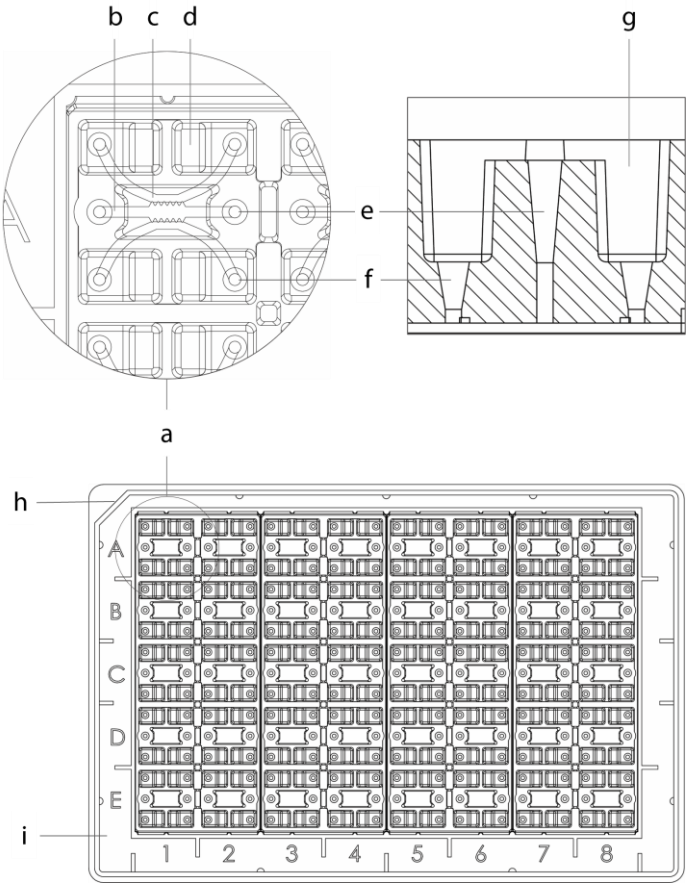
INTRODUCTION

Angio*Prime* is an assay-ready angiogenesis product in the idenTx 40 platform. The idenTx 40 plate is pre-seeded with HUVEC/TERT2 cells in the top media channel, and filled with Collagen type I hydrogel in the gel channel. The channels are filled with specialized shipping media to ensure cell stability and health during shipping at 4-8°C for up to 4 days. This streamlined solution enables consistent sprout formation and rapid evaluation of factors regulate angiogenic activity.

This protocol covers the basic handling of the assay-ready product– cellular recovery upon delivery, changing of medium, and immunostaining of cells.

SCHEMATIC

The following schematic shows the 3D presentation of idenTx 40. This nomenclature will be used extensively in this protocol.



Nomenclature:

a : site (40 sites per plate)
b : gel channel
c : media channel
d : trough
e : gel inlet
f : media inlet
g : media port

h : chamfer
i : reservoir

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TEMPERATURE DATA RETRIEVAL

Angio*Prime* is a temperature and time sensitive assay kit. To assess the shipment temperature profile, simply scan the provided Timestrip indicator (TL520) through the eTimestrip smartphone application. TL520 is a single use device that provides an immediate indication for temperature conditions (2–8°C range) using built-in LED lights. The TL520 can log and store up to 3800 temperature data points. The application works with all NFC enabled smartphones. Report can be exported in PDF and CSV files.

MATERIALS

- ETimestrip app

1. Check if LED light blinks every 30 seconds. This indicates continuous recording of temperature.
2. Once Angio*Prime* is unboxed, immediately break the TL520 tab to stop temperature measurement.

Note *TL520 temperature indicator is placed along with the idenTx 40 plate.*

3. Scan the QR code to download eTimestrip mobile application

Available on the App Store

GET IT ON Google Play

4. Place phone above the Timestrip to view the data (enable the mobile NFC function)

! Critical *Make sure to place the mobile phone close to the device.*

? Troubleshoot *If that does not work, check to see if NFC is enabled in your Connection Preferences (Android users). If it still not working, restart app and phone and retry step 2.*

5. When the mobile phone is close to the device, it will automatically synchronize the time while reading the device data.
6. Click the up arrow in the upper right corner to select the desired report format to export the data report

