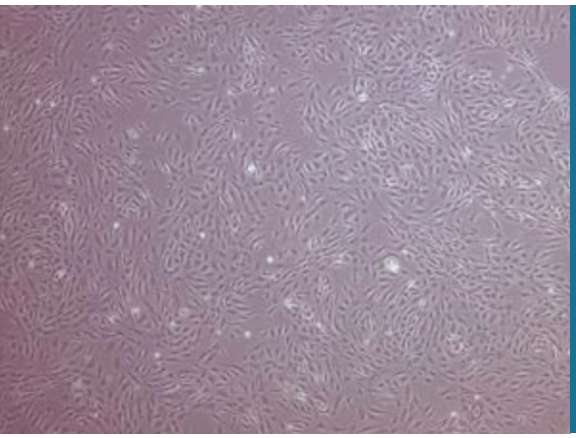
Instructions for use for TNCbio's Defined Endothelial cell growth kit XF501



Instructions for use for the serum-free culture of human endothelial cells under defined medium conditions with TNCbio's cell growth kit XF501.

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MEDIUM PREPARATION:

1. Bring all kit components to 37°C.
2. Add the Serum-Free-Supplement to the medium.
3. Add the other supplements to the medium.
4. Keep the prepared medium at room temperature before use.

The prepared medium can be stored at 4°C up to 6 weeks.

COATING OF FLASKS OR DISHES:

Coat sterile culture dishes or flasks with human or recombinant fibronectin. Dilute the fibronectin to a concentration of 1 ug/cm^2 or 10 ug/ml and coat your cell culture vessels with a minimal volume. Allow to air dry for 45 minutes under a laminar flow and then aspirate the excess solution before seeding the cells.

THAW THE CELLS:

1. Prepare a 15 ml tube with 10 ml growth medium and store it until use in the laminar flow cabinet.
2. Place the vial of frozen cells in a 37°C water bath for approximately 1 minute with moderate agitation until only a little bit of ice is still visible. Ensure that the vial is not completely submerged and water does not enter the vial.
3. Remove the vial from the water bath and wipe with 70% isopropanol or ethanol to sterilize.

4. Transfer cells into a 15 ml tube with a sterile 2 ml pipette.
5. Rinse the cryovial with 1 ml of medium to recover the remaining cells and add to the 15 ml tube.
6. Swirl gently after adding each portion.
7. Centrifuge the 15 ml tube containing the cells at 200x g for 5 minutes at room temperature.
8. After centrifugation, remove the supernatant and discard.
9. Resuspend the cell pellet in 10 ml of endothelial cell growth medium.
10. Count the cells using trypan blue exclusion.

Optional:

Add DNase at 1 ug/ml to prevent cell clumping.

NOTE: *Thawing is very stressful for each cell and therefore it is recommended after thawing and after the transfer of the cells to 15 ml medium, to take an aliquot for counting and seed the cells immediately in 2 T25 flasks. Place them in the incubator during counting. After one day not all cells have attached due to stress during the first few minutes after thawing. Therefore less cells might get attached compared with the number of cells counted as vital cells.*

CELL CULTIVATION:

1. Calculate seeding cell number (e.g.: T25 flask – 25cm^2, seeding density: 1x 10^4/cm^2 = seed 250 000 cells per T25 flask)
2. Seed the resuspended cells according to the calculation.
3. Add the prepared defined endothelial cell medium to a T25 flask to reach a final volume of 10 ml per flask.
4. Cultivate in an incubator at 37°C and 5% CO^2.
5. The cells will attach within 12 hours after seeding.
6. Change the medium every 48 hours.
7. After reaching 80% confluence passage the cells. (PD time will be between 30 and 72 hours)
8. Passage cells and sub-culture following the instructions for sub cultivation, as mentioned below.

User protocol for the Sub cultivation from endothelial cells cultivated with TNCbio's defined Endothelial Cell Growth Medium

When cells are approximately 70-80% confluent they should still be in the log phase of growth and will require sub culturing.

NOTE: *avoid cells to become over confluent since they will start to die and may not be recoverable.*

PREPARATIONS

- Prepare the endothelial cell growth medium as mentioned on page 1. And pipette 10 ml of the prepared endothelial cell growth medium in a 50 ml centrifuge tube.
- Prepare dissociation solution. TNCbio strongly recommends Accutase.
- Prepare fibronectin coated culture flasks following the instructions on page 1.
- Label flasks properly mentioning cell line, passage number, split ratio, date etc.
- Note the details of the sub culturing in the culture record log sheet.

EXPANSION OF CULTURED ENDOTHELIAL CELLS:

1. Take the flask with the cultured cells and carefully pour off the medium into a waste container.
   NOTE: *take care to avoid contamination*
2. Pipette under sterile conditions enough PBS into the flask to rinse the cells and get rid of remaining medium and proteins in the residue. Tip flask gently a few times to rinse the cells and carefully pour or pipette the PBS from the flask into the waste container.
3. Pipette enough dissociation reagent (Accutase) to cover the flask bottom covering the cell layer. (e.g.: in a 25 cm^2 flask approx 2 ml Accutase solution)
4. Shuffle flask gently to ensure all cells are in contact with the dissociation enzyme.
5. As soon as cells have detached collect them in the prepared 50 ml tube.
6. Centrifuge the cell suspension for 5 minutes at 200 g, without brake.
7. Remove very carefully the supernatant as much as possible.
8. Resuspend the cell pellet gently with 6 ml prepared endothelial cell growth medium using a 1ml serological pipette. Avoid too much shear stress.
9. Count the cells
10. Pipette 10 000 cells/cm^2 into coated flasks and adjust with endothelial cell growth medium to the required volume. (e.g.: in a 25 cm^2 flask approximately 5 – 10ml)
11. Leave the cells overnight to recover and settle.