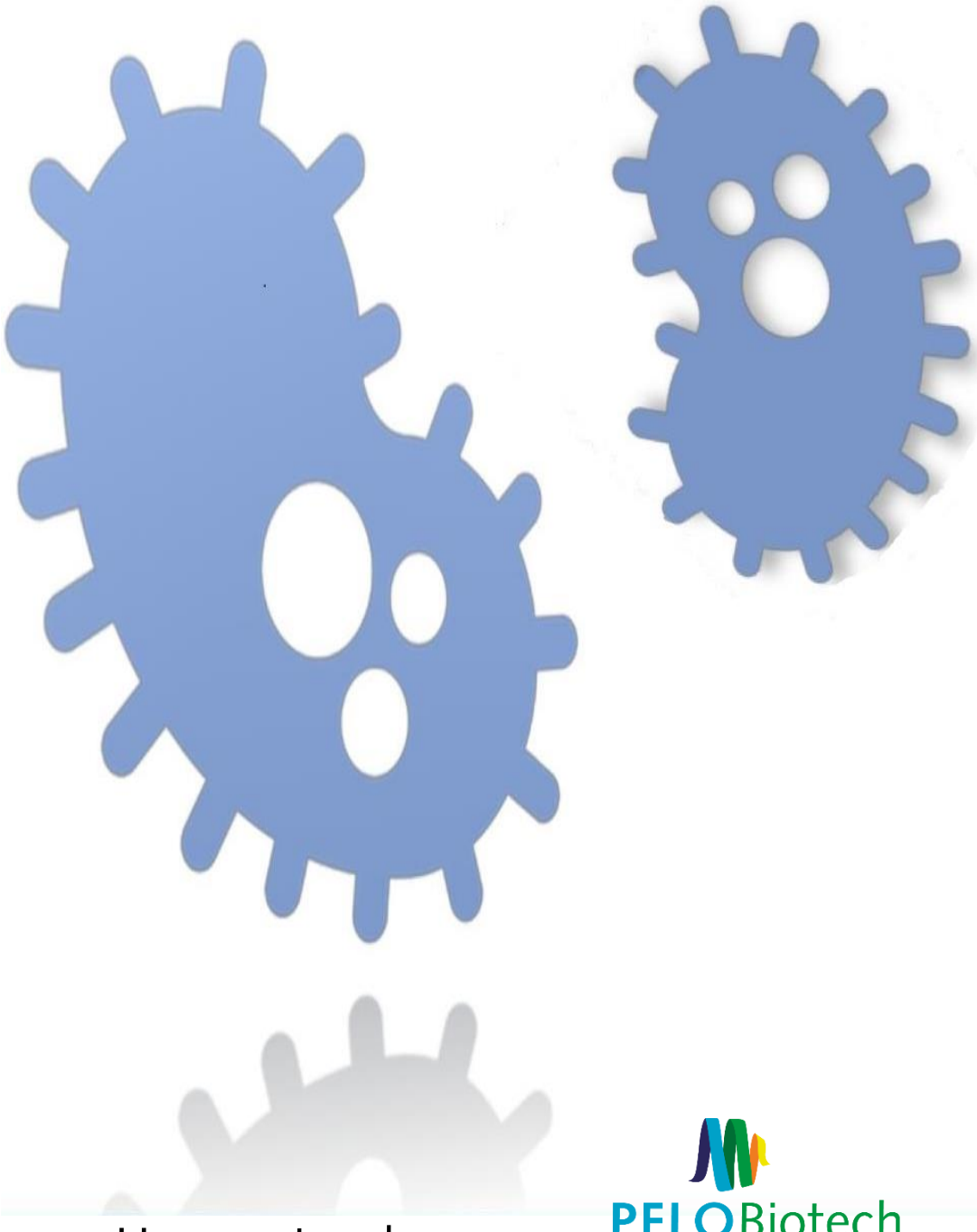


PELOBiotech
More than Competence⁴ Cells

User protocol:
Culture of endothelial cells

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User protocol for Endothelial cells (please have a look at the data sheet and CoA for cell and lot specific instructions).

