3D Network Formation Assay Kit

(3D-NF)
Cat #8718

Introduction

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. Because of its 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 Network Formation Assay Kit is an inclusive kit that utilizes purified collagen type I to mimic the intricacies of angiogenesis more closely with a focus on anastomosis (see Figure 1 at end of protocol).

Kit Components

Cat #	# of vials	Name	Quantity	Storage
8718-a	2	Collagen I from rat tail, 4 mg/mL	10 mL	2-8 °C
8718-b	1	Buffer A, 10X	5 mL	2-8 °C
8718-c	1	Buffer B	1 mL	2-8 °C
8718-d	3	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	1	3D Medium - basal - serum free	100 mL	2-8 °C
8052	1	3D Growth Supplement	1 mL	-20°C
0573	1	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-NF is tested for the formation of HUVEC cord networks according to the included protocol. All components are negative for bacterial and fungal contamination.

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

Shipping
8718-a, 8718-b, 8718-c, 8718-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 or on ice until ready for use.

- We recommend making about 500 µL of extra gel to account for gel lost during pipetting.
- Gel polymerization is affected by temperature.
- All work is to be done in a sterile flow hood to maintain sterility; surfaces should be decontaminated prior to entry and no components should be opened outside of the sterile area.
- Protocol details instructions to make 1 mL of collagen gel.
    - Each "sandwich" requires 2 gel layers of 400 µL each.
    - Please scale appropriately.
- Included HUVEC vial is guaranteed to contain at least $5x10^5$ viable cells immediately upon thawing.
    - Each well or each sandwich requires $7x10^5$ viable cells; please consider this when preparing cell cultures for assaying and scale appropriately.
        - For reference, 1 confluent T-75 flask will contain roughly $4x10^6$ HUVEC (Note: this is only an estimate).
    - We do not recommend extensive sub-culturing of cells prior to 3D assaying; sub-culturing can select for 2D growing characteristics, which can affect 3D assaying efficacy.
    - 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 5 sandwiches; 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 A4 and complete 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: preparation time ~5 hr, designed for 24-well plates

Protocol details instructions to make 1 mL of collagen gel. Each "sandwich" requires 800 µL of gel per well. Please scale appropriately. Kit components required for Section B: HUVEC (#8000), Collagen I (8718-a), Buffer A (8718-b), Buffer B (8718-c), sterile H_2O (8718-d), and complete 3D Medium - serum free (8001, 8052, and 0573).

B1. When desired amount of HUVEC have been achieved, gather necessary materials to prepare 3D assay: kit components, ice, pipettes, tubes, tips, 24-well plate(s), 37 °C/5% CO2 humidity incubator, $7x10^5$ cells per intended sandwich (from Section A), complete 3D Medium, complete ECM (from Section A), trypsin/EDTA (not included), DPBS (not included), and a trypsin neutralizing solution (not included).

1.1. To prepare 3D Medium – serum free, add 1 mL 3D Growth Supplement (8052) and 1 mL pen/strep (0573) to 100 mL 3D Medium (8001).

1.1.1. Store prepared medium at 2-8°C when not in use; use at room temperature with assay.

B2. Prepare a 24-well plate(s) for plating.

B3. Prepare gel components by combining 500 µL collagen (Cat. #8718-a), 100 µL Buffer A (Cat. #8718-b), and 388.5 µL sterile H_2O (Cat. 8718-d#) in a tube.

3.1. Mix contents well with gentle pipetting after adding each reagent; avoid bubbles.

3.2. If possible, keep everything on ice while combining.

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

B4. To the mixture from Step 4, add 11.5 µL Buffer B (Cat. #8718-c).

4.1. Mix well with gentle pipetting; avoid bubbles.

4.2. BE QUICK; gel starts to polymerize immediately with the addition of Buffer B.

B5. Aliquot 400 µL gel from Step B4 to 1 well of a 24-well plate.

5.1. Repeat as necessary for desired number of wells.

5.2. Mixture from Step B4 prepared as described is enough for 2 wells of a 24-well plate, or 2 400-µL aliquots.

5.3. Tip: dispense gel into the middle of the well to ensure even coating of the well bottom.

B6. Carefully transfer the plate to a 37 °C/5% CO_2 incubator and let the gel polymerize there for 1 hr undisturbed.

B7. Return gel components (Cat. #8718-a, 8718-b, 8718-c, 8718-d) to their storage units until Step B13.

B8. While the gel is polymerizing, trypsinize and count HUVEC intended for assaying.

8.1. Ensure that trypsin is deactivated using a trypsin neutralizing solution (recommended: ScienCell™ Trypsin Neutralization Solution, Cat. #0113); alternatively, complete ECM may be used to neutralize trypsin.