Note *The report can be exported in PDF or Excel format.*

CELLULAR RECOVERY UPON DELIVERY ⏱ TIMING 60 min

MATERIALS

Reagents

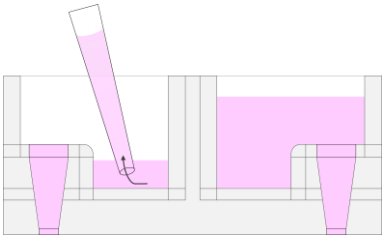
- Sterile 1X PBS
- 13 ml of Control Medium

7. On Friday, peel open the idenTx 40 plate from the packaging inside a biosafety cabinet (BSC).

8. Open the lid of the Angio*Prime* idenTx 40 and aspirate media if necessary (from potential spillage during shipping)

9. Use sterile tweezers to peel off the transparent film from the bottom right corner tab.

10. Remove Shipping Medium from all 4 ports by carefully placing the tip of an aspirator at the troughs.

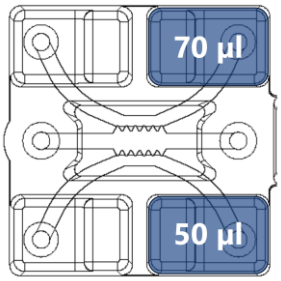


! Critical *Do NOT aspirate medium from inlets to avoid accidental removal of medium from the channels.*

11. Gently distribute 4 ml of sterilized 1X PBS across the perimeter of the reservoir.

12. Aliquot 13 ml of Control Medium into a 15 ml centrifuge tube and store the remaining medium at 4°C.

13. Add 70 µl and 50 µl of Control Medium into one port of the top media channel (pre-seeded with HUVEC/TERT2) and bottom media channel respectively as shown in diagram on the right.

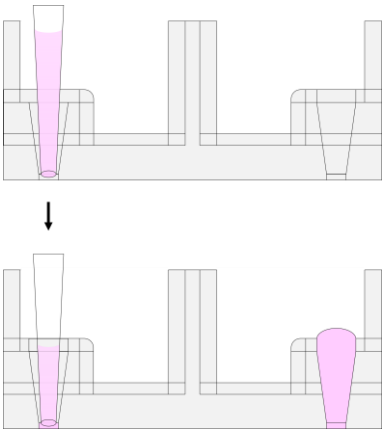


14. Place the Angio*Prime* idenTx 40 in a 5% CO_2 incubator at 37°C for 30 min.

15. Ensure that the medium builds up in the opposite connected port to indicate a successful medium change.

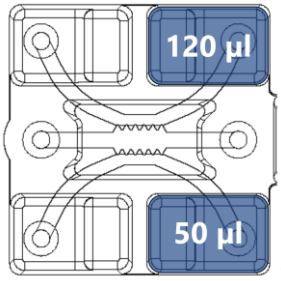
! Critical *Ensure that there is medium flow in both top and bottom media channels.*

? Troubleshoot *If there is no medium build-up in the opposite connected port, gently add 10 µl of medium directly into the media inlet of the blocked channel.*



16. Remove the medium from all 4 ports

17. Add 120 µl of Control Medium into one port of the top media channel (pre-seeded with HUVEC/TERT2) and 50 µl of Control Medium into one port of the bottom channel as shown diagram on the right.



18. Place the Angio*Prime* idenTx 40 back into the 5 % CO_2 incubator at 37°C and allow the cells to recover over the weekends (Saturday and Sunday) without additional medium change.

ANGIOGENESIS INDUCTION ⏱ TIMING 15 min

MATERIALS

Reagents

- Pre-warmed Control Medium
- Pre-warmed Angiogenesis Medium

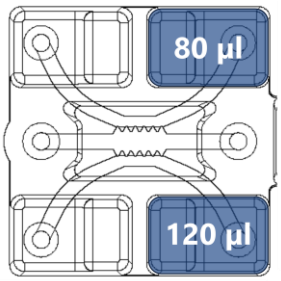
19. Pre-warm Control Medium and Angiogenesis Medium at 37°C for not more than 30 min.

20. Remove medium from all 4 ports.

Reminder *Do NOT aspirate medium from inlets.*

21. For **negative control**, add 80 µl of Control Medium into the top channel and 120 µl of the same media into the bottom channel.

    (Optional) Add pro-angiogenic factors in Control Medium to test for compound efficacy.



