Rev. 1

ScienCell™ Research Laboratories

3D Network Formation Assay Kit

(3D-NF)

Catalog #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)	5×10^5	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:

- We recommend making about 500 µL of extra gel to account for gel lost during pipetting.
- Keep all kit components on ice until ready to use. Gel polymerization is affected by temperature.
- 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.
- We do not recommend extensive subculturing of cells prior to 3D assaying; subculturing can select for 2D growing characteristics, which can affect 3D assaying efficacy.

A. Initiating HUVEC

1. Thaw HUVEC (Cat# 8000) vial following the instructions detailed in the product sheet for Cat# 8000.
2. If more cells are required, cells can be subcultured following the instructions detailed in the product sheet for Cat# 8000.
3. Determine how many cells are needed for your experiments and calculate accordingly.
    a. Please note that each well or gel dot requires about $7.5x10^5$ viable cells; scale appropriately.

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.

1. When desired amount of HUVEC have been achieved from Section A, gather necessary materials to prepare 3D assay: Cat# 8718-a, Cat# 8718-b, Cat# 8718-c, Cat# 8718-d, $7x10^5$ cells per intended sandwich (from Section A), complete 3D Medium, complete ECM (from Section A), 0.05% Trypsin/EDTA, DPBS, and Trypsin Neutralization Solution.
2. Prepare complete 3D Medium-serum free by adding 1 mL 3D Growth Supplement (Cat# 8052) and 1 mL pen/strep (Cat# 0573) to 100 mL bottle of Cat# 8001.
    a. Store prepared medium at 2-8°C when not in use; use at room temperature with assay.

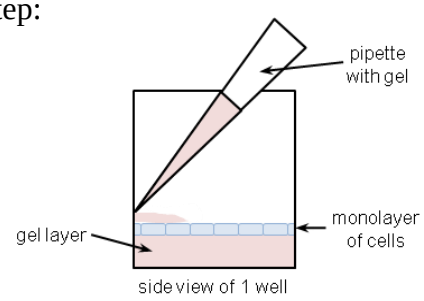
3. Obtain an uncoated 24-well plate(s) for plating. Prepare gel components in a separate tube by combining the following components listed in the table below. Example shown is for 1 mL collagen gel; please scale appropriately. Maintain all components on ice during this step.

Making Collagen Gels (for 1 mL gel)

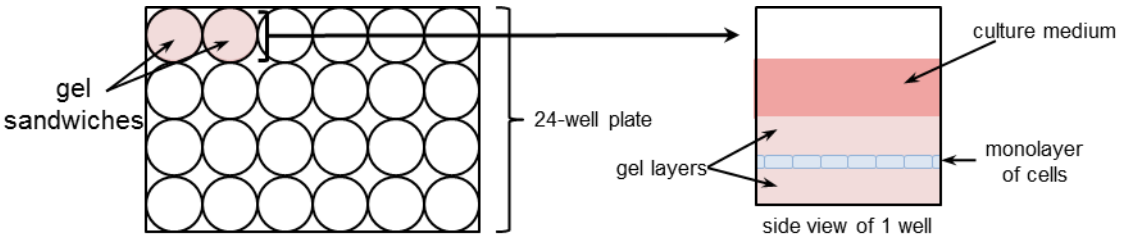
Component	Volume
Collagen I (Cat#8718-a)	500 µL
Buffer A (Cat#8718-b)	100 µL
Sterile H_2O (Cat#8718-d)	338.5µL