Materials required

- Endothelial Cell Growth Medium (e.g. Cat# PB-MH-100-2100, PB-MH-100-2190, PB-MH-100-2199, PB-MH-100-4090 or PB-MH-100-4099), Please check out Tab.: 1 to choose the right medium.
- Speed Coating solution (Cat# PB-LU-000-0002-00)
- Passaging Solutions (e.g. Passage Pack (Cat# PB-900028) or Cell Passaging Solution (Accutase) (Cat# PB-PAACUTASE))
- Freezing Medium (defined and animal free) (Cat# PB-PACRY-50))
- Cell Culture Lab

Tab.: 1

Further products	
Catalogue #	**Materials**
PB-LU-000-0003-100-f	Collagen type I
EC1-R105	Collagen Type I, Human
PB-LU-508-0000	Human plasma derived fibronectin
PB-LH-000-0000-50-1	Cell Culture Freezing Medium (FBS)
PB-LH-000-0000-49-1	Cell Culture Freezing Medium (NON-FBS)
PB-PACRY-50	Freezing medium defined and animal free

Culture of endothelial cells

General note

- All cell culture procedures must be conducted in a bio-safety cabinet.
- Use aseptic technique to prevent microbial contamination.
- Cryo-preserved cells must be stored in liquid nitrogen or seeded immediately upon arrival.
- Although medium is supplied sterile, please make sure to handle it under sterile conditions.

Prepare cell culture media

1. Warm the Basal Medium and Supplement(s) to 37°C.
2. Please wipe the outside of all bottles with 70% alcohol to sterilize.
3. Add Supplement(s) to the Basal Medium.
4. Keep Growth Medium at 37°C.
5. Coat flask with 2 mL Speed Coating Solution (SCS) per T25 flask and make sure whole surface is covered with SCS. Five minutes later dispose SCS by aspiration. Rinse the T25 flask with 5 mL PBS (Room Temperature, RT) twice. Flasks are now ready to be used.
6. Pre-equilibrate the culture flasks at 37°C, in a 5% CO_2 humidified incubator.

⚠ Please Note: A minimum of 10-15 mL medium has to be used in order to dilute out the cryoprotectant. The supplemented medium can be stored at 4°C up to 4-6 weeks!

Thawing the cells

Cells are all different in their needs so please have a look at the data sheet oir Tab.: what the optimal seeding density has to be!

1. Prepare a 15 mL tube with 10 mL growth medium and store it until use under the laminar flow cabinet.
2. Place the vial of frozen cells in a 37°C water bath for roughly 1 minute with moderate agitation until only a little bit of ice is 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. Using a sterile 2 mL pipette, transfer cells into a 15 mL tube.
5. Rinse the Cryovial with 1 mL of primary cells medium to recover the remaining cells. Add to the 15 mL tube.
6. Add further medium (9 mL from the aliquot you did earlier) in small amounts to the 15 mL tube. Swirl gently after adding each portion.
7. Centrifuge 15 mL tube containing the cells at 200 x g for 5 minutes at room temperature.

8. After centrifugation, remove supernatant and discard carefully without disturbing the pellet.
9. Resuspend cell pellet in a known volume (~ 10 mL) of culture endothelial cell medium.
10. Count the cells using a haemocytometer (Cat# 521-10-20) (or equivalent) to determine the number of viable cells per mL (see in the table below) and transfer for initial seeding.

⚠ Optional: Add DNase at 1 µg/mL final to prevent cell clumps.



The expert's advice:
Thawing is very stressful for cells and therefore I would recoomend to use ThawStar® (Cat# AST-645 or AST-650) for reproducible thawing of cells.

