PEPROTECH®
OUR SUPPORT, YOUR DISCOVERY

Instruction Manual

PeproGrow™ Endothelial Media Products

Maintenance Media for
EPCs and Endothelial Cells

Product Use Limitations: Not for human use.

Made in the United States.

Cultivation of EPCs and Endothelial Cells Using PeproGrow™ Endothelial Media Products

A. Introduction

PeproTech offers three separate endothelial cell culture media formulations developed for the *in vitro* cultivation of: endothelial progenitor cells (EPCs) derived from bone marrow or peripheral blood (PeproGrow™ EPC Medium); endothelial cells from large vessels (PeproGrow™ MacroV Medium); and endothelial cells from small vessels (PeproGrow™ MicroV Medium). These media formulations maintain outstanding endothelial cell morphology and function, and increase the activity of endothelial nitric oxide synthase (eNOS), which account for a specific, crucial marker for endothelial cells. By doing this, these media provide an optimal cell culture environment for macrovascular and microvascular endothelial cells, as well as for EPC; growing cells at rates that exceed commercially available media.

PeproGrow™ Endothelial Media Products are supplied as kits containing both a 500mL bottle of basal medium and a separate bottle of frozen growth supplement bottle that contains various essential growth factors and components for endothelial cell growth. Adding the growth supplement to the basal medium results in the complete culture medium. PeproGrow™ Endothelial Media Products do not contain antibiotics, antimycotics, antifungal, or phenol red, as these components can cause cell stress and masking effects that may reduce complete medium shelf life and influence experimental results.

PeproGrow™ Endothelial Media Products should be prepared under strict sterile conditions, and stored in the dark to prevent light damaging effects. These products are not intended for use in humans.

B. Materials and Reagents

1. PeproGrow™ Endothelial Media Products:

Kit/Components	Catalog Number:	Size:
PeproGrow™ EPC Medium Kit	**700-EPC**	
PeproGrow™ Endothelial Cell Basal Medium	ENDO-BM	500mL
PeproGrow™ EPC Growth Supplement	GS-EPC*	75mL
PeproGrow™ MacroV Medium Kit	**700-MacroV**	
PeproGrow™ Endothelial Cell Basal Medium	ENDO-BM	500mL
PeproGrow™ MacroV Growth Supplement	GS-MacroV*	25mL
PeproGrow™ MicroV Medium Kit	**700-MicroV**	
PeproGrow™ Endothelial Cell Basal Medium	ENDO-BM	500mL
PeproGrow™ MicroV Growth Supplement	GS-MicroV*	35mL

*Contains FBS.

2. Cell Lines:

Cell lines can be purchased from the researcher's vendor of choice or suppliers such as Lonza, PromoCell, Clonetics™, Cell-Systems, etc. The list of recommended cell types for PeproGrow™ Endothelial Media Products are listed below.

PeproGrow™ EPC Medium is recommended for Endothelial Progenitor Cells:

Human Endothelial Progenitor Cells (hEPCs)
Mouse Endothelial Progenitor Cells (mEPCs)

PeproGrow™ MacroV Medium is recommended for Macrovascular Endothelial Cells:

Human Umbilical Vein Endothelial Cells (HUVECs)
Human Umbilical Artery Endothelial Cells (HUAECs)
Human Aortic Endothelial Cells (HAoECs)
Human Pulmonary Artery Endothelial Cells (HPAECs)
Human Saphenous Vein Endothelial Cells (HSaVECs)
And other large vessel (macrovascular) endothelial cells

PeproGrow™ MicroV Medium is recommended for Microvascular Endothelial Cells:

Human Coronary Artery Endothelial Cells (HCAECs)
Human Pancreatic Microvascular Endothelial Cells (HPaMECs)
Human Dermal Microvascular Endothelial Cells (HDMECs)
Human Pulmonary Microvascular Endothelial Cells (HPMECs)
Human Dermal Lymphatic Endothelial Cells (HDLECs)
Human Brain Microvascular Endothelial Cells (HBMECs)
And other small vessel (microvascular) endothelial cells

3. Please refer to the appendix for additional materials and reagents.

C. Preparation of Basal Medium and Growth Supplement for Complete Culture Medium

1. Warm the basal medium in a water bath at 25ºC; the 500mL basal medium will take approximately 10 to 15 minutes to warm from 2ºC to 25ºC. If possible, cover the basal medium to protect it from exposure to light while warming and also after removal from the water bath.

