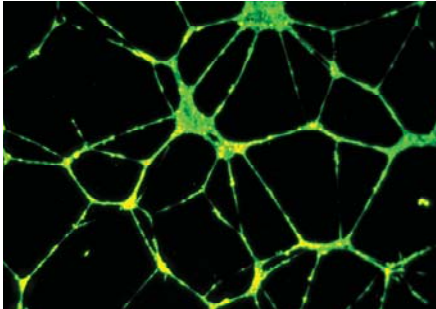


## Assay Methods

### Protocol: Endothelial Cell Tube Formation Assay



Angiogenesis is characterized by a number of cellular events including endothelial cell migration, invasion and differentiation into capillaries. In vitro endothelial tube formation assays are used as a model for studying endothelial differentiation and modulation of endothelial tube formation by antiangiogenic agents. Image acquisition and quantification of fluorescently labeled cells can be achieved by using MetaMorph<sup>®</sup> software coupled with an automated imager. In the following procedure, assay conditions and cell labeling for quantification of endothelial cell tube formation have been optimized to maximize the fluorescent signal while minimizing the cytotoxic effects of calcein AM on human microvascular endothelial cell lines (HMEC-1 and HMVEC), and primary human umbilical vein endothelial cells (BD<sup>™</sup> HUVEC-2). Results may vary depending upon the cells, dye used, and the specific experimental conditions.

#### Materials:

- BD Matrigel<sup>™</sup> matrix, 10 ml (BD cat. no. 354234); recommended concentration of BD Matrigel lot: 10 mg/ml or greater.
- BD Falcon<sup>™</sup> 24-well flat-bottom standard tissue culture-treated plate (BD cat. no. 353047).
- Endothelial cell culture medium (e.g., EGM-2 MV, Lonza cat. no. CC-4147).
- Fetal bovine serum or appropriate growth factor as an angiogenesis stimulator.
- Hanks' Balanced Salt Solution (HBSS) (e.g., Life Technology cat. no. 14025-092).
- Fluorophore (e.g., BD<sup>™</sup> Calcein AM Fluorescent Dye, 10x50 µg, BD cat. no. 354216).
- DMSO.
- Humidified tissue culture incubator, 37°C, 5% CO<sub>2</sub> atmosphere.
- Endothelial cells such as HMEC-1, HMVEC, or BD HUVEC-2 cells (BD cat. no. 354151).
- Laminar flow tissue culture hood.
- Automated imager, fluorescence microscope, tube quantification software.

#### Procedure:

##### 1.0 Reconstitution of BD Matrigel matrix

Color variations may occur in frozen or thawed vials of BD Matrigel matrix, ranging from straw yellow to dark red due to the interaction of carbon dioxide with the bicarbonate buffer and phenol red. Variation in color is normal, does not affect product efficacy, and will disappear upon equilibration with 5% CO<sub>2</sub>.

- 1.1 Thaw BD Matrigel matrix overnight on ice at 4°C.
- 1.2 Once thawed, swirl vial to ensure that material is evenly dispersed.
- 1.3 Spray top of vial with 70% ethanol and air dry.
- 1.4 Keep product on ice and handle using sterile technique.
- 1.5 Dispense material into appropriate aliquots, using pre-cooled pipets, tips, and tubes, and refreeze immediately. Avoid multiple freeze thaws.

#### Precaution

BD Matrigel matrix will gel rapidly at 22°C to 35°C. Thaw overnight at 4°C on ice. Keep product on ice before use, and use pre-cooled pipets, tips, and tubes when preparing BD Matrigel matrix for use.

## 2.0 Coating Procedure

*NOTE: Once gelled, BD Matrigel™ matrix should be used immediately. We recommend using BD Matrigel matrix with a protein concentration of at least 10 mg/ml. The concentration of BD Matrigel matrix is lot-specific and can be found on the Certificate of Analysis. You can pre-screen matrigel lots and order lot-specific vials of BD Matrigel matrix with your preferred protein concentration by contacting BD Biosciences.*

- 2.1 Thaw BD Matrigel matrix as recommended. Using cooled pipets, mix it to homogeneity.
- 2.2 Keeping 24-well culture plates on ice, add 0.289 ml of chilled BD Matrigel matrix (10 mg/ml) per well. This quantity should be sufficient to cover the entire growth surface easily. If BD Matrigel matrix needs to be diluted to 10 mg/ml, dilute with serum free medium.
- 2.3 Avoid air bubbles in BD Matrigel matrix while pipetting the liquid into each well. If air bubbles get trapped in the wells, centrifuge the plate at 300 x g for 10 minutes in a centrifuge that has been pre-cooled to 4°C.
- 2.4 Incubate plates at 37°C for 30-60 minutes.
- 2.5 The plates are now ready to use.

