

## Tube Formation Assay in the $\mu$ -Plate 96 Well 3D

When endothelial cells are seeded onto a basement membrane-like surface (e.g., Matrigel®), they form capillary-like structures *in vitro*, which recapitulates angiogenesis.

A tube formation assay can give insights into pro- or anti-angiogenic effects of specific substances. It is performed by first seeding single cells and then observing and imaging the tube formation over time with several readout possibilities (e.g., tube length and the number of loops). The expression “tubes” describes the cords of cells that are visible in a formed network. It does not indicate, specifically, that the cords have a lumen. Typically, tubes are formed within a few hours, making the tube formation assay a rapid tool for angiogenesis quantification.

This Application Note is a step-by-step protocol explaining a tube formation assay using the  $\mu$ -Plate 96 Well 3D with endothelial cells (human umbilical vein cells, HUVECs) on Matrigel®.

The  $\mu$ -Plate has a specialized geometry for easy, convenient, and reproducible tube formation assays. Our technology is a “well-in-a-well” geometry that allows for a minimum amount of gel, 10  $\mu$ l, per well, with no gel meniscus formation. The design ensures the formation of a uniformly thick gel matrix on which all cells are in one optical plane, creating reproducible cell culture conditions and high-quality phase contrast or fluorescence microscopy images.

### Related Documents

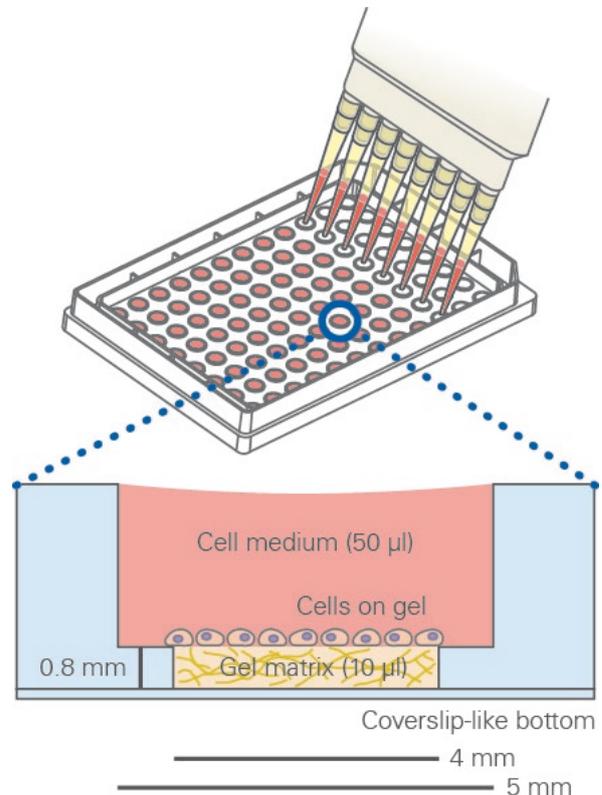
[Application Note 19: Tube Formation Assay in the  \$\mu\$ -Slide 15 Well 3D \(PDF\)](#)

[Application Note 27: Data Analysis of Tube Formation Assays \(PDF\)](#)

[Application Guide Angiogenesis Assays \(PDF\)](#)

[Application Guide Live Cell Imaging \(PDF\)](#)

[Instructions  \$\mu\$ -Plate 96 Well 3D \(PDF\)](#)



The “well-in-a-well” feature avoids gel meniscus formation.

For high-throughput tube formation assays with the  $\mu$ -Slide 15 Well 3D, see [Application Note 19, “Tube Formation Assay in the  \$\mu\$ -Slide 15 Well 3D” \(PDF\)](#).

## 1. Material

### 1.1. Reagents and Buffers

- Human umbilical vein endothelial cells (HUVEC, C-12203, PromoCell)
- Collagen IV (354233, Corning)
- 0.05 M HCl (X942.1, Carl Roth)
- Endothelial Cell Growth Medium (PromoCell, C-22010)
- PBS (14190144, Gibco)
- Accutase (A1110501, Gibco)
- Matrigel® Growth Factor Reduced, Phenol Red-Free (Corning® #356231), 10 µl per well
- Optional: Calcein AM (PromoKine, PK-CA707-80011)

### 1.2. Equipment

- [µ-Plate 96 Well 3D](#), ibiTreat (89646, ibidi)
- Scale paper for checking the optimal gel volume
- Ice or appropriate cooling racks
- Standard cell culture equipment (pipettes, sterile working bench, cell culture incubator, culture flasks, hemocytometer, etc.)
- Multichannel pipette with tips
- PCR tube strips (for aliquot preparation)
- Inverted microscope (optionally with fluorescence and appropriate filter sets for calcein staining)

## 2. Preparation of the 3D Gel

Perform all the following protocol steps under sterile conditions.

