INTRODUCTION

The idenTx 40 is a sterile, single-use, and stackable plate, with a throughput size of 40. The perimeter of the plate serves as a reservoir to provide a humidified environment. This protocol covers the basic handling of the idenTx 40 – the preparation and filling of collagen gel, hydrating and coating of media channels, seeding cells, changing medium and staining cells. The formation of an endothelial monolayer is used here as illustration.

Protocols for the opentrons can be created with both protocol designer (https://designer.opentrons.com/) and python API.

All the protocols have been created with protocol designer which contains drag and drop graphical interface along with visual labware and liquid management.

Protocols created for the idenTx 40 include preparation of reagents and filling for all 40 sites of the idenTx 40. If needed, with protocol designer, the volumes for each reagent used as well as filling of the sites needed can be edited.

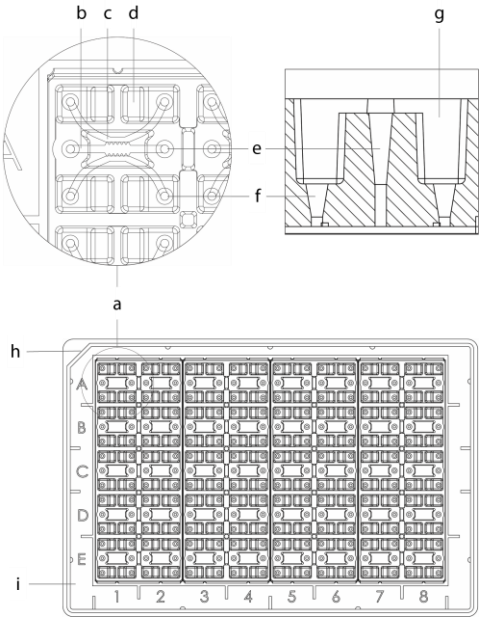
It is advised to import the protocol files onto protocol designer website first to view the starting deck state and prepare accordingly before exporting and uploading onto opentrons app for running of the protocol.

Reminder:

1) Always set the temperature module temperature on Opentrons app first to allow the temperature to set.
2) Temperature modules can only be kept on the deck's side slots and not in the centre.
3) Each time a new protocol is loaded onto the app to run, remember to shift the pippete tips to the starting position. *Opentrons always picks up the first tip from A1.
4) Make sure everything placed on the deck is fit tightly and does not shift.
5) If there is a need to add steps, duplicate the steps needed. If new steps are created, make sure the tip position and flow rate are followed according to Table 5.

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 : chamber
i : reservoir

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PREPARING & FILLING COLLAGEN GEL

MATERIALS

Reagents

- 10 X PBS with phenol red (see REAGENT SETUP; Life Technologies, Cat. No. 70011044)
- Sodium hydroxide solution, 0.5 M (see REAGENT SETUP; Sigma-Aldrich, Cat. No. 221465)
- Sterile deionized water (Thermo Water Purifying System)
- Collagen type I, rat tail (Corning Life Science, Cat. No. 354236)
- Cell culture medium (Lonza, Cat. No. CC3202)

Others

- 1.5 ml microcentrifuge tube
- Pocket size pH meter (Hach, Cat. No. H138) or pH papers (Sigma Aldrich, Cat. No. 37144)
- idenTx 40

Opentrons Equipment

- Temperature Module (GEN2)
- Opentrons 24 Well Aluminum Block with NEST 1.5 mL Snapcap
- Opentrons 96 tip rack 300ul
- Opentrons 96 tip rack 20ul

Opentrons protocols available

- 2.5mg_ml Collagen preparation without cells (500ul) and filling for idenTx 40
- 2.5mg_ml Collagen preparation with cells (500ul) and filling for idenTx 40

Reminder:

1) Turn on the HEPA filter module to high and wipe down the deck's surface and sterilize each item before bringing placing it into the deck.
2) Set the temperature module to 4 °C on the Opentrons app under modules and wait for the temperature to cool down before starting the run.
3) For each run, import the respective files onto https://designer.opentrons.com/.
4) The pipettes, module and equipment and reagent placement on the deck has been preselected. Under file, liquids and design, you can check the pipette, module, equipment and reagent selection. Under design, click starting deck state and arrange the deck accordingly with the various equipment and reagents ensuring that they are placed in the exact positions as stated
5) Finally, check the final deck state to check the wells that would have been filled.
6) Export the protocol and upload it onto the Opentrons App and start the run.