## 3.0 Endothelial Cell Tube Formation Assay

- 3.1 Prepare the endothelial tube formation plate as directed above.
- 3.2 Culture endothelial cells with desired endothelial cell medium to desired confluence. For BD™ HUVEC-2, HMVEC, and HMEC-1, 70-80% confluence is recommended.

*NOTE: Primary cells should be low passage number (e.g., HUVECs should not be passaged more than 5 times).*

- 3.3 Prepare endothelial cell suspensions by trypsinizing the cell monolayers and resuspending the cells in culture medium with 5-10% serum or with your desired angiogenesis promoters at  $4 \times 10^5$  cells/ml when using BD HUVEC-2, HMVEC or HMEC-1 cells. Additional testing agents such as inhibitory agents can be included at this step as well.

*NOTE: Most endothelial cell media does not contain a sufficient concentration of serum to deactivate trypsin. The use of a trypsin neutralizing solution is recommended.*

- 3.4 Add 300  $\mu$ l of the cell suspension ( $1.2 \times 10^5$  cells of BD HUVEC-2, HMVEC, or HMEC-1) to each well.
- 3.5 Incubate the angiogenesis assay plate for 16-18 hours at 37°C, 5% CO<sub>2</sub> atmosphere.

#### 4.0 Measurement of Tube Formation - Labeling with BD Calcein AM Fluorescent Dye

*NOTE: Each well in the 24-well plate requires 300  $\mu$ l of BD™ Calcein AM dye at 8  $\mu$ g/ml in Hanks Balanced Salt Solution (HBSS). We recommend preparing 9.0 ml of dye solution per plate to account for pipetting losses. If using 50  $\mu$ g vials of BD Calcein AM, two vials will be needed. HBSS is recommended since the use of culture medium results in auto-hydrolysis of the label, giving an unacceptably high backgrounds. This section may be performed under non-aseptic conditions.*

- 4.1 Prepare BD Calcein AM solution at 8  $\mu$ g/ml. For each plate, 9.0 ml of BD calcein AM dye solution will be needed. If using two 50  $\mu$ g vials, measure 12.5 ml of HBSS and warm to 37°C. Add 20  $\mu$ l of DMSO to each 50  $\mu$ g vial of BD calcein AM and then transfer both vials' contents to total HBSS volume of 12.5 ml resulting in a solution of 8  $\mu$ g/ml.
- 4.2 Following incubation (step 3.5), carefully remove medium from the plates. Be careful not to disturb tubes that may have formed in the BD Matrigel™ matrix. This can be accomplished by gently aspirating the medium using a Pasteur pipet.
- 4.3 Wash the plate with HBSS by adding 750  $\mu$ l of HBSS to each well. Remove HBSS as described in 4.2.
- 4.4 Repeat the wash once.
- 4.5 Label cells by adding 300  $\mu$ l/well of 8  $\mu$ g/ml BD calcein AM in HBSS and incubate plates for 30-40 minutes at 37°C, 5% CO<sub>2</sub>.
- 4.6 Remove the labeling solution as in 4.2.
- 4.7 Wash the plates twice with HBSS as in 4.3.
- 4.8 The plate is now ready for image acquisition using an automated imager or for taking pictures using a fluorescent microscope.

*NOTE: Once hydrolysis occurs, BD calcein AM leaks out of cells resulting in a higher background. Labeled plates can be stored at 4°C for 1-2 hours with minimum increase in background.*

- 4.9 Process the acquired images with the desired hardware and software. We use Gen-1 Cell-based Screening System and MetaMorph® software to automatically acquire images and measure tube length.
- 4.10 If an automated image acquisition instrument is not available, it is possible to use a fluorescent microscope that is capable of taking pictures manually, and process images using either MetaMorph or another equivalent software.

*NOTE: Various researchers have measured a number of parameters such as tube length, tube areas, or branch points. In the angiogenesis system: endothelial cell tube formation (BD cat. no. 354149 and 354150), tube formation is measured using the MetaMorph Software system. Some other commonly used imaging software packages for measuring the extent of tube formation include BD Pathway™ 855 Bioimager with sophisticated image and data analysis algorithms; Image-Pro® Plus (Media Cybernetics [www.mediacy.com](http://www.mediacy.com)) and NIH Image (<http://rsb.info.nih.gov/nih-image/index.html>).*



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