Reminder *Only add media into one port of each top and bottom media channels.*

22. For **positive control**, add 80 µl of Angiogenesis Medium into the top channel and 120 µl of the same media into the bottom channel.

    (Optional) Add anti-angiogenic factors in Angiogenesis Medium to test for compound efficacy.

Note *The direction of interstitial flow occurs from the bottom media channel to top media channel.*

23. Keep the idenTx 40 plate in a 5 % CO_2 incubator at 37°C overnight.

24. Repeat Angiogenesis Induction (steps 20–23) daily for the next 3 days.

IMMUNOSTAINING ⏱ TIMING 2 d

MATERIALS

Reagents

- 4% Formaldehyde (Sigma Aldrich, Cat. No.158127)
- 0.1% Triton X-100 (Sigma Aldrich, Cat. No. T8787)
- Blocking buffer (Life Technologies, Cat. No. B-10710)
- 1X PBS (Life Technologies, Cat. No. 70011044)
- Alexa Fluor™ 568 Phalloidin (Thermo Fisher, Cat No. A12380)

Cell Fixation ⏱ **TIMING 20 min + 15 min incubation**

25. Remove the medium from all 4 ports.
26. Add 120 µl of 1X PBS into one of the ports of the top channel and 80 µl in one of the ports of the bottom channel. Observe for fluid build-up in the opposite connected port to indicate a successful reagent exchange. Repeat this for the other channel.
27. Remove 1X PBS from all ports.
28. Repeat wash once more.
29. Add 100 µl of 4% formaldehyde into one of the ports of the top channel and 80 µl in one of the ports of the bottom channel.
30. Incubate for 15 min at RT.
31. Remove 4% formaldehyde from all ports by using a micropipette.

! Critical *Dispose 4% formaldehyde and the 1st subsequent wash in a designated waste bottle.*

32. Wash the plate by adding 100 µl 1X PBS in one of the ports in the top channel and 80 µl 1X PBS in one of the ports in the bottom channel. Observe for reagent exchange.
33. Remove 1X PBS from all ports and repeat washing once more.

▪ **PAUSE POINT** *Fixed cells can be kept for up to 1 month (depending on target antigens) at 4°C as long as the PBS in the ports does not dry up.*

Cell Permeabilization ⏱ **TIMING 10 min + 20 min incubation**

(Optional: Permeabilization is only necessary when cell-impermeable fluorescent probes are used, e.g. Phalloidin)

34. Remove 1X PBS from all 4 ports.
35. Add 100 µl of 0.1 % Triton X-100 into one of the ports. Repeat for the other channel with 80 µl of 0.1 % Triton X-100.

36. Incubate for 20 min at RT.

37. Wash once with 1X PBS as described in step 25–26 of cell fixation.

Blocking ⏱ **TIMING 15 min + 60 min incubation**

(Optional: Blocking is only necessary for immunofluorescent staining)

38. Remove 1X PBS from all 4 ports carefully by placing the tip of an aspirator at the troughs.

39. Add 100 µl of blocking buffer into one of the ports. Repeat for the other channel with 80 µl of blocking buffer.

40. Incubate for 1 h at RT.

Primary Antibody Staining ⏱ **TIMING 18 h**

(Optional: Primary antibody staining is only necessary for immunofluorescent staining)

41. Prepare antibodies according to the manufacturer's recommendations.

42. Remove blocking buffer from all 4 ports carefully by placing the tip of an aspirator at the troughs.

! Critical *Do not wash with 1X PBS after the blocking step.*

43. Add 80 µl of antibodies into one of the ports. Add 50 µl of antibodies into one of the ports of the other channel.

44. Seal idenTx 40 with parafilm and incubate overnight at 4°C.

45. Remove the primary antibodies from all 4 ports (or use a micropipette to recycle the antibodies) at the troughs. You may reuse the antibodies depending on the antigen and antibody.