1. Import the file **2.5mg_ml Collagen preparation without cells (500ul) and filling for idenTx 40** or **2.5mg_ml Collagen preparation with cells (500ul) and filling for idenTx 40** onto protocol designer (https://designer.opentrons.com/).

2. The volumes and steps have been added for the preparation of 500ul of 2.5mg/ml collagen as well as filling of all 40 sites.

Reminder *Make sure the color of 10X PBS with phenol red is homogeneous before use as the color of 10X PBS with phenol red changes when it is about to freeze.*

Table 1 An example of collagen gel recipe

Reagents	Volume
10X PBS	50 µl
Collagen	382 µl
0.5 M NaOH	13 µl
Deionized water	55 µl

3. Place the 10X PBS with phenol red, collagen stock solution, 0.5 M NaOH solution, media and deionized water on the assigned wells of the Opentrons 24 well aluminum block with nest 1.5ml Snapcap. Make sure all of them are in 1.5ml microcentrifuge tubes.

Reminder *Make sure the color of 10X PBS with phenol red is homogeneous before use as the color of 10X PBS with phenol red changes when it is about to freeze.*

4. Remove the idenTx 40 from the packaging. Place the idenTx 40 (Pharmaplate) on the deck as well.

5. It is encouraged to check the color of the mixed solution after the mixing steps and ensure that the collagen solution has been mixed thoroughly. Phenol red is a pH indicator itself and the desired color for pH7.4 is faint pink.

? Troubleshoot *See Table 2 if collagen has not been mixed thoroughly.*

6. 5ul of collagen will be filled for each site.

! Critical *Limit the volume of collagen solution to 5ul to prevent the collagen solution from overflowing into the media channels.*

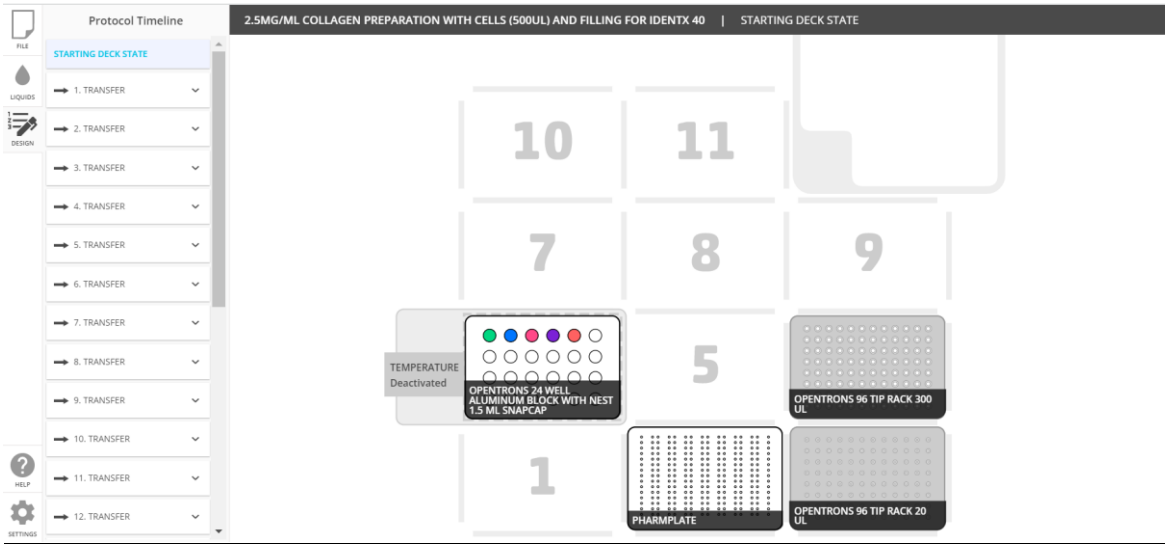
7. After the run is complete, check that all wells are filled properly with no leakage under the microscope.

