3D Endothelial–Pericyte Coculturing Kit

(3D-EPC)
Cat #8728

Product Description

Blood vessels are responsible for transporting blood throughout the body as part of the circulatory system. Their main components are endothelial cells, which comprise the inner lining, and mural (or perivascular) cells, which are largely responsible for regulating vasoconstriction, pruning, permeability, and vascular maturation. There are two main types of mural cells: vascular smooth muscle and pericytes. Pericytes wrap around capillary vessels and communicate with other cells using their long cytoplasmic processes. Vessels that lack or have lost pericyte investment are shown to become hemorrhagic or hyperdilated. Unlike vascular smooth muscle cells, pericytes have roles beyond scaffolding evidenced by their direct communication and physical contact with endothelial cells, and their density differs with respect to their function and capillary bed. Among their many functions, pericytes most notably contribute to blood brain barrier functionality and their loss has been found to contribute to neurodegenerative diseases and stroke. ScienCell™'s 3D Endothelial-Pericyte Coculturing Kit (3D-EPC) combines both pericytes and endothelial cells in one 3-dimensional *in vitro* assay that utilizes a defined serum-free medium to mimic the vasculature more closely in a controlled environment (see Figure 1 at end of protocol).

Kit Components

Cat #	# of vials	Name	Quantity	Storage
8728-a	1	Collagen I from rat tail, 4 mg/mL	20 mL	2-8 °C
8728-b	1	Buffer A, 10X	5 mL	2-8 °C
8728-c	1	Buffer B	1 mL	2-8 °C
8728-d	1	sterile H_2O	15 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
1200-3D	1	Human Brain Vascular Pericytes (HBVP-3D)	1 x 10^5	liquid nitrogen
1201-3D	1	Pericyte Medium – 3D	100 mL	2-8 °C
1252-3D	1	Pericyte Growth Supplement – 3D	1 mL	-20°C
0002-3D	1	Fetal Bovine Serum	2 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	2	Penicillin/streptomycin Solution	1 mL	-20°C

Additional Materials Recommended (not included)

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
0413	Poly-L-Lysine, 10 mg/mL

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

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

Shipping
8728-a, 8728-b, 8728-c, 8728-d are shipped on gel ice; 1001-b, 8001, and 1201-3D are shipped at room temperature; 8000, 1200-3D, 1052, 0025, 0503, 1252-3D, 0002-3D, 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 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 so 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 recommend plating HUVEC onto fibronectin-coated culture vessels at 2 µg/cm^2. Note: Bovine plasma fibronectin (cat #8248) is not included.
- Included HBVP vial (cat #1200-3D) contains $1x10^5$ viable cells immediately upon thawing.
    - Each well or each gel dot requires $1.5x10^4$ viable cells; please consider this when preparing cell cultures for assaying and scale appropriately.
        - For reference, 1 confluent T-25 flask will contain about $1.0x10^6$ HVBP (Note: this is only an estimate).

- We recommend plating HBVP onto poly-L-lysine-coated culture vessels at 2 μg/cm^2. Note: poly-L-lysine (cat #0413) is not included.

- 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.

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 rinse 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^{o}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. Initiating HBVP:

Kit components required for Section B: HBVP (#1200-3D) and complete PM (Cat. #1201-3D, 1252-3D, 0002-3D, and 0503).

B1. Prepare a sterile culture vessel. We recommend plating directly into 2 poly-L-lysine-coated T-25 flasks with poly-L-lysine at 2 μg/cm^2, depending on the number of assays to be performed. Each

confluent T-25 flask should yield about $1.0x10^6$ HBVP for roughly 65 gel dots; please scale accordingly. To obtain a 2 $\mu g/cm^2$ poly-L-lysine-coated culture vessel, add 5 ml of sterile water to a T-25 flask and then add 5 µl of poly-L-lysine stock solution (10 mg/mL) (Cat. #0413). Leave the vessel in a 37°C incubator overnight (or for a minimum of 1 hour).

B2. Prepare complete pericyte medium (PM) (Cat. #1201-100, 1252-3D, 0002-3D, and 0503) by decontaminating external surfaces with 70% ethanol, transferring components to a sterile field, aseptically transferring the supplements (Cat. #1252-3D, 0002-3D, and 0503) to the basal medium (Cat. #1201-100) with a pipette, and rinse the supplement tubes with medium to recover the entire volume.