WARNING: Repeated warming of the entire bottle over extended periods of time may impair the medium and reduce the shelf life.

2. After the basal medium has warmed, gently shake the basal medium for 30 seconds.

3. Thaw the growth supplement in a water bath at 25ºC just before use. For reference, the smallest of the three available growth supplements, PeproGrow™ MacroV Growth Supplement (25mL), will take about 30 to 45 minutes to warm from -20ºC to 25ºC.

4. Wipe the outside of both the basal medium and the growth supplement with a disinfecting solution such as 70% ethanol.

5. After thawing, gently pipette the growth supplement up and down, or gently invert the vial.

6. Using a sterile technique in a laminar flow culture hood, transfer the volume of basal medium listed in the chart below and the volume of growth supplement listed below to a 500mL 0.2µm size pore filter.

Basal Medium Volume	Growth Supplement	Volume
425mL	PeproGrow™ EPC Growth Supplement	75mL
475mL	PeproGrow™ MacroV Growth Supplement	25mL
465mL	PeproGrow™ MicroV Growth Supplement	35mL

7. Add antibiotics, antifungal, antimycotics, and/or phenol red to the filter if desired.

8. Filter all the components together, label the bottle with both the date of mixture and the newly calculated expiration date (four to six weeks from date of mixture), and store at 2ºC to 8ºC in the dark.

9. Remove only the necessary amount of medium for each immediate use and warm to 37°C in a water bath prior to culturing EPCs or endothelial cells.

Storage/Stability: Keep Medium and Growth Supplement in the dark.

Product Form	Temperature	Storage Time
Liquid Basal Medium	2°C to 8°C; Keep in the dark. Do not freeze.	12 months
Growth Supplement	-20°C to -80°C; Keep in the dark.	12 months
Complete Medium after preparation	2°C to 8°C; Keep in the dark.	4-6 weeks

Avoid repeated warming cycles or heating the medium above 37ºC.
Avoid repeated freezing cycles of the growth supplement.

General Recommendations:

- PeproGrow™ Endothelial Media should be used in a sterile environment.
- Penicillin-Streptomycin Amphotericin (PSA) can be added separately according to a manufacturer's instructions and acceptable concentrations for endothelial cell growth.
  - The Biological Industries Penicillin-Streptomycin Amphotericin B solution (Catalog # 03-033-1) antibiotic/antifungal mix solution is composed of 10,000 units/mL Penicillin G Sodium Salt, 10mg/mL Streptomycin Sulfate and 25µg/mL Amphotericin B. This can be added before filtering the growth supplement/basal medium at a 1:100 dilution (5ml Pen/Strep/AmphB solution into 500mL basal medium + growth supplement). All components should be filtered together.

D. Cell Well(s)/Cell Plate Preparation using Fibronectin

NOTE: It is recommended to plate endothelial cells onto fibronectin-coated wells or plates. Fibronectin enhances the endothelial cell-to-cell adherence and attachment to the bottom of the wells or plates so that endothelial cells form a monolayer. Adhere the fibronectin to the bottom of the well prior to adding cells.

1. Completely cover the bottom of the well with phosphate buffered saline (PBS) (e.g. add 0.7mL PBS/well for a 6-well plate, or 0.3mL PBS/well for a 24-well plate).
2. Dilute the fibronectin 1:100 (when the fibronectin vial concentration is 1.0mg/mL) in PBS and add to the bottom of the well (e.g. 7µL/well for a 6-well plate, or 3µL/well for a 24-well plate).
3. Incubate the fibronectin for 40 minutes at room temperature, or 20 minutes at 37°C, to allow the reagent to spread and attach to the bottom of the well.
4. After the incubation, discard the PBS and immediately plate the cells.