Determining the cell viability and yield

To determine cell viability and yield from the cell suspension in step 10, please follow these instructions:

1. Please ensure a uniform cell suspension (do not vortex).
2. Take 100 µL of the cell suspension and mix it in a 1.5 mL tube with 100 µL of Trypan Blue solution and 800 µL of PBS (1/10 dilution) and keep the rest of the cell suspension in the laminar flow hood until seeding. Dilution can be modified considering the cell pellet. Please work quickly.
3. Gently homogenize the solution by inverting the tube with the cells and the Trypan Blue solution (do not vortex).
4. Add an aliquot of 10 µL with the Trypan Blue/cell mixture into a cell counting chamber.
5. Proceed to cell observation and counting under the microscope. Dead cells will appear blue.
6. In order to determine cell viability and yield, count the living and dead cells and use the formula below:

Viability (%) = (Live cell count/Total cell count) x 100
Total Cell number = Viable cell count/Quadrants counted x Dilution factor x 10 ^{4} x Current volume(mL)*
** This factor is applicable when it is used a Hemocytometer*
When you know the viable cell number calculate seeding cell number for your cell culture flask (e.g.: T-25- 25 cm^2, seeding density: 1x10^4 Cells/cm^2 or higher; e.g. seed 250.000 per T25).

Seeding of Endothelial cells into cell culture flasks

1. The cells should be initially seeded with 10.000 – 20.000 cells/cm^2 on the precoated flasks and culture the cells at 37°C in a CO_2 incubator.
2. Examine the cells microscopically to check even distribution of cells in each flask, and transfer to a CO_2 incubator. Do not disturb culture for the next 16 hours to allow cell attachment.
3. Medium replacement after 24 hours, replace medium over a cell free surface of the flask (please use only pre-warmed and pre-equilibrated medium) and add fresh medium 5mL per T25 or 15 mL per T75 over a cell free area of the flask. Never replace or add medium over the cell layer.
4. Re-feed cells every 48 hours until they become confluent. Ideally passage the cells when they are sub-confluent and still actively dividing. Normally after 80% confluence will be reached you should passage cells. (population doubling time under defined conditions can be between 30 up to 72 hours.)

Passaging of primary cells

For the detachment of the cells Trypsin / EDTA, TrypLE or Accutase can be used. Below please find a summarized overview of their properties.

If you are interested in passaging under defined conditions please contact us.

Accutase**: (Cat# PB-PAACCUTASE)** is a mixture of proteolytic and collagenolytic enzymes isolated from crustaceans; it is mammary component free; for some cell types no need for inactivation or removal during passaging; is very gentle for cells, thus most of the surface proteins are intact after passaging; after use Accutase has to be stored at 4°C; Accutase is inactivated automatically after 1 h at 37°C.
Trypsin / EDTA**: (PB-900028 – Passage Pack)** is a solution of proteolytic enzymes containing Trypsin, Chymotrypsin and Elastase isolated from porcine pancreas that shows lot-to-lot variability in activity; is commonly used in cell culture; is available from different suppliers; has to be inactivated (e.g. FBS alone, FBS supplemented medium or trypsin inhibitor from soybean); compared with Accutase more cell culture experience of the user is necessary, because cells can be irreversibly damaged by too high trypsin concentrations or too long incubation times.

Culture of endothelial cells

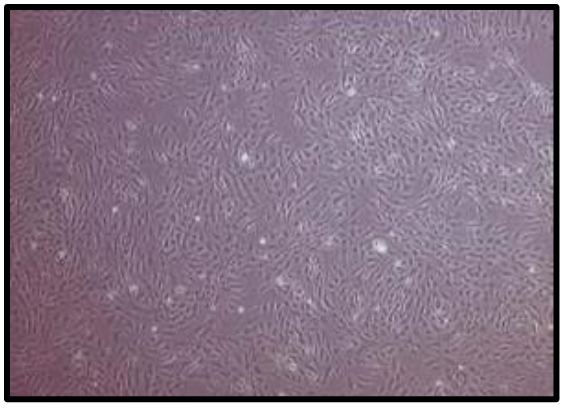
Prearrangement

1. Remove PELOBiotech's Cellovation-medium for endothelial cells from the fridge and warm it up to room temperature; best way is using a water bath at 37°C.
2. Prepare e.g. Accutase solution.
3. Open the medium bottle and fill 10 mL of the complete Cell Growth Medium in a 50 mL centrifuge tube.
4. Prepare Speed Coating solution coated culture flasks (see instructions on the second page).
5. Please make sure flasks are labelled with the cell line, passage number, split ratio, date, operator initials and the number of the cells.
6. Write down the details of the sub-culturing in the culture record log sheet.

The expert's advice:



You should use Accutase to avoid over-trypsinization.



