the cells reach 90 - 100% confluency or when they start to peel.

Note: Wells that are not fully confluent at the time of passaging can be combined as necessary to reach the equivalent of one confluent well.

1. For each well to be passaged, coat a T-25 cm^2 flask with ACF Cell Attachment Substrate (see section 3.2).
2. Passage cells using the Animal Component-Free Cell Dissociation Kit as follows:
   a) Warm ACF Enzymatic Dissociation Solution and ACF Enzyme Inhibition Solution to room temperature (15 - 25°C). Do not incubate at 37°C.
   b) Wash each well twice with 2 mL of PBS.
   c) Add 0.5 mL of ACF Enzymatic Dissociation Solution to each well and incubate at 37°C for 4 - 5 minutes.
   d) Gently tap the plate to detach cells. If less than 90% of the cells have detached, incubate at 37°C for an additional 1 - 2 minutes and tap again.
   e) Add 0.5 mL of ACF Enzyme Inhibition Solution to each well. Pipette up and down to create a single-cell suspension and to lift any remaining cells. Add the cell suspension from each well to individual 15 mL tubes.
   f) Wash each well with 2 mL of PBS and add the wash to the corresponding tube from step e).
   g) Centrifuge tubes at 300 x *g* for 5 minutes with the **brake on**.
   h) Remove and discard the supernatants. Resuspend each cell pellet in 4 - 5 mL of complete EC-Cult™ Medium.
   i) Transfer one cell suspension to one coated T-25 cm^2 flask (prepared in step 1). Use a new coated flask for each cell suspension.

      Note: At this point, the cell density is approximately 10,000 - 15,000 cells/cm^2.
   j) Incubate flasks at 37°C and 5% CO_2. When cells are ready to be expanded, proceed to section 6.0. If cells have not reached confluency in 3 days, perform a full medium change.

Note: Cells from the first passage may contain contaminating monocytes, macrophages, or hematopoietic stem cells. With further passaging, these other cell types will be selected out.

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6.0 Passaging or Expansion of ECFCs

The following protocol provides general instructions for the passaging/expansion of ECFCs beyond the first passage (section 5.2 or 5.3). Passage cells at 90% confluency; refer to Figure 3 for a representative image.

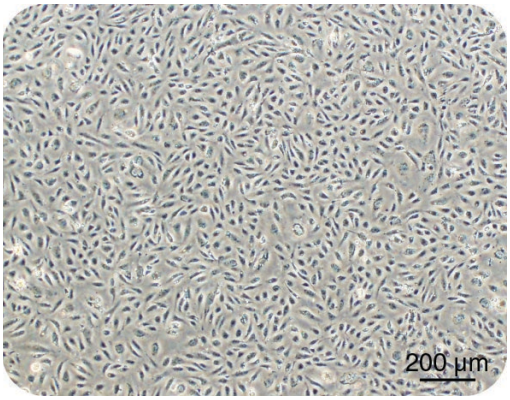

Figure 3. Monolayer of Established ECFCs at Passage 2

1. Coat the desired culture vessel(s) for expansion (e.g. T-25 cm^2 or T-75 cm^2 flask) with ACF Cell Attachment Substrate (see section 3.2).
2. Detach cells using the Animal Component-Free Cell Dissociation Kit as follows:
   a) Warm ACF Enzymatic Dissociation Solution and ACF Enzyme Inhibition Solution to room temperature (15 - 25°C). Do not incubate at 37°C.
   b) Wash the well or flask twice with PBS.
   c) Add ACF Enzymatic Dissociation Solution to the well or flask according to Table 2.

Table 2. Recommended Volume of ACF Enzymatic Dissociation Solution or ACF Enzyme Inhibition Solution for Various Cultureware

CULTUREWARE	VOLUME OF ACF ENZYMATIC DISSOCIATION SOLUTION **OR** ACF ENZYME INHIBITION SOLUTION
24-well plate	0.2 mL/well
6-well plate	0.5 mL/well
T-25 cm^2 flask	1 mL/flask
T-75 cm^2 flask	2 mL/flask

   d) Incubate at 37°C for 4 - 5 minutes.
   e) Gently tap the cultureware to detach cells. If less than 90% of cells have detached, incubate at 37°C for an additional 1 - 2 minutes and tap again.
   f) Add ACF Enzyme Inhibition Solution to the well or flask according to Table 2.

g) Pipette up and down to create a single-cell suspension and to lift any remaining cells. Add the cell suspension from one well or flask to one 15 mL conical tube.

h) Wash the well or flask with PBS and add the wash to the corresponding tube from step g).

i) Centrifuge the cell suspension at 300 x *g* for 5 minutes with the **brake on**.

j) Remove and discard the supernatants. Resuspend each cell pellet in complete EC-Cult™ Medium as follows:

- For one well of a 6-well plate, resuspend in 0.5 mL
- For a T-25 cm^2 flask, resuspend in 1 mL
- For a T-75 cm^2 flask, resuspend in 2 mL

3. Obtain a viable cell count using Trypan Blue and a hemocytometer.
4. Add cells to coated plates or flasks at the desired density in complete EC-Cult™ Medium. The optimal density is 10,000 cells/cm^2 for cells to reach confluency in 2 - 4 days.