E. Endothelial Cell Cultivation

NOTE: The following are suggested protocols for selected cell types. Please refer to the manufacturer of purchased cells or the researcher's standard laboratory protocols as needed.

I. Cell Culture of EPCs (Endothelial Progenitor Cells) with PeproGrow™ EPC Medium

NOTE: Endothelial progenitor cells (EPCs) are primitive cells within the endothelial lineage. These cells are derived from bone marrow. EPCs migrate into the blood stream and can differentiate into several types of mature vascular endothelial cells. Upon receiving cryopreserved cells, immediately transfer cells from dry ice to liquid nitrogen and store the cells in liquid nitrogen until ready for cell culture. EPCs can either be isolated from human samples using a well-defined protocol or delivered at the 4^{th} passage, either as cryopreserved or proliferating cells in culture flasks (cryovials available from several suppliers, such as LONZA and PromoCell, usually contain 500,000 cells in 1mL volume). In general, the cryopreserved EPCs are guaranteed to further expand for at least 4-5 population doublings.

Additional EPC Information: EPCs play a crucial role in revascularization and angiogenesis. These cells are involved in tumor growth, metastasis, and collateral formation due to tissue ischemia. *In vivo*, EPCs invade breast and ovarian cancer cell clusters, whereas human microvascular endothelial cells (HMVECs) do not. Interestingly, EPCs share more similarity to human tumor endothelial cells in their gene expression patterns than human umbilical vein endothelial cells (HUVECs) or HMVECs. Alteration in EPC number and function has also been observed in the pathogenesis of aging and smoking-related diseases, and a variety of cardiovascular diseases such as coronary artery disease (CAD), ischemia, pulmonary hypertension,

cerebral vascular disease, acute myocardial infarction, diabetes mellitus, arthritis, and wound healing. EPCs are used in these research and drug discovery areas, as well as in research related to cancer, cutaneous wound healing, and skin regeneration (Hill JM, N Engl J Med. 2003 Feb 13;348(7):593-600).

Properties of EPCs:

- EPCs are progenitor cells, which are not terminally differentiated mature endothelial cells, such as HUVECs and HMVECs.
- EPCs are capable of differentiating into specific subtypes of endothelial cells, such as vein endothelial cells, microvascular endothelial cells, and aortic endothelial cells.
- The gene expression pattern is more similar to mature endothelial cells than endothelial cells from tumors.
- EPCs specifically migrate to tumor sites.
- The number and function of EPCs in the human blood is altered during pathogenesis of a variety of human diseases, such as cancer, inflammatory diseases, diabetes mellitus and cardiovascular diseases.

1. Culturing EPCs

a. Prepare PeproGrow™ EPC Medium according to "C. Preparation of Basal Medium and Growth Supplement for Complete Culture Medium."

b. Warm the medium in a 37°C water bath.

c. Wipe the outside of the frozen cell vial with 70% ethanol. Quickly thaw the frozen cells in the 37°C water bath.

d. Aseptically transfer the cell suspension into a 15mL conical tube. Rinse the vial with 1mL of the medium and transfer to the 15mL tube.

e. Add enough medium for a total volume of 5mL, and then gently resuspend the cells.

f. Dispense the cell suspension into the desired cell culture plate. A seeding density of 20,000 - 25,000 cells/cm^2 is recommended. Do not centrifuge the cells, as this can damage the cells more-so than the effects of residual DMSO in the culture.

g. Incubate the cells at 37°C with 5% CO_2 and 95% air in a humidified incubator. Change the medium the next day to remove unattached cells, and then every other day thereafter. A healthy cell culture appears with a cobblestone-like morphology, and the cell count should be doubled after two to three days in culture.

