gibco by life technologies

Angiogenesis Starter Kit

Description

Angiogenesis—the formation of new blood vessels from existing vasculature—is an integral part of both normal and pathological processes. Endothelial cells are the key cell type involved in this process. During angiogenesis, these cells disrupt the surrounding basement membrane and migrate toward an angiogenic stimulus, where they proliferate and re-organize to form the necessary three-dimensional vessel structure.

The Angiogenesis Starter Kit contains media and reagents optimized for culturing Human Umbilical Vein Endothelial Cells (HUVEC) on Geltrex® LDEV-Free Reduced Growth Factor Basement Membrane Matrix to model the formation of endothelial cell tube networks. The following protocol provides instructions for performing the tube formation assay, one of the most well-established *in vitro* assays for the formation of three-dimensional vessels.

Kit Name/Components	**Cat. no./** Part no.	**Amount**	**Storage**	**Shelf life***
Angiogenesis Starter Kit includes:	**A14609-01**	**1 kit**		
Human Umbilical Vein Endothelial Cells (HUVEC)	C-003-5C	1 mL ($\geq 5 \times 10^5$ viable cells/mL)	Liquid nitrogen vapor phase	2 years
LVES (50X) Large Vessel Endothelial Supplement	A14608-01	11 mL	–20°C to –5°C	12 months
Medium 200	M-200-500	500 mL	2°C to 8°C; Protect from light	12 months
Geltrex® LDEV-Free Reduced Growth Factor Basement Membrane Matrix	A14132-02	5 mL	–80°C to –20°C	18 months

* Shelf life duration is determined from Date of Manufacture.

Product use

For Research Use Only. Not for use in diagnostic procedures.

Important information

- Store LVES (50X) Large Vessel Endothelial Supplement in a non-frost free freezer. Avoid multiple freeze/thaw cycles.
- Do not freeze Medium 200.
- Avoid multiple freeze/thaw cycles of Geltrex® LDEV-Free Reduced Growth Factor Basement Membrane Matrix.

Safety information

Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Caution: Human origin materials are non-reactive (donor level) for anti-HIV 1 & 2, anti-HCV, and HB_sAg. Handle in accordance with established bio-safety practices.

Prepare LVES-supplemented Medium 200

To support the plating and proliferation of human large vessel endothelial cells, including HUVEC, supplement Medium 200 with LVES (included in the kit). LVES is an endothelial supplement optimized for angiogenesis applications, improved cell health, and increased growth rates.

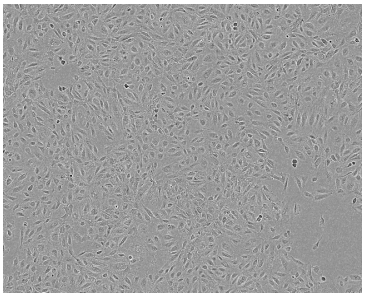
1. Thaw the LVES (50X) solution in a 37°C water bath or overnight at 4°C. If thawed in a water bath, do not leave the vial at 37°C after the solution has thawed.
2. Aseptically transfer the entire contents of the thawed LVES solution to the bottle of Medium 200, tightly cap the bottle, and swirl to mix. Avoid causing medium to foam.
3. Store the supplemented medium in the dark at 4°C. Do **not** freeze supplemented medium. When stored properly, LVES-supplemented Medium 200 is stable for 1 month.

Initiate cultures from cryopreserved HUVEC

We recommend seeding cells recovered from cryopreservation at a density of 2.5×10^3 viable cells/cm^2. One vial of HUVEC containing $\geq 5 \times 10^5$ viable cells can be used to seed three T-75 (75 cm^2) culture flasks.

1. Rapidly thaw a frozen vial of HUVEC in a 37°C water bath.
2. Take out the cryotube from water bath when only a tiny ice crystal is left and transfer it into a biosafety hood. Disinfect the outside of the cryotube with 70% isopropyl alcohol.
3. Open the vial and pipet the cell suspension up and down with a 1-mL pipette to disperse the cells.
4. Remove 20 µL of thawed cells from the vial and determine the number of viable cells per mL using your preferred method (e.g., Trypan blue and a hemocytometer, Countess® Automated Cell Counter).
5. Dilute the contents of the vial (1 mL) to a concentration of 1.25×10^4 viable cells/mL using LVES-supplemented Medium 200.
6. Add 15 mL of cell suspension to each T-75 culture flask.
7. Following inoculation, swirl the medium in the flasks to distribute the cells. HUVEC attach to culture surfaces quickly, and if the medium is not distributed immediately following inoculation, the cells may grow in uneven patterns.
8. Incubate the cultures at 37°C in a humidified atmosphere of 5% CO_2. For best results, do not disturb the culture for at least 24 hours after the culture has been initiated.
9. Change culture medium 24–36 hours after seeding and every other day thereafter, until the culture is at ~ 80% confluency (4 to 6 days). A representative image of HUVECs at ~80% confluency is shown in Figure 1 (next page). Note that some irregularly sized and shaped cells may be observed.