4. Mix contents well with gentle pipetting after adding each reagent; avoid bubbles.
5. To the collagen mixture from Step 3, add 11.5 µL Buffer B (Cat# 8718-c). BE QUICK. Gel starts to polymerize with addition of Buffer B. Mix well with gentle pipetting and avoid bubbles.
6. Aliquot 400 µL gel from Step 5 to 1 well of a 24-well plate. Repeat as necessary for desired number of wells.
    a. Mixture from Step 5 prepared as described is enough for 2 wells of a 24-well plate, or 2 400-µL aliquots.
    b. Tip: dispense gel into the middle of the well to ensure even coating of the well bottom.
7. Carefully transfer the plate to a 37 °C/5% CO_2 incubator and let the gel polymerize there for 1 hr undisturbed.
8. Return gel components (Cat. #8718-a, 8718-b, 8718-c, 8718-d) to 2-8^{o}C.
9. While the gel is polymerizing, harvest HUVEC from culturing vessel following instructions provided in Cat# 8000.
    a. Once cells have been harvested and counted, aliquot $7x10^5$ cells for each intended gel sandwich and resuspend in 500 µL complete ECM per sandwich.
        i. Example: for 2 sandwiches isolate $1.4x10^6$ cells and resuspend in 1 mL complete ECM.
        ii. Concentration of cells should now be $7x10^5$ cells per 500 µL ECM.
        iii. If necessary, centrifuge cells at 1,100 rpm (2 rcf) for 5 minutes to concentrate cells.
        iv. Set aside on ice or keep at 4°C until almost ready for use.
        v. Bring cells to room temperature 5 minutes before gels finish incubating.
        vi. Adequately resuspend cells prior to use to ensure even distribution.
10. After 1 hour incubation of plate, aliquot 500 µL of cell suspension from Step 9 onto each gel layer.
    a. Dispense cell suspension gently and drop-wise onto middle of gel and ensure even distribution.
    b. Aggressive dispensing can disrupt gel integrity.
11. Return the gel plate with cells to the incubator and allow the cells attach to the gel for 1-3 hours.
    a. If unsure of attachment time, overnight incubation is permissible.
12. After cells attach, gently remove medium from wells using a pipette.
    a. Make sure to remove all medium; cells will not dry immediately because of the gel.
    b. If medium is not completely removed, gel sandwich layers may not adhere to each other.
    c. Using a vacuum aspirator may disrupt or dislodge the gel and/or cells.
13. Repeat Steps 3 through 6, except this time in Step 6, slowly add the second layer of gel off to the side of the well to avoid disrupting or dislodging cells.
    a. Aggressively depositing the second layer of gel in this step can lift cells off the matrix.

b. 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.
c. Illustrated depiction of this step:



14. Incubate the plate undisturbed at 37 °C/5% CO_2 for 3 hours in the tissue culture incubator.
15. After 3 hours, add room temperature 3D Medium gently and carefully down the side of the well.
    a. Forceful addition of medium can damage the gel sandwich.
    b. Cold media can disrupt the integrity of the gel.
    c. Final construction of the gel sandwich will approximate this diagram:



16. Maintain the assay in a 37 °C/5% CO_2 humidity incubator and change media every other day.
    a. Remove media using a pipette, as vacuum aspiration can dislodge the gel.
17. Observe; assay typically peaks around day 5.

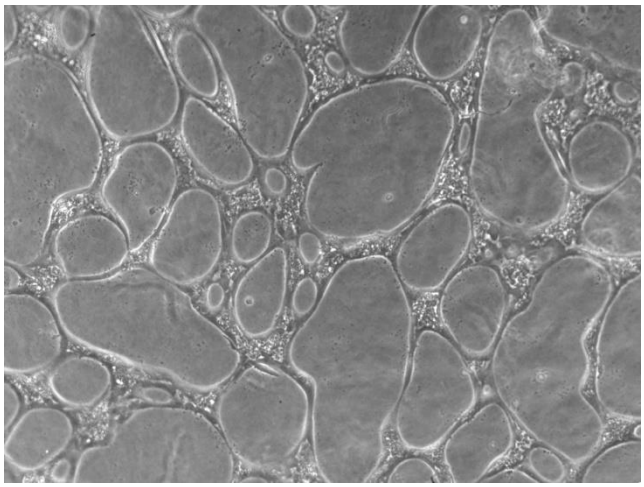


Figure 1. Day 4, 10x light microscope image of HUVEC cord network.