2. Sub-culturing EPCs

NOTE: Subculture the cells when they reach approximately 90% confluence.

a. Warm each bottle of PBS, 0.05% trypsin/EDTA, and the medium in a 37°C water bath.

b. Rinse the cells with PBS.

c. Incubate the cells with the trypsin/EDTA solution until about 90% of the cells begin to detach. Monitor the cells with a microscope and avoid over-trypsinization.

d. Add fetal bovine serum equal to $1/10^{th}$ the volume of the trypsin/EDTA to neutralize trypsin. Gently shake the culture plate to mix.

e. Gently resuspend the cells and transfer the cells into a 15ml conical tube.

f. Centrifuge the cell suspension at 400g for 5 minutes at room temperature.

g. Carefully remove the supernatant without disturbing the cell pellet. Resuspend the cells in 5mL medium.

h. Count the cells and plate these in a new culture vessel at the density of about 20,000-25,000/cm^2.

i. Incubate the cells at 37°C with 5% CO_2 and 95% air in a humidified incubator.

j. Change the medium every other day.

II. Cell Culture of HUVECs (Human Umbilical Vein Endothelial Cells) or other macrovascular endothelial cells with PeproGrow™ MacroV Complete Medium

NOTE: HUVECs originate from the umbilical vein, either from isolated laboratory protocols or commercially cryopreserved cells purchased from a supplier, such as Clonetics™, PromoCell, Cell-Systems, or other vendors.

General Recommendations:

- HUVECs can be serially propagated for 30-70 population doublings. However, after about 5-7 passages the cells gradually start to increase in size, grow more slowly, and lose specific functions. Experiments should be performed with cells between passages two through four.
- Cells should be grown on tissue culture plastic flasks, plates or dishes coated with fibronectin or gelatin. Recombinant human fibronectin is available through Akron, Sigma or other vendors. Gelatin is prepared as a 1% solution in Phosphate Buffered Saline (PBS) and sterilized by autoclaving.

- Coat dishes by covering with a thin layer of 1% gelatin or 10µg/mL fibronectin, and incubate at room temperature for at least 40 minutes or in an incubator for 20 minutes. Aspirate the coating medium, and then seed cells immediately onto the dish.

1. Passaging HUVECs

NOTE: Seeding Cells: Ampoule (500,000 cells/1mL volume) is for seeding cells into two 75mm fibronectin-coated dishes.

a. Grow the cells until 80% confluent. This may take 4 or more days.

b. Aspirate the medium and wash the cells with PBS.

c. For a T75 T-flask, add 1.5mL of trypsin solution (0.05% trypsin, 0.53mM EDTA) to the flask.

d. Add 4mL medium to the flask.

e. Split the cells 1:4 into newly coated dishes.

2. Storing HUVECs

NOTE: HUVECs can be stored for extended periods of time in liquid nitrogen.

a. Wash the cells in PBS and trypsinize according to standard laboratory protocol.

b. Stop trypsin with 5X volume of medium.

c. Centrifuge the cells for 5 minutes at 400g.

d. Resuspend the cells in 0.5mL medium.

e. Cool the cells on ice.

f. Dilute the cell suspension with 0.5mL of 20% DMSO in medium to a final concentration of 10% DMSO.

g. Transfer the cell suspension to a cryotube and slowly freeze to -70°C using a specialized freezing container, or by wrapping the tubes heavily in tissue and placing these overnight in a -70°C freezer.

h. Transfer the vial of cells to liquid nitrogen.

3. Recovering HUVECs

a. Thaw the cells rapidly in a 37°C water bath.

b. Dilute the cells with 9mL of culture medium.

c. Centrifuge the cells for 5 minutes at 400g.

d. Resuspend the cells in culture medium and seed into appropriate flasks/plates.

III. Cell Culture of HMVECs (Human Microvascular Endothelial Cells) with PeproGrow™ MicroV Complete Medium

NOTE: HMVECs are commercially available through suppliers, such as Clonetics™, PromoCell, Cell-Systems, or other vendors.