Passage of cells using Accutase (PB-PAACCUTASE):

1. Cells should be passaged when they reach 70 to 80% confluency (depending on the cell type). Do not allow the cells to become 100% confluent.
2. When ready, carefully pour off media in a swift uniform motion from flask of the required cells into waste. Taking care not to increase contamination risk with any drips.
3. Using sterile technique, pour/pipette enough sterile PBS into the flask to give cells a wash and get rid of any medium with protein content in the residual culture media. Rotate flask in a circular motion gently a few times to rinse the cells and carefully pour/pipette the PBS back out into waste pot.
    a. Using pipette, add enough Enzyme (Cell Passaging Solution (Accutase solution) (PB-PAACUTASE) to cover the cells at the bottom of the flask.
    b. e.g. in 25 cm^2 flask approx 2 mL.
    c. 75 cm^2 flask approx. 5 mL
    d. 175 cm^2 flask approx. 10 mL.
4. Incubate at 37°C until cells are rounded and start to detach. Check for cell detachment under the microscope!

5. Then gently tap the culture vessel to detach any remaining cells.
6. When using Accutase: add two volumes of primary cell culture medium to the detached cells (or PBS w/ 2 % FBS). When using Trypsin / EDTA: add the same volume of Trypsin inhibitor.
7. Rinse the bottom of the culture vessel 2 to 3 times with the suspension to remove all cells and to separate them into a single cell suspension.
8. Transfer the cell suspension to a centrifugation tube.
9. Centrifuge the cells 5 min, 200 x g at room temperature.



The expert's advice:
Please note that the RPM to reach 200 g depends on the diameter of the rotor that is used.

10. Aspirate supernatant very carefully, because the pellet is not as fixed as it would be if you used a higher centrifugation speed e.g. 500 g and resuspend the pellet in 1 to 2 mL primary cell culture medium.
11. Count the cells and seed viable cells according to the seeding density listed in the data sheet.
12. Cells should become confluent within 4 to 7 days (when split at a 1:3 ratio without counting).

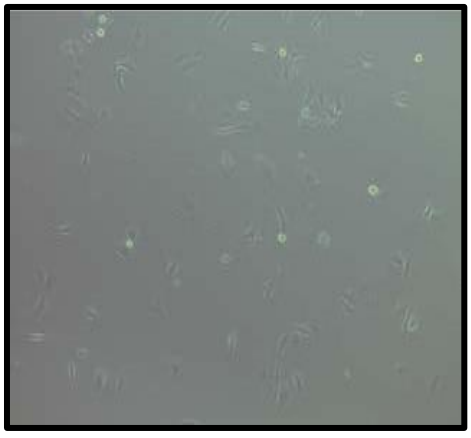
Passage of cells using the Passage Pack (PB-900028 Passage Pack):

1. Remove and discard primary cell culture medium from the 80% confluent T-25 flasks.
2. Wash cells twice with 10 mL of Hanks balanced salt solution (HBSS).
3. Use warm (37°C) Trypsin-EDTA (1X) solution and add 2 mL to a T-25 flask.
4. As soon as cells have detached **(the flask may require a few firm gentle taps)**, add Trypsin Neutralization Solution or add 10 mL of primary cell culture medium to the flask (medium supplemented with 5-10 % FBS will neutralize the trypsin).

⚠ **Over-trypsinization causes irreversible damage to cells**

5. Centrifuge 15 mL tube containing the cells at 200 x g for 5 minutes at room temperature.
6. After centrifugation, decant and discard the supernatant.
7. Resuspend cell pellet in a known volume (~ 1 mL) of primary cell culture medium.
8. Count the cells using Trypan Blue exclusion method.
9. Seed cells with seeding density listed in the data sheet and in the table below.
10. Add 10 mL of primary cell culture medium to a T25 flask
11. Change medium every two days.

After some hours it should look like the following picture:



⚠ **To prevent contamination**, it is crucial to handle cells with sterile technique in sterile conditions. In case you lack confidence in your technique, it would be wise to use antibiotics like penicillin streptomycin (100 µg/mL). However, if you experience recurrent contamination, it is advisable to opt for gentamycin (0.5 to 50 µg /mL) or tetracycline (12.5-15.0 µg/ml) rather than penicillin or streptomycin as antibiotic-resistant bacteria may be the cause of contamination.