Figure 1 HUVECs cultured to ~80% confluent density.



Endothelial Tube Formation Assay (*In Vitro* Angiogenesis)

Day 1:

Seed cells

1. Seed HUVEC at 2.5×10^3 viable cells per cm^2 in a culture flask using LVES-supplemented Medium 200 (for example, 15 mL total volume of a cell suspension with 1.25×10^4 viable cells/mL are needed for a T-75 flask).
2. Change culture medium 24–36 hours after seeding and every other day thereafter, until the culture is at ~ 80% confluency (4–6 days; see Figure 1).

   Note: Passage number may impact assay performance; for best results, we recommend using HUVECs passaged less than 4 times following thaw.

Day 4 or 5:

Thaw Geltrex® Matrix

3. One day before performing the tube formation assay, place the vial containing the Geltrex® LDEV-Free Reduced Growth Factor Basement Membrane Matrix at 4°C to thaw overnight.

   Note: Refrigerator temperatures may vary; therefore, thaw the Geltrex® Matrix on ice in a refrigerator.

Day 6 or 7:

Prepare Geltrex® Matrix-coated vessel

Geltrex® Matrix irreversibly gels within 5–10 minutes above 15°C; work quickly to coat the culture vessels. We recommend keeping thawed Geltrex® solution on ice to ensure that it remains soluble. Pre-chilling culture plates or flasks (15–20 minutes at 4°C) and placing them on a pre-chilled (4°C) cool pack in a biosafety hood will also extend the working time for vessel coating.

The following procedure is designed for coating one standard 24-well plate; adjust the volumes appropriately for different formats.

4. Mix the thawed Geltrex® solution by slowly pipetting the solution up and down; be careful not to introduce air bubbles.
5. Add 0.1 mL of undiluted Geltrex® solution to each well of a 24-well plate (i.e., 50 µL of Geltrex® solution per cm^2 growth surface). It is helpful to use a large bore pipette tip (e.g. for a 1-mL micropipetter) for transfer and to use the tip to gently spread Geltrex® solution to coat each well. Incubate the coated plate at 37°C for 30 minutes to allow the matrix to solidify. Time the completion of cell harvest and counting to coincide with this incubation (typically takes 15–20 minutes).

   Notes: 50 µL of Geltrex® Matrix per cm^2 is sufficient for culture flasks, dishes, or culture plates with larger well sizes (e.g., 12-well, 24-well); 100 µL per cm^2 is necessary for smaller well sizes (e.g., 96-well). Due to its high viscosity, bubbles may be retained in Geltrex® coating. These can be removed by gently squeezing a wash bottle (with straw removed) containing 100% ethanol to blow the vapor over the well surface.

Harvest cells

The following procedure is designed for one T-75 flask (75 cm^2); adjust the volumes appropriately for different formats. Do not warm any of the reagents prior to use.

6. Remove all culture medium from the flask containing HUVEC at ~ 80% confluency (see Figure 1).
7. Wash the cells by adding 10 mL of DPBS without Calcium and Magnesium and swirling the flask gently. Remove and discard the wash buffer.
8. Add 3 mL of TrypLE™ Express reagent to the T-75 flask, and tilt the flask in all directions to evenly distribute the reagent. Incubate the HUVEC in TrypLE™ Express reagent for 5 minutes at room temperature.

   Note: Trypsin/EDTA Solution (Cat. no. R-001-100) and Trypsin Neutralizer Solution (Cat. no. R-002-100) may be substituted for TrypLE™ Express reagent. See HUVEC product manual for details.
9. After incubation, check the flask under the microscope for cell detachment. Firmly tap the flask as necessary to facilitate complete cell detachment.
10. Add 9 mL of supplemented Medium 200 to the flask and collect the cell suspension in a 50-mL conical tube.
11. Firmly tap the flask, rewash it with an additional 9 mL of supplemented Medium 200 and collect the remaining cells in the same 50-mL conical tube.
12. Centrifuge the tube containing dislodged HUVEC at $180 \times g$ for 7 minutes to pellet the cells.
13. Remove the supernatant from the tube, being careful not to dislodge the cell pellet.
14. Add 5 mL of supplemented Medium 200 to the cell pellet and resuspend by pipetting up and down several times.
15. Remove 20 µL of the cell suspension from the tube and determine the number of viable cells per mL using your preferred method (e.g., Trypan blue and a hemocytometer, Countess® Automated Cell Counter).

Perform tube formation assay

The following procedure is designed for seeding cells into one Geltrex® Matrix-coated 24-well plate; adjust the volumes appropriately for different formats.