8. Distribute up to 6 ml of sterilized water across the perimeter of the reservoir to ensure full coverage.

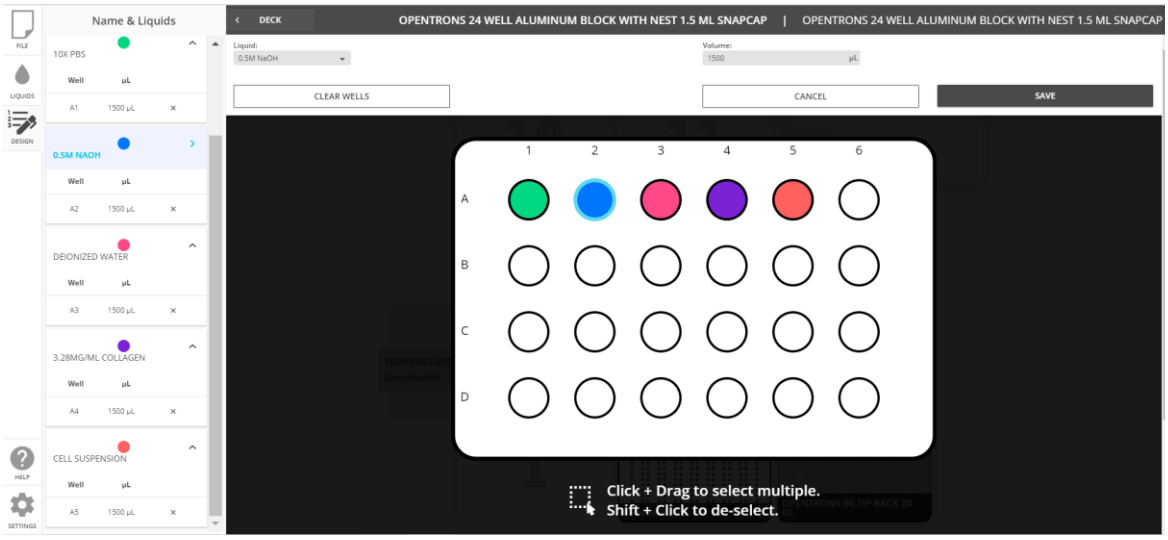
! Critical *Be careful not to tilt the plate when the reservoir is filled to avoid spilling of water into the sites.*

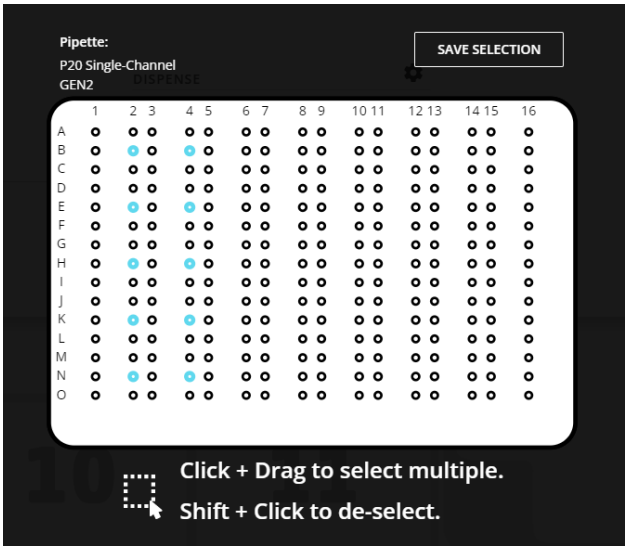
9. Incubate the idenTx 40 in a 37°C incubator for 30 min to allow the polymerization of collagen.

! Critical *Be careful while handling to avoid spilling of gel into the media channels.*