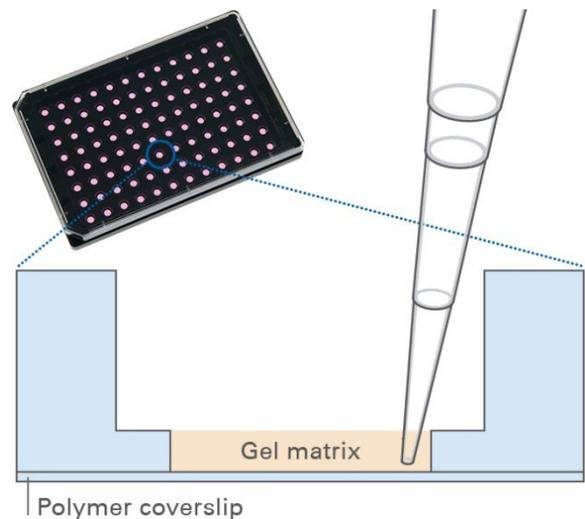
### 2.1. Gel Application

**Important Note:** Always use precooled pipet tips (4 °C) for pipetting the gel!

1. The day before the experiment, place the Matrigel® on ice in the fridge at 4 °C to let it slowly thaw overnight.
2. On the day of the experiment, place the Matrigel® on ice in the laminar flow hood. Aliquot the required amount in PCR tube strips, including exceed. Keep on ice.
3. Under the hood, remove the µ-Plate 96 Well 3D from the sterile packing.



4. Put a scale paper into the hood below the  $\mu$ -Plate for volume adjustment. Make sure the distance between the scale paper and the wells is ca. 1–2 cm.
5. With the multichannel pipette, apply 10  $\mu$ l of gel to each inner well of the  $\mu$ -Plate 96 Well 3D. Hold the pipet tip upright in the middle of the well. This prevents the gel from flowing into the upper well.
6. Close the  $\mu$ -Plate with the lid.

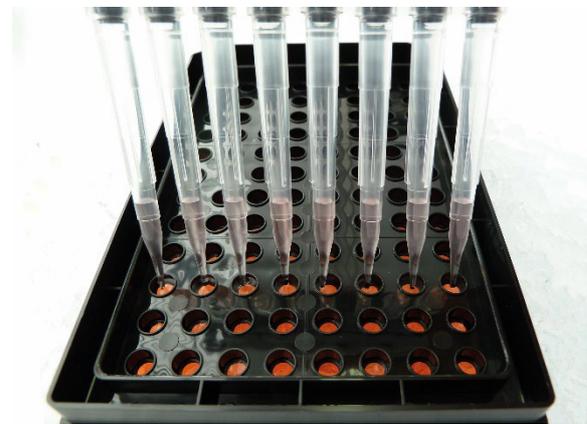


### Important Notes for Pipetting the Gel

Always use precooled pipet tips (4 °C) for pipetting the gel.

To avoid air bubbles in the gel, make sure the Matrigel<sup>®</sup> solution is properly mixed before filling the well. Make three up and down movements (10  $\mu$ l) with the pipet while leaving the tip in the gel. Then transfer 10  $\mu$ l to each of the wells.

Work quickly to avoid evaporation effects.



### Adjusting the Gel Volume

A meniscus-free gel with a plain surface is crucial for optimal imaging. To achieve this, the gel volume of each inner well needs to be precisely 10  $\mu$ l. Due to the high viscosity of Matrigel<sup>®</sup>, it might be necessary to adjust the pipet to more or less than 10  $\mu$ l.

To control the amount of gel, place the  $\mu$ -Plate 1–2 cm above a scale paper and observe the scale paper through the filled wells. There is no magnification or minimization effect with a volume of precisely 10  $\mu$ l. If the grid of the scale paper appears reduced, the pipetting volume needs to be increased. If the grid is magnified, the pipetting volume must be decreased.

## 2.2. Gelation

1. To minimize evaporation, fill the side reservoirs of the  $\mu$ -Plate with sterile water. Fill only half of the reservoirs' volume to avoid overflowing the liquid into the wells.
2. Put the  $\mu$ -Plate into the incubator for polymerization (30–60 min).
3. In the meantime, prepare the cell suspension (Chapter 3).