B3. Aspirate the poly-L-lysine solution and add 5 ml of complete medium to the culture vessel. Leave the vessel in the sterile field and proceed to thaw the cryopreserved cells.

B4. Thaw the cryopreserved vial of HBVP (#1200-3D) in a 37°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.

B5. 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 PM from Step A2.

B6. Gently resuspend and transfer the thawed HBVP into the prepared culture vessel with the appropriate amount of PM for the vessel size. We recommend using 5 mL complete PM per T-25 flask.

B7. 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.

B8. 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.

B9. Refresh the culture medium the next day to remove residual DMSO and unattached cells.

B10. 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. #1200.

C. 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 C: HUVEC (#8000), HBVP (#1200-3D), Collagen I (8728-a), Buffer A (8728-b), Buffer B (8728-c), sterile H_2O (8728-d), and prepared 3D Assay Medium - serum free (8001, 8052, and 0573).

C1. When desired amount of HUVEC and HBVP have been achieved from Sections A and B, 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), $1.5x10^4$ HBVP per intended gel dot (from Section B), 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.

C2. Wash both HUVEC and HBVP attached to culturing vessels with 1x DPBS.

C3. Detach both HUVEC and HBVP from culturing vessels 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 for HUVEC and HBVP cultures, respectively.

C4. Aliquot $7.5x10^4$ HUVEC for each intended embedded gel dot in an appropriately sized tube.

C5. To each of those HUVEC-containing tube(s) from Step C4, add $1.5x10^4$ HBVP for each intended embedded gel dot.

5.1. Example 1: For 1 75- µL gel dot, aliquot $7.5x10^4$ HUVEC and $1.5x10^4$ HBVP into the same one tube.

5.2. Example 2: For 5 75-µL gel dots, aliquot $3.75x10^5$ HUVEC and $7.5x10^4$ HBVP into the same one tube.

5.3. Note: The ratio of HBVP to HUVEC must be maintained.

5.3.1. HBVP density affects tubule formation and gel rigidity.

C6. Spin down cells at 2 rcf for 5 minutes and set aside on ice.

6.1. Leave the excess media with the cell pellet to remove just prior to collagen addition before plating.

C7. Obtain an uncoated 24-well plate(s) for plating and bring it into the hood.

C8. Prepare gel components in a separate tube by combining 625 µL collagen I (8728-a), 100 µL Buffer A (8728-b), and 225 µL sterile H_2O (8728-d).

8.1. Note: This step makes 1 mL of gel.

8.1.1. The final proportions of HUVEC/HVBP/gel should be $7.5x10^4$ HUVEC/$1.5x10^4$ HBVP/75 µL gel for each 75- µL gel dot.

8.1.2. Please scale appropriately.

8.2. Mix contents well with gentle pipetting after adding each reagent and avoid bubbles.

8.3. If possible, keep everything on ice while combining components.

C9. Retrieve pelleted cells from step C6 and remove excess medium.

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

C10. To the mixture from step C8, add 50 µL Buffer B (8728-c).

10.1. Mix well with gentle pipetting and avoid bubbles.

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

C11. Add the appropriate amount of gel to cells to obtain a final ratio of $7.5x10^4$ HUVEC/$1.5x10^4$ HBVP/75 µL gel.

11.1. Mix well by pipetting; avoid bubbles.

11.2. Example 1: For 1 75-µL gel dot, add 75 µL gel mix from Step C10 to a tube containing $7.5x10^4$ HUVEC and $1.5x10^4$ HBVP

