3D Embedded Tubule Formation Kit

(3D-ETF)
Cat #8708

Product Description

Angiogenesis is the formation of new blood vessels from preexisting vessels. As such, angiogenesis research is relevant in numerous contexts such as organ development, tissue repair, wound healing, and tumor progression. At its most basic, it is a complex multistep physiological process that involves cell survival, proliferation, migration, extracellular matrix degradation, altering cell-cell adhesion, cellular differentiation, network formation, lumen formation, and pruning. Due to the complexities, angiogenesis is difficult to study in a 2-dimensional *in vitro* system, which inherently lacks multiple aspects of the physiological angiogenic microenvironment. ScienCell™'s 3-dimensional Embedded Tubule Formation Kit (3D-ETF) is an inclusive kit that utilizes purified collagen type I to mimic all the intricacies of the angiogenic process more closely, including the lumen formation step (see Figure 1 at end of protocol). Gels can be fixed and stained for quantitative assessment.

Kit Components

Cat #	# of vials	Name	Quantity	Storage
8708-a	1	Collagen I from rat tail, 4 mg/mL	10 mL	2-8 °C
8708-b	1	Buffer A, 10X	1.5 mL	2-8 °C
8708-c	1	Buffer B	1 mL	2-8 °C
8708-d	1	sterile H_2O	5 mL	2-8 °C
8000	1	Human Umbilical Vein Endothelial Cells (HUVEC)	5 x 10^5	liquid nitrogen
1001-b	1	Endothelial Cell Medium - basal	500 mL	2-8 °C
1052	1	Endothelial Cell Growth Supplement	5 mL	-20°C
0025	1	Fetal Bovine Serum	25 mL	-20°C
0503	1	Penicillin/streptomycin Solution	5 mL	-20°C
8001	3	3D Medium - basal - serum free	100 mL	2-8 °C
8052	3	3D Growth Supplement	1 mL	-20°C
0573	3	Penicillin/streptomycin Solution	1 mL	-20°C

Not Included: Additional Recommended Materials

Cat #	Product Name
0183	0.05% Trypsin/EDTA (T/E)
0113	Trypsin Neutralization Solution (TNS)
0303	Dulbecco's Phosphate-Buffered Saline (DPBS)
8248	Bovine Plasma Fibronectin

Quality Control
3D-ETF is tested for the formation of lumen-containing HUVEC tubules according to the included protocol. All components are negative for bacterial and fungal contamination.

Product Use
3D-ETF is for research use only. It is not approved for human or animal use, or application in clinical or *in vitro* diagnostic procedures.

Shipping
8708-a, 8708-b, 8708-c, 8708-d are shipped on gel ice; 1001-b and 8001 are shipped at room temperature; 8000, 1052, 0025, 0503, 8052 and 0573 are shipped on dry ice.

Procedure:

Important notes before starting: Keep all kit components chilled on ice until ready for use.

- We recommend always making about 500 µL of extra gel to account for gel lost during pipetting.
- Gel polymerization is affected by temperature.
- All work should be performed in a sterile flow hood to maintain sterility; no components should be opened outside of a sterile working environment.
- Decontaminate external surfaces of component receptacles with 70% ethanol prior to entering the sterile working environment.
- Protocol details instructions to make 1 mL of collagen gel; please scale appropriately
    - Each "gel dot" requires 75 µL of gel per well.
    - Please scale appropriately.
- Kit is designed for 75 µL embedding dots in 24-well plates.
- Included HUVEC vial (Cat. #8000) contains $5x10^5$ viable cells immediately upon thawing.
    - Each well or each gel dot requires $7.5x10^4$ viable cells; please consider this when preparing cell cultures for assaying and scale appropriately.
        - For reference, 1 confluent T-75 flask will contain about $4x10^6$ HUVEC (Note: this is only an estimate).
    - We do not recommend extensive sub-culturing of cells prior to performing 3D assays. Sub-culturing can select for 2D growing characteristics, which can affect 3D assaying efficacy. Note: Sub-culturing reagents such as 0.05% T/E solution (Cat. #0183) and DPBS (Cat. #0303) are not included.
    - We recommend plating HUVEC onto fibronectin-coated culture vessels at 2 µg/cm^2. Note: Bovine plasma fibronectin (Cat. #8248) is not included.