### 3. Cell Seeding

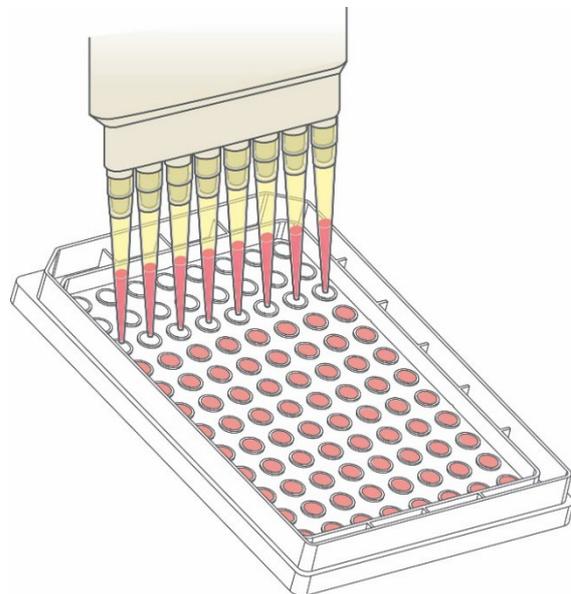
The number of cells seeded on the gel surface is a crucial parameter for obtaining reliable results from a tube formation assay. The cell type and size determine the cell seeding number and must be optimized before starting an experimental series.

Please read the [Instructions](#) before working with the  $\mu$ -Plate 96 Well 3D, ibiTreat. Perform all steps under sterile conditions. Before starting the experiment, prepare the HUVECs in a standard cell culture flask (e.g., T75, coated with 6.7  $\mu$ g/ml Collagen IV in 0.05 M HCl for 1 h) with the cells adherent at the bottom. The cells should be healthy and optimally subconfluent on the day of the experiment.

It is important to work swiftly during the whole procedure so the wells will not dry out.

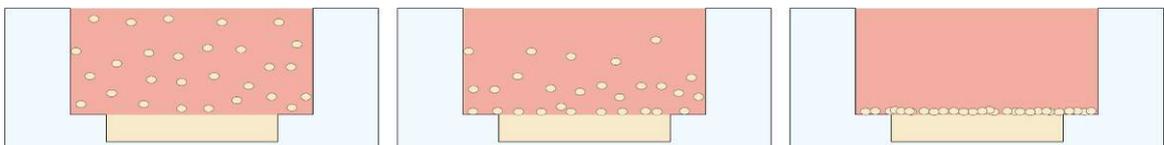
If not stated otherwise, all given volumes are per well, and all incubation steps are at room temperature.

1. Treat the cultured HUVECs with Accutase for 1–2 min for detachment.
2. Harvest the cell suspension, centrifuge and dilute it in a low amount of culture medium for counting; the amount depends on the required cell concentration.
3. Count the cells. Counting should be performed as accurately as possible and always in the same way to have the same number of cells in all wells. For a final cell number of 10,000 cells per well, adjust the cells to a final concentration of  $1.4 \times 10^5$  cells/ml in culture medium.
4. Take the  $\mu$ -Plate 96 Well 3D with the gel from the incubator and put it under the flow hood.



*Pipetting cells into the  $\mu$ -Plate 96 Well 3D using a multichannel pipette.*

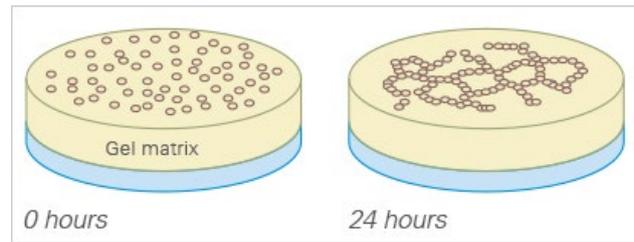
5. Before adding the cell suspension to the wells, mix it well by pipetting up and down several times. Apply 70  $\mu$ l into each upper well. Keep the pipet tip upright, and do not touch the gel with the pipet tip.



*Sinking process of cells. After some minutes, all cells have fallen to the ground.*

6. Cover the  $\mu$ -Plate with the supplied lid. Optionally, [ibiSeal 71 x 107 \(10875\)](#), a self-adhesive cover film, can be used to cover the wells for meniscus flattening and improved phase contrast microscopy.