16. Dilute the cells in supplemented Medium 200. We recommend a seeding density of approximately 25,000 cells per cm^2.

    Note: For example, an average 24-well plate has a culture area of ~1.9 cm^2 per well, yielding a total culture area of ~46 cm^2 and requiring ~1.9×10^6 viable cells to seed the entire plate. Recommended seeding volume per well of a 24-well plate is 0.25 mL; therefore, 1.9×10^6 viable cells would need to be resuspended in a minimum volume of 6 mL of complete medium to seed an entire 24-well plate. A typical cell yield from a single, ~80% confluent T-75 flask is ~3×10^6 viable cells.
17. Gently add cells to the Geltrex® Matrix-coated plate (0.25 mL of cell suspension per well of a 24-well plate). Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

    Note: Incubation times may vary, but HUVECs usually form well-developed tube networks after 14–18 hours under these conditions. After 24 hours, endothelial cells typically undergo apoptosis.

Day 7 or 8:

Visualize cells

18. *Optional:* At the end of the incubation period, you may stain the cells to label the tubules using a cell-permeable dye (e.g., Calcein, AM).

    Note: You may also use fluorescent Calcein AM dyes with different spectral properties such as Calcein Blue, AM (Cat. no. C1429) and CellTrace™ Calcein Red-Orange, AM (Cat. no. C34851).

    a. Prepare stock solution of Calcein AM as described in product manual.

    b. Prepare working dye solution at 2 mg/mL by diluting it in DBPS with Calcium and Magnesium. You will need 0.25 mL of dye solution per well of a 24-well plate (i.e., 6 mL of dye solution to label entire 24-well plate); adjust the volumes appropriately for different plate formats.

    c. Carefully remove the medium from the plate(s) by holding the plate(s) at a slight angle and gently aspirating the medium with a sterile Pasteur pipette, taking care not to disrupt tubules.

    d. Gently wash the plate with 1 mL/well of DPBS with Calcium and Magnesium and aspirate as described above.

    e. Add the freshly prepared dye solution to the cells at 0.25 mL/well and incubate for 30 minutes at 37°C, 5% CO_2, protected from light.

    f. Gently aspirate the dye-containing solution and wash the cells with 1 mL/well of DBPS with Calcium and Magnesium.

    g. Add 0.25 mL/well of supplemented Medium 200. The cells are now ready for imaging.

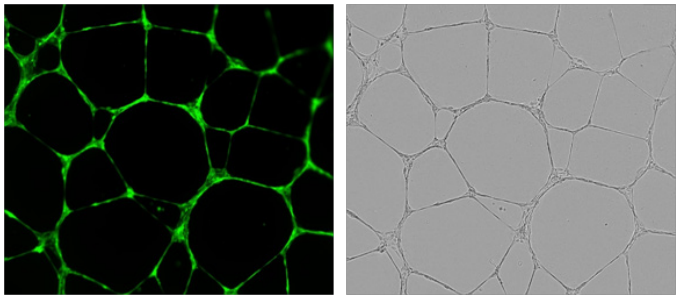
    Note: Cells can be imaged in DPBS with Calcium and Magnesium. We recommend imaging the cells within 1 hour of labeling. Calcein AM is not "fixable" and will be effluxed from cells.

19. Visualize the cells:

    - If a fluorescent dye was used, cells may be visualized using a fluorescence microscope or an automated imaging system (e.g. FLoid™ Cell Imaging Station) with the appropriate filter set. See Calcein AM product manual or the Life Technologies website for excitation and emission spectral data.
    - If a fluorescent dye was not used, cells may be visualized directly using a light microscope.

Expected results

Figure 2 HUVECs were seeded on a 24-well polystyrene plate coated with Geltrex® Matrix (50 µL/cm²) at 25,000 viable cells/cm² using LVES-supplemented Medium 200 and incubated at 37°C and 5% CO_2. At 16 hours post-seeding, the cells were stained with 2 µg/mL of Calcein, AM, incubated for 30 minutes at 37°C, 5% CO_2, and then imaged at 4X magnification (left panel). Right panel is the phase contrast image of the field shown on the left.



Related products

Product	Cat. no.
TrypLE™ Express (1X), no Phenol Red	12604
Dulbecco's PBS (DPBS) without Calcium and Magnesium	14190
Dulbecco's PBS (DPBS) with Calcium and Magnesium	14040
Trypsin/EDTA Solution	R-001-100
Trypsin Neutralizer Solution	R-002-100
Calcein AM	C3099
Calcein Blue, AM	C1429
CellTrace™ Calcein Red-Orange, AM	C34851
Countess® Automated Cell Counter	C10227
FLoid™ Cell Imaging Station	4471136

Explanation of symbols and warnings

The symbols present on the product label are explained below:

MM-YYYY	LOT		
Use By:	Batch code	Keep away from light	Temperature Limitation
REF			STERILE A
Catalog number	Consult instructions for use	Caution, consult accompanying documents	Sterilized using aseptic processing techniques

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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