General Recommendations:

- HMVECs can be serially propagated for 20-40 population doublings. However, after about 5-7 passages the cells gradually start to increase in size, grow more slowly, and lose specific functions. Experiments should be performed with cells between passages two through four.
- Cells should be grown on tissue culture plastic flasks, plates or dishes coated with either with fibronectin or gelatin. Recombinant human fibronectin is available through Akron, Sigma or other vendors. Gelatin is prepared as a 1% solution in Phosphate Buffered Saline (PBS) and sterilized by autoclaving.
- Coat dishes by covering with a thin layer of 1% gelatin or 10µg/mL fibronectin, and incubate at room temperature for at least 30 minutes. Aspirate the coating medium, and then seed cells immediately onto the dish.

1. Passaging HMVECs

NOTE: Seeding Cells: Ampoule (500,000 cells/1mL volume) is for seeding cells into two 75mm fibronectin-coated dishes.

a. Grow the cells until 80% confluent. This may take 4 or more days.

b. Aspirate the medium and wash the cells with PBS.

c. For a T75 T-flask, add 1.5mL of trypsin solution (0.025% trypsin, 0.53mM EDTA) to the flask.

d. Add 4 mL medium to the flask.

e. Split the cells 1:4 into newly coated dishes.

2. Storing HMVECs

NOTE: HMVECs can be stored for extended periods of time in liquid nitrogen.

a. Wash the cells in PBS and trypsinize according to standard laboratory protocol.

b. Stop trypsin with 5X volume of medium.

c. Centrifuge the cells for 5 minutes at 400g.

d. Resuspend the cells in 0.5mL medium.

e. Cool the cells on ice.

f. Dilute the cell suspension with 0.5mL of 20% DMSO in medium serum to a final concentration of 10% DMSO.

g. Transfer the cell suspension to a cryotube and slowly freeze to -70°C using a specialized freezing container, or by wrapping the tubes heavily in tissue and placing these overnight in a -70°C freezer.

h. Transfer the vial of cells to liquid nitrogen.

3. Recovering HMVECs

a. Thaw the cells rapidly in a 37°C water bath.

b. Dilute the cells with 9mL of culture medium.

c. Centrifuge the cells for 5 minutes at 400g.

d. Resuspend the cells in culture medium and seed into appropriate flasks/plates.

Appendix I: Reagents and Materials

Reagents and Materials for Cell Culture

1. PeproGrow™ Endothelial Media; Refer to "C. Preparation of Basal Medium and Growth Supplement for Complete Culture Medium"

2. Penicillin-Streptomycin Amphotericin B solution (Biological Industries catalog number 03-033-1)

3. Cell culture plate

4. Cell culture dish

5. T Flasks

6. Fibronectin (Akron, Sigma or other commercially available alternative)

7. Trypsin

8. DMSO

9. Phenol Red

Preparation and addition of Antibiotic/Antifungal Solution to Cell Culture Medium

NOTE: The addition of antibiotics, antimycotics, antifungal, and phenol red can cause cell stress and masking effects that may reduce complete medium shelf life and influence experimental results.

1. It is recommended to use Penicillin-Streptomycin Amphotericin B solution (Biological Industries catalog number 03-033-1).
2. Dilute the antibiotic/antifungal solution 1:100 into the total complete medium at step #7 of "C. Preparation of Basal Medium and Growth Supplement for Complete Culture Medium."

Appendix II: Figures and Descriptions

Cell Protocol Summary for Figures (thawing, seeding and analysis of cells):

EPCs were isolated from peripheral blood of a volunteer in a collaborator's laboratory according to their isolation protocol. The cells were seeded onto fibronectin-coated 6X well-plates, and incubated in a humidified incubator at 37ºC for 7 days with PeproGrow™ Endothelial Medium and a competitor's medium. The media was changed every other day for 7 days.