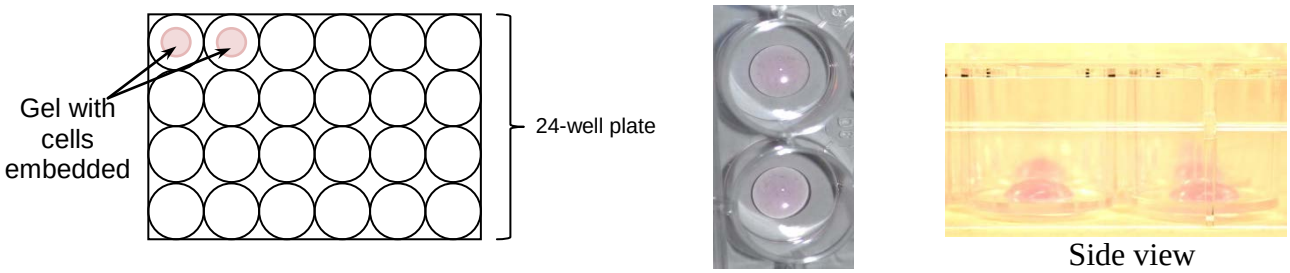
11.3. Example 2: For 5 75-µL gel dots, add 375 µL gel mix from Step C10 to a tube containing $3.75x10^5$ HUVEC and $7.5x10^4$ HBVP

C12. Carefully pipette 75 µL of the cells/gel mixture from Step C11 to the middle of 1 well of a 24-well plate.

12.1. Once gel dots have been plated, avoid tilting or moving the plate.

12.2. Note: Do not panic if gel spreads to sides of well; assay will still perform as described but visualization and subsequent staining may be inhibited.

12.3. The gel dots with embedded cells will approximate this diagram (left) and photo of gel dots properly plated (right):



C13. Leave plate undisturbed in hood for 5 minutes.

C14. Carefully place the plate in a 37 °C/5% CO_2 humidity incubator and let the gel polymerize undisturbed for 1 hour.

C15. After polymerization, gently add room temperature or warm complete 3D Medium from Step C1.1 to each gel dot well drop-wise and down the side of the well.

15.1. Aggressive addition of media can dislodge the gel dot.

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

15.3. Add enough media so that the gel dot is entirely covered (typically around 700 µL).

C16. Maintain the assay in a 37 °C/5% CO_2 humidity incubator and change the medium every other day.

16.1. Do not use a vacuum aspirator as aggressive aspiration can dislodge the gel.

16.2. Remove media using a pipette by hand.

C17. Observe cells; assay typically peaks around days 7-9 but can be maintained past day 9. See Figure 1 below for tubule formation.

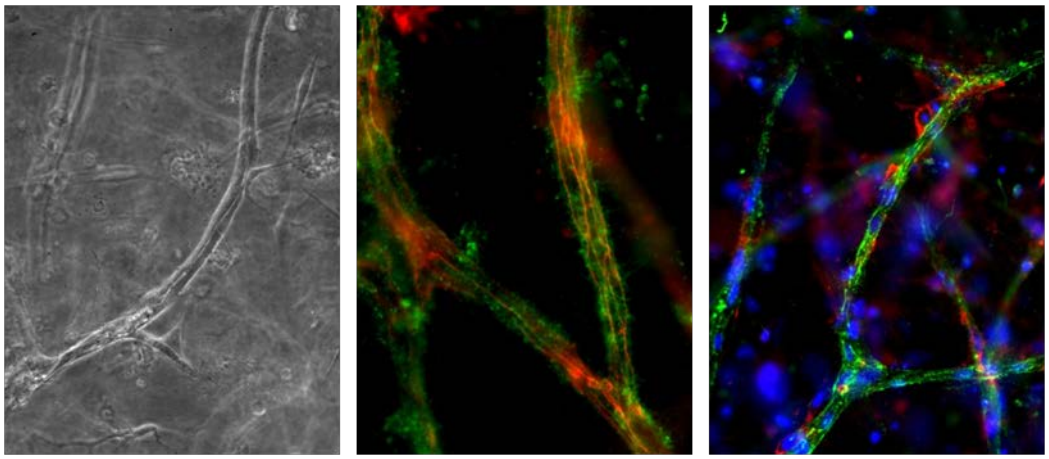


Figure 1. (Left) Phase contrast image of lumen-containing tubule, day 9, 200x. (Center) Fluorescence image of multicellular tubule showing endothelial marker CD31 staining in green and tight junction protein ZO-1 staining in red, day 9, 400x. (Right) Fluorescence image of multicellular tubule showing endothelial marker VWF staining in green, pericyte marker NG-2 staining in red, and DAPI staining in blue, day 9, 200x.