46. Wash the channels with 1X PBS 3-5 times, with a 5 min incubation between each wash.

Secondary Antibody Staining ⏱ ***TIMING 30 min + 60 min incubation***

(Optional: Secondary antibody staining is only necessary for immunofluorescent staining)

47. Prepare the secondary antibodies according to the manufacturer's recommendations.

48. Add 80 µl of antibodies into one of the ports and 50 µl of antibodies into the other channel.

49. Incubate for 1 h in the dark at RT.

! Caution *Cover the idenTx 40 with aluminium foil to minimize photo-bleaching.*

50. Remove the secondary antibodies from all 4 ports (or use a micropipette to recycle the antibodies) at the troughs.

51. Wash the channels with 1X PBS 3 times, with a 5 min incubation between each wash.

QUANTIFICATION OF ANGIOGENIC SPROUTING ⏱ TIMING Variable

In order to quantify the extent of angiogenesis in idenTx 40, we recommend labelling the cells with appropriate fluorophores (such as VE-cadherin or Phalloidin staining that is specific to endothelial cells) to visualize them. Bright field, phase contrast and epifluorescence microscopy are all compatible with idenTx 40 but three-dimensional imaging techniques such as confocal microscopy is preferred due to the nature of this assay. The following quantification methods use images taken from confocal microscopy as illustrative examples.

SPROUT LENGTH AND NUMBER OF BRANCHING POINTS

The sprout length is an informative metric that can describe how fast (when the period of culture is taken into consideration) and how far the sprouts have extended into the 3D gel region. It can also tell the complexity of the sprouts especially when the sprout length is analyzed together with the number of branching points. The higher number of branching points indicates the greater complexity of a sprout.

52. We recommend using confocal images of endothelial cells that are either fluorescently tagged or stained to quantify the sprout length and the number of branching points.

53. Pre-process the images if necessary. Depending on the image quality, you may apply a Gaussian filter to smoothen the edges and fill up the gaps between the bright signals around the cell membrane if the endothelial cells are stained for cell–cell junction protein (e.g. VE-cadherin).

Reminder *You need to optimize the pre-processing steps based on the stained proteins/organelles and the image quality. Other pre-processing techniques that may help achieve more accurate estimation of sprout length and number of branching points including noise reduction through despeckle and background subtraction. You should always pre-process your images as a whole*

54. Try all threshold methods on at least three individual stacks of images. Choose the method that segments your data best and produces the closest estimation to the original images. Threshold the pre-processed images to binarize the images.

55. Use Skeletonize3D plugin (http://fiji.sc/Skeletonize3D) in ImageJ to find the centerlines (also known as skeleton) of objects in the input image.

56. Use AnalyzeSkeleton plugin (http://fiji.sc/AnalyzeSkeleton) in ImageJ to analyze the skeletons you have generated. This yields information including the average and maximum branch length (Sprout length), the number of branches and the number of actual junctions (branching points).

Reminder *You may perform a 3D projection on a stack of images to generate a 2D image before the image binarization only if the information across the different planes along the z-axis is not important for your application.*

SPROUT AREA

The sprout area measures the extent of endothelial cell coverage. The sprout area is best analyzed together with the number of branching points and sprout length to understand the sprout morphologies. In combination, the sprout area and sprout length can help derive the mean tubular diameter which is another useful metric for angiogenesis studies.

57. We recommend using confocal images of endothelial cells that are either fluorescently tagged or stained to quantify the sprout length and the number of branching points.

58. Pre-process the images if necessary. Depending on the image quality, you may apply a Gaussian filter to smoothen the edges and fill up the gaps between the bright signals around the cell membrane if the endothelial cells are stained for cell–cell junction protein (e.g. VE-cadherin).

Reminder *You need to optimize the pre-processing steps based on the stained proteins/organelles and the image quality. Other pre-processing techniques that may help achieve more accurate estimation of sprout length and number of branching points including noise reduction through despeckle and background subtraction. You should always pre-process your images as a whole*

59. Try all threshold methods on at least three individual stacks of images. Choose the method that segments your data best and produces the closest estimation to the original images. Threshold the pre-processed images to binarize the images.

60. Use Isosurface function in BoneJ plugin (http://bonej.org/) in imageJ to estimate the 3D surface area

Reminder *The 3D visualization of the images and the estimation of the 3D surface area are greatly affected by the resampling and threshold values that are chosen for BoneJ plugin. You should vary these two values systematically and choose the combination based on the rendered images that are closest to your actual data.*

61. You can then derive the mean tubular diameter by dividing the sprout area by the sprout length

Reminder *Be cautious when deriving a number based on two estimated values as the discrepancies may be high. We recommend using this method ONLY IF the diameters of lumina are largely homogenous as validated through analyzing the cross-sectional images.*