HUVECs and HDMECs were thawed in sterile conditions and centrifuged in the commercial media of the purchased cells (12 minutes, 400g, and 23ºC). The supernatant was discarded, and the pellet was resuspended in each cell type's PeproGrow™ Endothelial Medium and a competitor's medium. Cells were then seeded onto fibronectin-coated 6X well-plates and incubated in a humidified incubator at 37ºC for 5 days. The media was changed every other day for 7 days.

On the 7th day of cell cultivation, each cell type was detached, lysed, and analyzed for its proliferation ability by XTT assay (Biological Industries, Cell Proliferation Kit XTT, catalog number 20-300-1000) and for endothelial nitric oxide synthase expression (eNOS antibody).

Figure 1.

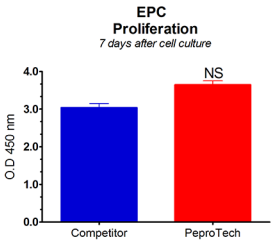
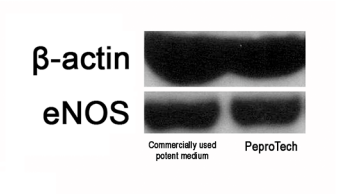


Figure 2.



Figures 1 and 2: EPC Proliferation. EPCs were seeded onto fibronectin-coated plates, and incubated for 7 days in the PeproGrow™ Endothelial Cell Basal Medium + PeproGrow™ EPC Growth Supplement, and a competitor's medium. The proliferation ability of EPCs was assessed 7 days after cell cultivation using the XTT assay according to the manufacturer protocol. The proliferation ability of EPCs was expressed as the average of optical density (O.D.) calculated using a plate reader for two independent assays run in triplicate. **Figure 2** represents a standard Western Blot assay.

Figure 3.

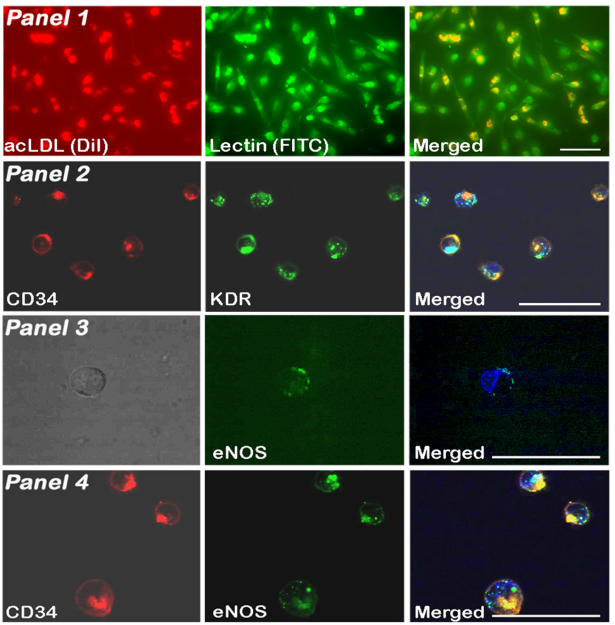


Figure 3: EPC Characterization. EPCs were cultured for 7 days. Each description correlates to images from left to right: Panel 1: Acetylated LDL uptake by adherent spindle-shaped EPCs, FITC-conjugated lectin UEA-1 binding to the surface of EPCs, and positive double-stained (merged image) EPCs for acetylated LDL uptake and lectin binding. Panel 2: Immunofluorescence detection of CD34 antigen (red), KDR (green) on the surface of EPCs, and merged image. Panel 3: Immunofluorescence detection of eNOS on a single non-stained EPC (green). Panel 4: Immunofluorescence detection of CD34 antigen on the EPCs surface (red), eNOS (green), and merged image. The EPCs nuclei were stained with the blue fluorescent DNA dye DRAQ5™.

Figure 4.