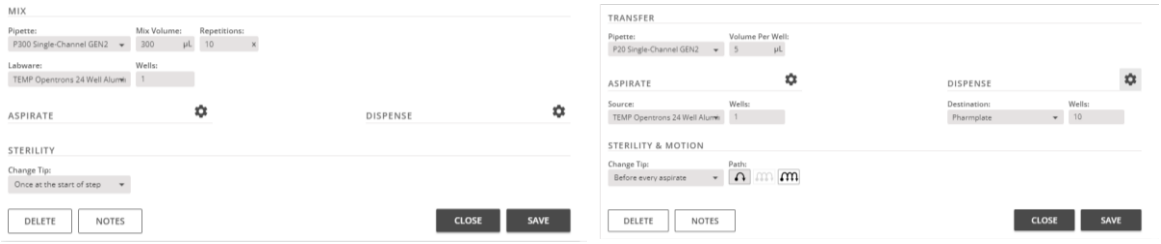


Starting deck state under the tab design





Well selection for collagen filling on idenTx 40. It has been preselected to fill 10 gel channels at a time, after which the collagen solution will be mixed before filling of next 10 gel channels. If filling of all 40 sites is not required, you will have to change the well selection.



Mixing step for collagen mixing before filling. Transfer step of dispensing collagen into idenTx 40 gel channel Remove these steps accordingly if you do not want to fill all 40 sites of the plate.

HYDRATING & COATING MEDIA CHANNELS

MATERIALS

Reagents

- Fibronectin (Sigma-Aldrich, Cat. No. F0895) or any coating reagent for your specific application

! Critical *Collagen coating is not suitable for collagen-filled idenTx 40 as the solvent of the collagen coating solution may dissolve the polymerized collagen.*

Others

- Collagen-filled *idenTx 40*

Opentrons Equipment

- Opentrons 10 tube rack with nest 4 x 50ml, 6 x 15ml conical
- Opentrons 96 tip rack 300ul
- Opentrons 96 tip rack 20ul

Opentrons protocols available

- Media inlet filling only (Coating solution) (idenTx 40)
- Media port filling only (75ul + 55ul) (idenTx 40)
- Media inlet and port filling (idenTx 40)

Reminder:

1) Turn on the HEPA filter module to high and wipe down the deck's surface and sterilize each item before bringing placing it into the deck.
2) For each run, import the respective files onto https://designer.opentrons.com/.
3) The pipettes, module and equipment and reagent placement on the deck has been preselected. Arrange deck accordingly.
4) Finally, check the final deck state to check the wells that would have been filled.
5) Export the protocol and upload it onto the Opentrons App and start the run.

10. If fibronectin coating is being used, use the protocol **Media inlet filling only (Coating solution) (idenTx 40)**. Incubate the media-channel-hydrated sites for 1 h in a 37°C incubator. Then use the protocol **Media port filling only (75ul + 55ul) (idenTx 40)**.
11. If coating is not required, media addition to port can be done immediately. For this, use the protocol **Media inlet and port filling (idenTx 40).**
12. 12ul of coating solution or media will be injected into each media channel. Due to surface tension, the injected coating solution will form a spherical cap at the opposite inlet.

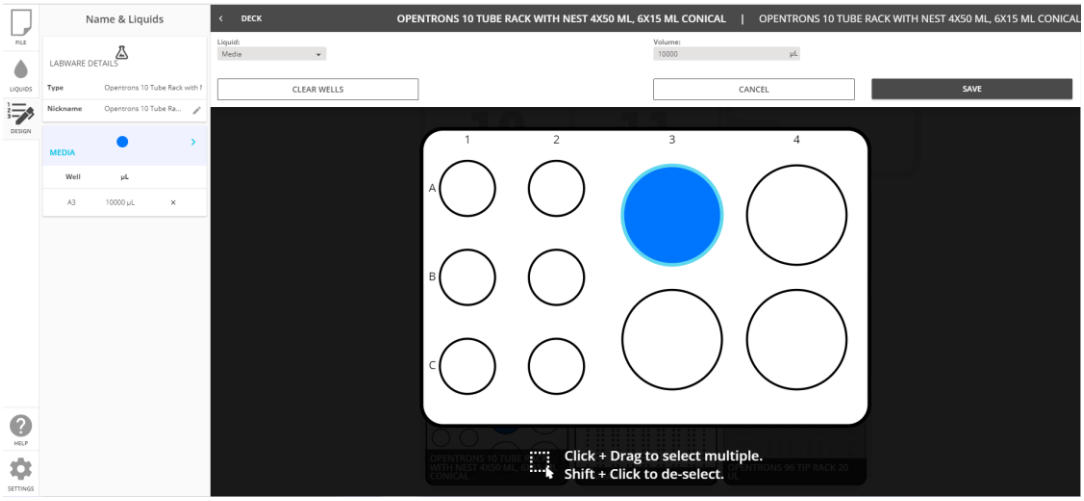
    ? Troubleshoot *See Table 2 for the alternative methods if spherical cap does not form at the opposite media inlet.*
13. For first media addition to port, 75 µl of medium will be added into one port and then 55 µl of medium will be added into the opposite port of the same media channel to flush out the coating solution.