   Note: Appropriate seeding densities range from 2,500 - 15,000 cells/cm^2 and may require optimization depending on the proliferative potential of each sample.
5. Incubate at 37°C and 5% CO_2. If the cells have not grown to confluency after 3 days, perform a full medium change.
6. Proceed to section 7.0 for cryopreservation of the remaining cells, if desired.

7.0 Cryopreserving and Thawing ECFCs

7.1 Cryopreserving Cells

The following protocol is for cryopreserving ECFCs using Cryostor® CS10, a serum-free, animal component-free, defined cryopreservation medium.

1. Detach cells as described in section 6.0 step 2.
2. Obtain a viable cell count using Trypan Blue and a hemocytometer.
3. Resuspend cells to a concentration of 0.5 - 1 x 10^6 cells/mL in cold (2 - 8°C) CryoStor® CS10. Mix thoroughly and transfer the suspension to a cryovial.
4. Cryopreserve cells using a standard slow rate-controlled cooling protocol (approximately -1°C/minute) or an isopropanol freezing container.
5. After 24 hours, transfer cryovials to liquid nitrogen for long-term storage (-135°C).

   Note: Long-term storage at -80°C is not recommended.

7.2 Thawing Cells

The following protocol is for thawing one vial of frozen ECFCs into a tissue culture flask for culturing. This protocol may be used for frozen ECFCs previously expanded in either serum-containing medium or complete EC-Cult™ Medium.

1. Coat a T-75 cm^2 tissue culture flask with ACF Cell Attachment Substrate (see section 3.2).
2. Warm (37°C) a 1 mL aliquot and a 2 mL aliquot of complete EC-Cult™ Medium in 15 mL conical tubes in a water bath or incubator. Place tubes in a biosafety cabinet along with the coated flask.

   Note: Ensure this step is completed before proceeding to step 3.
3. Remove a vial of 1 x 10^6 cryopreserved ECFCs from liquid nitrogen storage and immerse up to 2/3 of vial height in a 37°C water bath. Gently swirl until there is only a small amount of ice remaining (approximately 30 - 60 seconds). Avoid shaking the vial.
4. Wipe the outside of the vial with 70% ethanol or isopropanol prior to placing it in the biosafety cabinet.
5. Using a 1 mL pipette, slowly (dropwise) transfer thawed cell suspension to the 15 mL conical tube containing 1 mL of warm medium (prepared in step 2). Rinse the vial with the additional 2 mL of warm medium and add the wash to the 15 mL tube. Mix by gently swirling.

   Note: Do not centrifuge the cells.
6. OPTIONAL: Perform a viable cell count using Trypan Blue and a hemocytometer.
7. Add cells to the coated flask (prepared in step 1) at a density of > 10,000 cells/cm^2 (e.g. 1 x 10^6 cells per T-75 cm^2 flask). Do not exceed a density of 15,000 cells/cm^2.
8. Incubate at 37°C and 5% CO_2.
9. After 18 - 24 hours, remove medium and replace with fresh complete EC-Cult™ Medium.

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8.0 Troubleshooting

PROBLEM	POSSIBLE CAUSE	SOLUTION
No ECFC colony growth	• Harsh treatment of MNCs	• Remove and replace medium slowly (1 mL every 5 - 10 seconds) during the first week after plating
	• Low MNC seeding density	• Try higher seeding densities (e.g. 35 - 50 x 10^6 MNCs per well)
	• Reagents are outdated	• Use complete EC-Cult™ Medium within 15 days of preparation
	• Blood is too old	• Process umbilical cord blood as soon as possible. Process adult peripheral blood immediately.
ECFCs will not detach from the plate	• Cell adherence can be naturally very high, especially after 14 days in culture	• When ECFCs are passaged for the first time (from the 6-well plate), ensure the wells are rinsed with PBS twice before addition of dissociation solution. Allow the plate to incubate for 1 - 2 additional minutes. Tap the plates to dislodge the cells, and rinse thoroughly to ensure the majority of the cells have been lifted.
	• Low activity of dissociation solution	• Ensure dissociation solution is at room temperature (15 - 25°C) before use
ECFCs are not dividing, increase in size	• Cells are senescent	• Always seed ECFCs at a medium-to-high density (5,000 - 10,000 cells/cm^2). At a low seeding density, ECFCs experience stress, which can lead to premature senescence.
ECFCs are peeling from the plate	• Cell density is too high	• Lift cells and re-plate at a lower density. Do not exceed 10,000 - 12,500 cells/cm^2, unless plating freshly thawed cells, which require a density greater than 10,000 cells/cm^2.
	• Cells have been on the plate for too long	• Lift cells and re-plate using fresh ACF Cell Attachment Substrate
ECFC cultures grow unevenly	• Attachment substrate was not spread evenly	• Ensure wells are evenly covered
	• Uneven cell distribution during seeding	• Ensure the wells were coated correctly and that the cultureware was swirled in a “figure 8” or back-and-forth motion after seeding. Using a microscope, verify that the ECFCs are evenly distributed before placing the cells in the incubator.

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9.0 References

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TECHNICAL MANUAL

Culture of Human Endothelial Colony-Forming Cells (ECFCs) Using EC-Cult™-XF Culture Kit

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