A. Initiating HUVEC cells:

Kit components required for Section A: HUVEC (#8000) and complete ECM (#1001-b, 1052, 0025, and 0503).

A1. Prepare a sterile culture vessel. We recommend plating directly into 3 fibronectin-coated T-75 flasks with fibronectin at 2 µg/cm^2, depending on the number of assays to be performed. Each confluent T-75 flask should yield about $4x10^6$ HUVEC for roughly 50 gel dots; please scale accordingly. To obtain a 2 µg/cm^2 fibronectin-coated culture vessel, add 5 ml of sterile Dulbecco's phosphate buffered saline, Ca^{++}- and Mg^{++}-free (Cat. #0303) to a T-75 flask and then add 150 µl of fibronectin stock solution (Cat. #8248). Leave the vessel in a 37^{o}C incubator overnight (or for at least 2 hours).

A2. Prepare complete endothelial cell medium (ECM) (Cat. #1001-b, 1052, 0025, and 0503) by decontaminating external surfaces with 70% ethanol, transferring components to a sterile field, aseptically transferring the supplements (Cat. #1052, 0025, and 0503) to the basal medium (1001-b) with a pipette, and rinsing the supplement tubes with medium to recover the entire volume.

A3. Aspirate the fibronectin solution and add 15 ml of complete medium to the culture vessel. The fibronectin solution can be used twice. Leave the vessel in the sterile field and proceed to thaw the cryopreserved cells.

A4. Thaw the cryopreserved vial of HUVEC (#8000) in a 37^{o}C water bath with gentle rotation until contents are thawed.

Note: Dilution and centrifugation of cells after thawing are not recommended since these actions are more harmful to the cells than the effect of residual DMSO in the culture.

A5. Promptly remove the vial from the water bath upon thawing, decontaminate the vial's external surface with 70% ethanol, and transfer it to the sterile field containing both the prepared culture vessel from Step A3 and prepared ECM from Step A2.
A6. Gently resuspend and transfer the thawed HUVEC into the prepared culture vessel with the appropriate amount of ECM for the vessel size. We recommend using 15 mL ECM per T-75 flask.
A7. Replace the cap or lid of the culture vessel and gently rock the vessel to distribute the cells evenly within the culture vessel with gentle rocking and if necessary, loosen the vessel cap to allow gas exchange.
A8. Maintain the culture in a 37°C, 5% CO_2, humidity incubator and allow the cells to adhere without disturbance for at least 16 hours.
A9. Refresh the culture medium the next day to remove residual DMSO and unattached cells.
A10. Maintain the culture by changing the medium every three days thereafter. Optional: If subculturing is necessary, please refer to the protocol details included in the product sheet for Cat. #8000.

B. Preparation of 3D gel dots: preparation time ~1.25 hr, designed for 24-well plates
Protocol details instructions to make 1 mL of collagen gel. Each "gel dot" requires 75 µL of gel per well. Please scale appropriately. Kit components required for Section B: HUVEC (#8000), Collagen I (8708-a), Buffer A (8708-b), Buffer B (8708-c), sterile H_2O (8708-d), and prepared 3D Assay Medium - serum free (8001, 8052, and 0573).