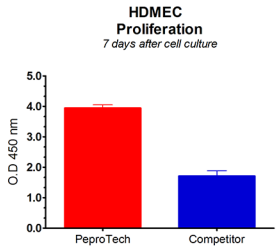
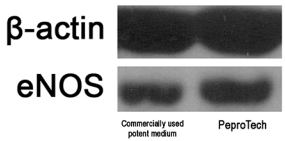


Figure 5.



Figures 4 and 5: HDMEC Proliferation. HDMECs were seeded onto fibronectin-coated plates and incubated for 7 days in the PeproGrow™ Endothelial Cell Basal Medium + PeproGrow™ MicroV Growth Supplement, and a competitor's medium. **Figure 4** represents the proliferative ability of HDMECs assessed 7 days after cell cultivation using the XTT assay following the manufacturer's protocol. The proliferation ability of HDMECs was expressed as the average of optical density (O.D.) calculated using a plate reader from two independent assays run in triplicate. **Figure 5** represents a standard Western Blot assay.

Figure 6:

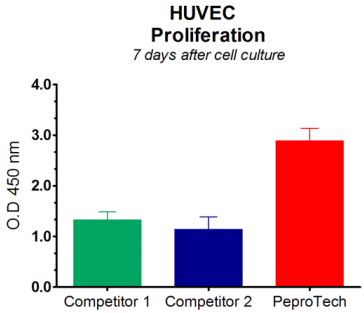
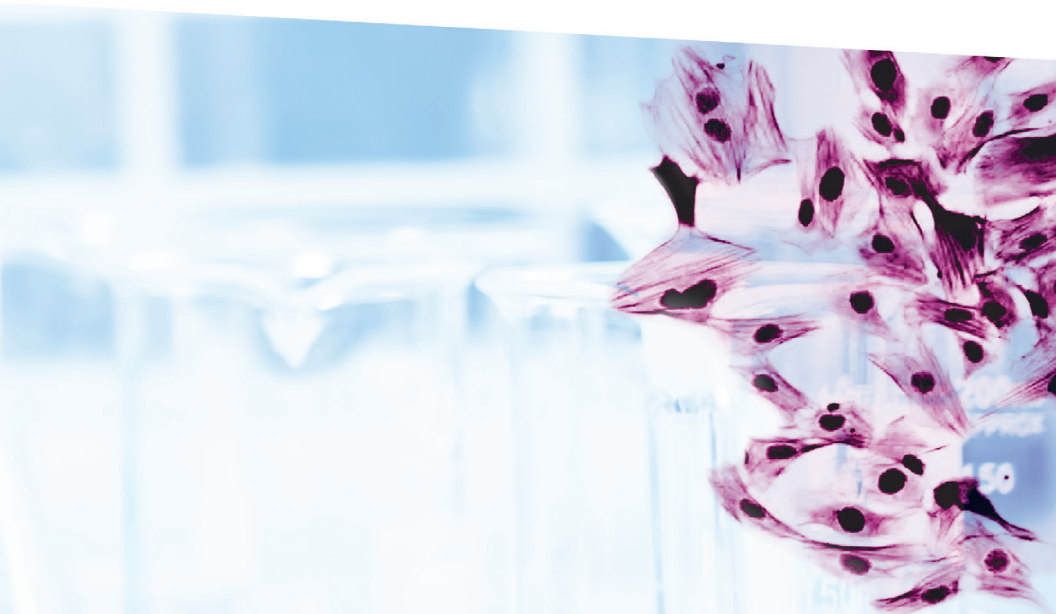


Figure 6: HUVEC Proliferation. HUVECs were seeded onto fibronectin-coated plates and incubated for 7 days in the PeproGrow™ Endothelial Cell Basal Medium + PeproGrow™ MacroV Growth Supplement, and two competitors' media. The proliferation ability of HUVECs was assessed 7 days after cell cultivation using the XTT assay following the manufacturer's protocol. The proliferation ability of HUVECs was expressed as the average of optical density (O.D.) calculated using a plate reader from two independent assays run in triplicate.



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