8.2. Isolate $7x10^5$ cells for each intended gel sandwich and resuspend in 500 µL complete ECM per sandwich..

8.2.1. Example: for 2 sandwiches isolate $1.4x10^6$ cells and resuspend in 1 mL complete ECM.

8.2.2. Concentration of cells should now be $7x10^5$ cells per 500 uL ECM.

8.2.3. If necessary, spin cells at 2 rcf for 5 minutes to concentrate cells.

8.2.4. Set aside on ice or keep at 4°C until almost ready for use.

8.2.5. Bring cells to room temperature 5 minutes before gels from Steps B5-B6 finish incubating.

8.2.6. Adequately resuspend cells prior to use to ensure even distribution.

B9. After the 1 hour incubation, retrieve gel plate (from Step 7) from incubator and return to sterile hood.

B10. Aliquot 500 µL of cell suspension from Step B8.2 onto each gel layer.

10.1. Dispense cell suspension gently and drop-wise onto middle of gel.

10.2. Aggressive dispensing can disrupt gel integrity.

10.3. Ensure that media and cells are evenly distributed over gel layer.

B11. Return the gel plate with cells to the incubator and let the cells attach to the gel.

11.1. Typically 1-3 hours is sufficient for attachment.

11.2. If unsure of attachment time, overnight incubation is permissible.

B12. After cells attach, gently remove medium from wells using a pipette by hand.

12.1. Make sure to remove all medium; cells will not dry immediately because of the gel.

12.2. If medium is not completely removed, gel sandwich layers may not adhere to each other.

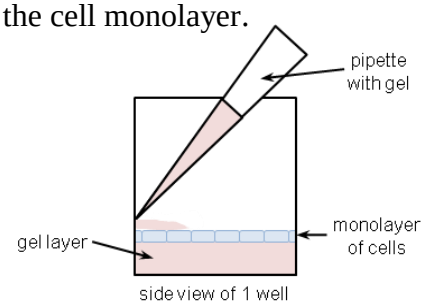
12.3. Using a vacuum aspirator may disrupt or dislodge the gel and/or cells.

B13. Repeat Steps B3 through B5, except this time in Step B5, slowly add the second layer of gel off to the side of the well to avoid disrupting or dislodging cells.

13.1. Aggressively depositing the second layer of gel in this step can lift cells off the matrix.

13.2. Avoid depositing the gel directly into the center of the well. Ensure that the second layer of gel evenly coats the first gel layer and the cell monolayer.

13.3. Illustrated depiction of this step:



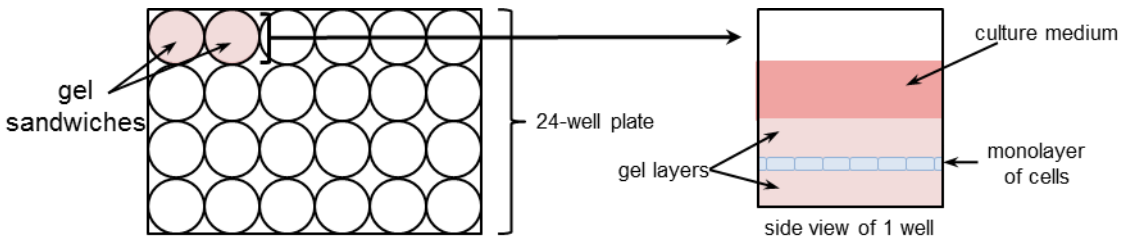
B14. Incubate the plate undisturbed at 37 °C/5% CO_2 for 3 hours in the tissue culture incubator.

B15. Bring the plate back to the sterile flow hood and add warm or room temperature 3D Medium from Step 3.1 gently and carefully down the side of the well.

15.1. Forceful addition of medium can damage the gel sandwich.

15.2. Cold media can disrupt the integrity of the gel.

15.3. Final construction of the gel sandwich will approximate this diagram:



B16. Maintain the assay in a 37 °C/5% CO_2 humidity incubator and change media every other day.
16.1. Do not use a vacuum aspirator; aggressive aspiration can dislodge the gel.
16.2. Remove media using a pipette by hand.
B17. Observe; assay typically peaks around day 5.

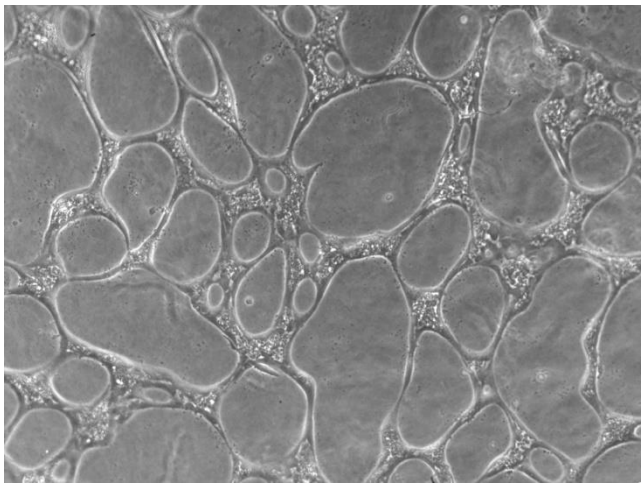


Figure 1. Day 4, 10x light microscope image of HUVEC cord network.