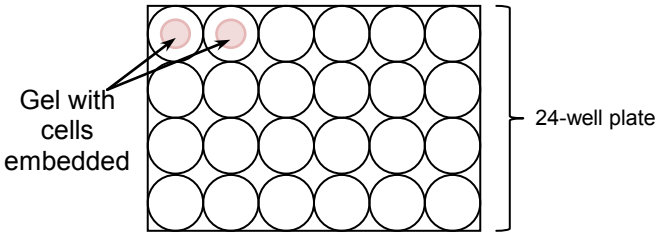
B1. When desired amount of HUVEC have been achieved from Section A, gather necessary materials to prepare 3D assay: required kit components (included), ice, pipettes, tubes, tips, 24-well plate(s), 37 °C/5% CO2 humidity incubator, $7.5x10^4$ HUVEC per intended gel dot (from Section A), trypsin/EDTA (not included), DPBS (not included), and a trypsin neutralizing solution (not included).
  1.1. To prepare 3D Assay Medium - serum free, add 1 mL 3D Growth Supplement (8052) and 1 mL pen/strep (0573) to 100 mL 3D Assay Medium - basal - serum free (8001).
    1.1.1. Store prepared medium at 2-8°C when not in use; use at room temperature with assay.
B2. Wash HUVEC attached to culturing vessel with 1x DPBS.
B3. Detach HUVEC from culturing vessel using trypsin/EDTA solution (T/E).
  3.1. We recommend using 0.05% T/E (Cat. #0103).
  3.2. Once detached, we recommend deactivating the trypsin using T/E neutralization solution (TNS) (not included) (Cat. #0113); alternatively, complete ECM may be used to neutralize trypsin.
B4. Aliquot $7.5x10^4$ cells for each intended embedded gel dot in an appropriately sized tube.
  4.1. Example: Aliquot $3.75x10^5$ cells for 5 75-µL gel dots or aliquot $7.5x10^4$ cells for 1 75- µL gel dot.
B5. Spin down cells at 2 rcf for 5 minutes and set aside on ice.
  5.1. Leave the excess media with the cell pellet and remove just prior to collagen addition before plating.
B6. Obtain an uncoated 24-well plate(s) for plating and bring it into the hood.
B7. Prepare gel components in a separate tube by combining 625 µL collagen I (8708-a), 100 µL Buffer A (8708-b), and 225 µL sterile H_2O (8708-d).
  7.1. Mix contents well with gentle pipetting after adding each reagent and avoid bubbles.
  7.2. If possible, keep everything on ice while combining components.
  7.3. Note: This step makes 1 mL of gel; please scale appropriately.
B8. Retrieve pelleted cells from Step B5 and remove excess medium.

********AFTER THIS NEXT STEP, BE AS QUICK AS POSSIBLE without sacrificing care********

B9. To the mixture from Step B7, add 50 µL Buffer B (8708-c).
  9.1. Mix well with gentle pipetting and avoid bubbles.
  9.2. BE QUICK; gel starts to polymerize immediately with addition of Buffer B.

B10. Add the appropriate amount of gel to cells to obtain a final ratio of $7.5x10^4$ cells/75 µL gel.
  10.1. Mix well by pipetting; avoid bubbles.

B11. Carefully pipette 75 µL of the cell/gel mixture from Step 10 to the middle of 1 well of a 24-well plate.
  11.1. Once gel dots have been plated, do not tilt or move plate in hood for 5 minutes. Doing so may cause gel to spread to the well edge and disrupt tubule formation.
  11.2. The gel dots with embedded cells will approximate this diagram (left) and photos of gel dots properly plated from the top (middle) and viewed from the side (right):



top view

side view

B12. Carefully place the plate in a 37 °C/5% CO_2 humidity incubator and let the gel polymerize undisturbed for 1 hour.

B13. After polymerization, gently add room temperature or warm complete 3D Medium from Step B1.1 to each gel dot well dropwise and down the side of the well.
  13.1. Aggressive addition of media can dislodge the gel dot.
  13.2. Cold media can disrupt the integrity of the gel.
  13.3. Add enough media so that the gel dot is entirely covered (typically around 700 µL).

B14. Maintain the assay in a 37 °C/5% CO_2 humidity incubator and change the medium every other day.
  14.1. Do not use a vacuum aspirator as aggressive aspiration can dislodge the gel.
  14.2. Remove media using a pipette by hand.

B15. Observe cells; assay typically peaks around day 6. See Figure 1 below for tubule formation.

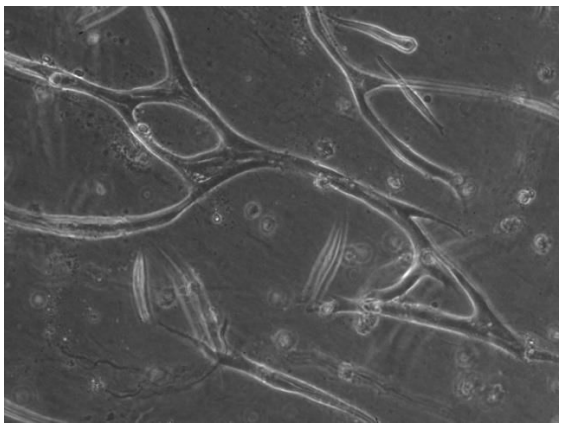


Figure 1. Day 5; phase contrast image of lumen-containing HUVEC tubule, 200x.