The expert's advice:
Using this cell suspension, pipette the recommended cells per cm^2 into new coated flasks. These flasks should then be topped up with culture media to required volume
e.g. in 25 cm^2 flask approx. 5-10 mL, 75 cm^2 flask approx. 10-30 mL, 175 cm^2 flask approx. 40-150 mL

Cryopreservation

Cell Freezing procedure:

Materials:

- Phosphate Buffered Saline (PBS)
- Cell Passaging Solution (PELOBiotech Cat#: PB-PAACUTASE) or Passage Pack (PB-900028 Passage Pack)
- Cold Freezing Media (CryoStor CS5 or 10; 5 or your homemade medium (10% dimethylsulfoxide, and 20% FBS, and 70% culture medium)), PB-205102 or PB-210102 each 100 mL) or any media mention in Tab.:1
- Labeled Cryovials (~3-5 per T-25 flask)
- T25 flask or T75 flask confluent cells

1. When ready, carefully pour off media from flask of the required cells into the waste. Taking care not to increase contamination risk with any drips.
2. Using sterile technique, pour/pipette enough sterile PBS into the flask to give cells a wash and get rid of any medium with protein content in the residual culture media. Tip flask gently a few times to rinse the cells and carefully pour/pipette the PBS back out into waste pot.
3. Using pipette, add enough Enzyme (Cell Passaging Solution (Accutase solution) (PB-PAACUTASE) to cover the cells at the bottom of the flask.
    a. e.g. in 25 cm2 flask approx. 2 mL
    b. 75 cm2 flask approx. 5 mL
    c. 175 cm2 flask approx. 10 mL
4. Roll flask gently to ensure Accutase comes in contact with all cells. Different cell lines require different incubation times.
5. As soon as cells have detached collect them in the prepared 50mL tube.

6. Centrifuge the cell suspension for 5 minutes at 200 g, without brake.
7. Remove supernatant with a sterile Pasteur pipette as much as possible but very carefully, because the pellet is not as fixed as if is using 500 g.
   Note: Remove as much culture media as possible, to reduce dilution of CryoStorTM solution.
8. Isolation: Add cold (2-8°C) CryoStor™. (e.g.: 0,5x10^6 cells – add 1 mL CryoStor™ solution)
   a. Cell concentrations: 0.5-10 x 10^6 cells/mL (higher cell density possible with testing). DMSO is pre-mixed in CryoStor™ - no additives are necessary.
9. PRE-FREEZE: Incubate cell suspension at 2-8°C for 8-10 minutes.
10. Immediately add 1 mL cell suspension into the labeled and at 4°C pre-cooled cryo tubes.
11. Place vials at -70-80 °C for 24 h, then transfer to -180.

SPECIAL NOTES:
Always use dry ice to transfer the cells to permanent storage to avoid temperature rise and cell damage. Cryovial contents can rise from -80˚C to over -50˚C in less a minute if exposed to room temperature air.

Tab.: 1

Cat #	Cells	Initial seeding density cells/ cm^2	Seeding density after passaging cells/ cm^2	Recommended media	Recommended coating
PB-CH-142-0011	Adipose Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-180-2011	Aortic Artery Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-155-4011	Bladder Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-110-4011	Brain Microvascular Endothelial Cells	20,000	20,000	PB-MH-100-4099	SCS

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Cat #	Cells	Initial seeding density cells/ cm^2	Seeding density after passaging cells/ cm^2	Recommended media	Recommended coating
PB-CH-151-4011	Cardiac Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-170-6111	Synovial Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-182-2011	Coronary Artery Endothelial Cells	10,000	6,000	PB-MH-100-4099	SCS
PB-CH-120-5811	Dermal Lymphatic Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-120-4011	Dermal Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-152-5211	Glomerular Microvascular Endothelial Cells	20,000	20,000	PB-MH-100-4099	SCS
PB-CH-190-2011	HUAEC - Umbilical Artery Endothelial Cells	20,000	10,000	PB-MH-100-2100, PB-MH-100-2190 or PB-MH-100-2199	SCS
PB-CH-190-8011	HUVEC - Umbilical Vein Endothelial Cells	10,000	6,000	PB-MH-100-2100, PB-MH-100-2190 or PB-MH-100-2199	SCS
PB-CH-190-8013	HUVEC - Umbilical Vein Endothelial Cells, pooled	10,000	6,000	PB-MH-100-2100, PB-MH-100-2190 or PB-MH-100-2199	SCS
PB-CH-190-8011-1	HUVEC - Umbilical Vein Endothelial Cells, single donor	5,000-10,000	5,000	PB-MH-100-2100, PB-MH-100-2190 or PB-MH-100-2199	SCS