7. The  $\mu$ -Plate 96 Well 3D is now ready for observation. After some minutes, the cells have sunk onto the gel surface. Tube formation begins and can be imaged in one focal plane without any disturbing gel meniscus. Optionally, and for better visibility of the tubes, a calcein staining can be performed before imaging.

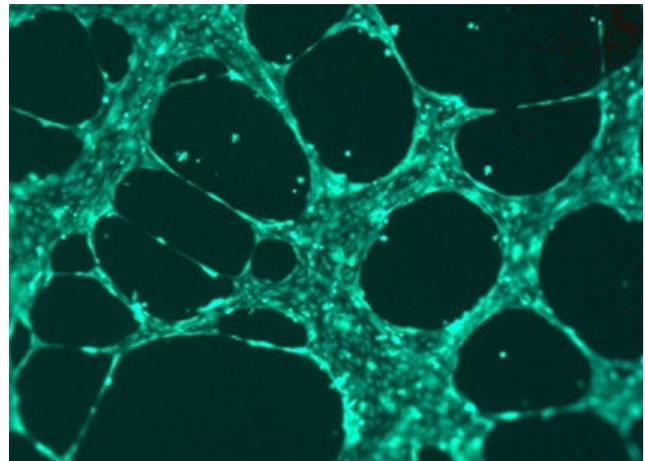


Schematic of tube formation on the Matrigel®.

#### 4. Optional: Calcein Staining

If visualization using fluorescence microscopy is desired, you can stain the living cells using calcein. Follow these steps after the tubes have formed:

- Right before staining, image the cells. This provides a comparison of the cell pattern before and after the staining to detect any staining artifacts that may occur.
- According to manufacturer's protocol, prepare 70  $\mu$ l calcein solution per well by mixing serum-free cell culture medium and calcein stock solution with a final concentration of 6.25  $\mu$ g/ml.
- Carefully discard the supernatant using a pipette. Take care not to damage the gel or the cell network.
- Add the calcein solution to the wells. Incubate in the dark for 30 minutes at room temperature.
- Wash 3 $\times$  gently with PBS. Slowly rinse the PBS over the side of the upper well. Do not pipette it directly onto the cells. Instead, remove it from the other side of the well, so that it gently rinses the cells.
- Image the cells using an inverted fluorescence microscope at 485/529 nm.



HUVEC network stained with calcein.

## 5. Image Acquisition

Tube formation can be imaged either using bright-field, phase contrast, or fluorescence microscopy (e.g., when staining with a live cell dye such as calcein, Chapter 4). Data collection on the microscope can be performed manually or automatically.

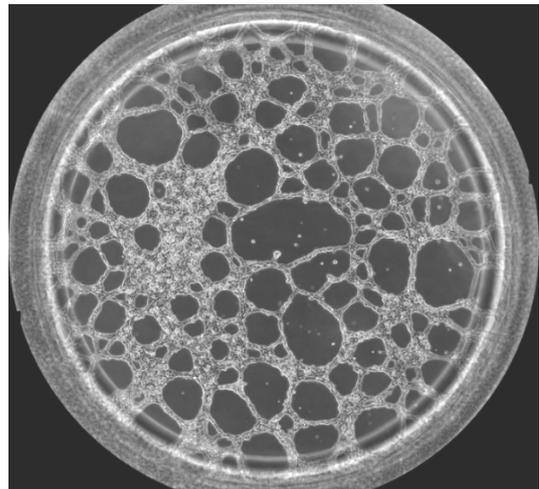
Especially when performing an experiment for the first time, we recommend recording a time-lapse video to determine the time dependency and the characteristics of the curve (e.g., maximum and stable phase). In subsequential experiments, single manual measurements are usually sufficient for investigating the effects of substances on tube formation.

### 5.1. Automatic Image Acquisition

Automatic image acquisition in a live cell imaging setup is the most convenient and reproducible way of collecting tube formation assay data. The [ibidi Stage Top Incubators](#) fit every standard inverted microscope and include CO<sub>2</sub>/O<sub>2</sub> control as well as actively controlled humidity.

Immediately after seeding the cells, put the  $\mu$ -Plate into the incubation chamber placed on an inverted microscope. Choose the section you want to observe, then start a time-lapse recording. For HUVECs, we recommend a 4 $\times$  or 10 $\times$  magnification and a time interval of 5 minutes between the single images. Use a software autofocus program to get sharp images over time. Keep in mind that cells can migrate into the gel and change the focal plane over time.