Culture of endothelial cells

Cat #	Cells	Initial seeding density cells/ cm^2	Seeding density after passaging cells/ cm^2	Recommended media	Recommended coating
PB-CH-187-2011	Iliac Artery Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-159-2011	Internal Mammary Artery Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-149-4911	Large Intestine Microvascular Endothelial Cells	20,000	20,000	PB-MH-100-4099	SCS
PB-CH-153-5811	Liver Lymphatic Microvascular Endothelial Cells	20,000	15,000	PB-MH-100-4099	SCS
PB-CH-153-5511	Liver Sinusoidal Microvascular Endothelial Cells	20,000	15,000	PB-MH-100-4099	SCS
PB-CH-150-4011	Lung Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-186-4011	Ovarian Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-147-4011	Pancreatic Microvascular Endothelial Cells	20,000	6,000-10,000	PB-MH-100-4099	SCS
PB-CH-157-4011	Prostate Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-150-5811	Pulmonary Lymphatic Microvascular Endothelial Cells	20,000	10,000-15,000	PB-MH-100-4099	SCS

Cat #	Cells	Initial seeding density cells/ cm^2	Seeding density after passaging cells/ cm^2	Recommended media	Recommended coating
PB-CH-160-8511	Retinal Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-181-8011	Saphenous Vein Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-148-4811	Small Intestine Microvascular Endothelial Cells	20,000	20,000	PB-MH-100-4099	SCS
PB-CH-170-6111	Synovial Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-162-4011	Tonsil Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS
PB-CH-158-4011	Uterine Microvascular Endothelial Cells	20,000	10,000	PB-MH-100-4099	SCS

What others also order (further products of interest):

For 2D Cultivation and in general

- Speed Coating solution (Cat# PB-LU-000-0002-00)
- Human Plasma Fibronectin Solution (HPF) (Cat# PB-LU-508-0000)
- Cell Passaging Solution (Accutase) (Cat# PB-PAACUTASE)
- Passage Pack (Cat# PB-900028)
- Freezing medium defined and animal free (Cat# PB-PACRY-50)
- CryoStor CS 2 (Cat# PB-202102)
- CryoStor CS 5 (Cat# PB-205102)
- CryoStor CS 10 (Cat# PB-210102)
- CRYO-GOLD (Serum- & protein-free) (Cat# PB-10003-01)
- CryoScarless DMSO-Free (Serum & DMSO-free) (Cat# CPL-A1)

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For 3D Cultivation

- 3D CoSeedis™ Chip200 - 6 chip pack for 200 organoids per chip (Cat# C200-6)
- 3D CoSeedis™ Chip680 -6 chip pack for 680 organoids per chip (Cat# C680-6)
- 3D CoSeedis™ Chip880 - 6 chip pack for 880 organoids per chip (Cat# C880-6)
- hPLMA (hPL derived hydrogel) (Cat# PB-PLL01-0100)
- HydroSeev (5 mg/mL) (Cat# PB-SVX-HY-10)
- iMatrix-411 (Cat# 892042)
- TissueSpec® Liver ECM Hydrogel (Cat# PB-MTSLV101)
- TissueSpec® Lung ECM Hydrogel (Cat# PB-MTSLG101)

If you have any question, please do not hesitate to contact us via Email.



Dr. Lothar Steeb,CSO
Lothar.steeb@pelobiotech.com

If you would like to know more about doing 3D cell culture please send an email to our expert Dr. Peter Frost to get started.



Dr. Peter Frost, CEO
peter.frost@pelobiotech.com