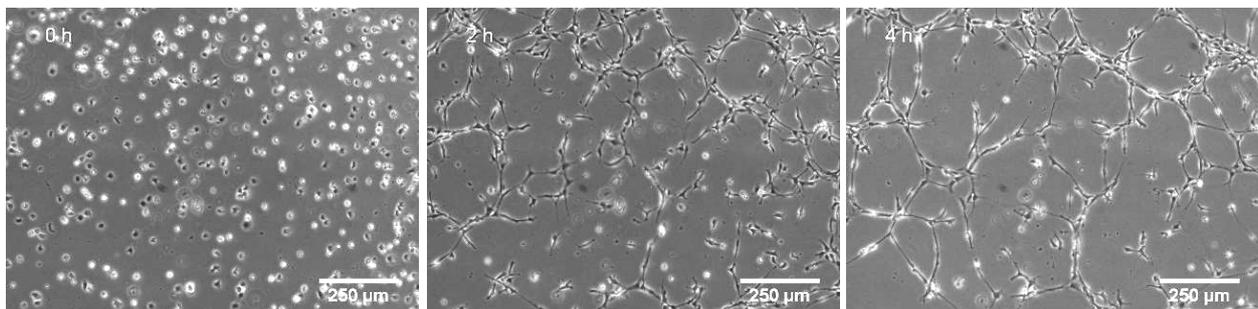
You can download the ["Live Cell Imaging" Application Guide \(PDF\)](#) for details and more live cell microscopy tips.



*Phase contrast image with a 10 $\times$  objective (arrangement of 5  $\times$  6 single images), showing an entire well (4 mm diameter) with HUVECs forming a cell network 10 hours after seeding.*

### 5.2. Manual Image Acquisition

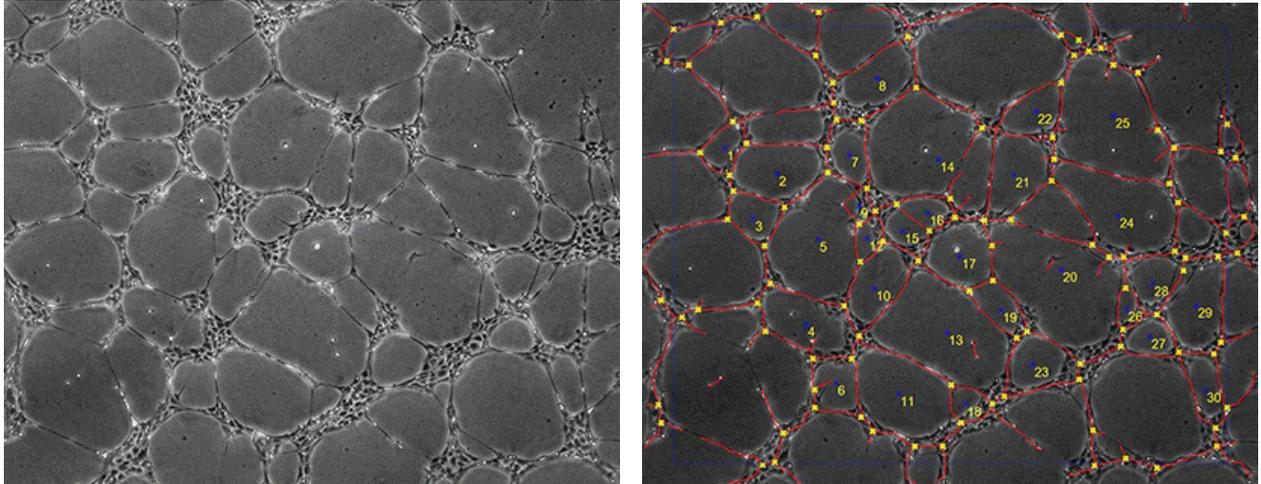
If you know the curve of the network formation, it is sufficient to collect images only at the points of interest. To incubate, place the  $\mu$ -Plate inside a humidity chamber within the incubator and then take out the  $\mu$ -Plate at distinct time points to manually collect images on the microscope.



*Time lapse images of a tube formation assay using HUVECs. Phase contrast microscopy with a 10 $\times$  objective at 0 h, 2 h, and 4 h after cell seeding.*

## 6. Image Analysis

To quantify tube formation, the images can be analyzed based on different parameters, such as tube length, loops, cell-covered area, or branching points. This can be performed manually using an image processing software (e.g., ImageJ) or using an automatic analysis software (e.g., Wimasis, ACAS, or FastTrack AI).



*Tube formation image before (left) and after (right) automatic image analysis.*