    ? Troubleshoot *See Table 2 for the alternative method if media does not settle in the troughs.*

    PAUSE POINT *The media-channel-hydrated plate can be kept in an incubator for not more than 2 days before the seeding steps depending on your application (In a single experiment, do not mix the media-channel-hydrated plate that are prepared at different time points because the properties of the collagen may change over time).*

    Reminder *Media channels must be hydrated with culture medium/coating solution after the polymerization of collagen. This is to prevent the collagen gel in the plate from drying up.*

    Reminder *If the coating solution has to be removed completely, remove all media from the ports and add 120 µl of medium into 1 port. Repeat this for another channel.*



Placement of 50ml centrifuge containing media for inlet and port filling. Ensure that volume of media present in tube is a maximum of 10ml when P20 is used for media inlet filling and maximum of 20ml when P300 is used for media port filling.

SEEDING CELLS

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 70011044)
- 0.25% trypsin with EDTA (Lonza, Cat. No. CC5012)
- Cell culture medium (Lonza, Cat. No. CC3202)

Others

- Collagen-filled and fibronectin-coated IdenTx 40
- Cells
- Centrifuge

Opentrons Equipment

- Opentrons 24 tube rack with Eppendorf 1.5ml safe-lock Snapcap
- Opentrons 10 tube rack with nest 4 x 50ml, 6 x 15ml conical
- Opentrons 96 tip rack 300ul
- Opentrons 96 tip rack 20ul

Opentrons protocols available

- Media removal with seeding into top and bottom media channels (idenTx 40)
- Seeding into top and bottom media channels (idenTx 40)
- Seeding into top media channels only (idenTx 40)
- Seeding into bottom media channels only (idenTx 40)
- Media removal only (idenTx 40)
- Media port filling only (100ul) (idenTx 40)

Reminder:

1) Turn on the HEPA filter module to high and wipe down the deck's surface and sterilize each item before bringing placing it into the deck.
2) For each run, import the respective files onto https://designer.opentrons.com/.
3) The pipettes, module and equipment and reagent placement on the deck has been preselected. Arrange deck accordingly.
4) Finally, check the final deck state to check the wells that would have been filled.
5) Export the protocol and upload it onto the Opentrons App and start the run.

14. Trypsinize cells as per protocol. Briefly, remove medium from the cell culture flask/dish and wash the endothelial cells with sterile 1X PBS for twice. Add enough 0.25 % trypsin with EDTA solution to cover the bottom of the culture flask/dish and incubate them for 2 min in a CO_2 incubator.

15. Perform a visual inspection to make sure the cells have detached from the substrate.

16. Add medium with FBS, at least 5 times the volume of trypsin, into the culture flask/dish to neutralize the activity of trypsin.

17. Transfer the cell suspension to a 15 ml tube and pellet the cells by centrifuging at 250 x*g* for 5 min at RT.

18. Re-suspend the cells in culture medium with densities ranging from 1 M to 3 M cells/ml, depending on cell types and applications. For seeding cells with Opentrons, 10ul will be needed for each media channel.

19. Use the protocol **Media removal with seeding into top and bottom media channels (idenTx 40).**

20. 70ul of media will be removed from all media ports. 10ul of cell suspension will be seeded into top media channel inlets first. After 30 mins incubation time, another 10ul of cell suspension will be seeding into bottom media channel inlets.

21. Visual inspection of the channel under a microscope is recommended. Therefore, pause the protocol after one inlet has been seeded. If the cell density or distribution is not optimal, add 120 µl of culture medium to 1 media port to flush out the cells. Adjust the cell concentration and repeat Steps 35 – 40.

22. Keep the idenTx 40 in a 5 % CO_2 incubator at 37°C for at least another 30 mins to allow the endothelial cells in the bottom channel to adhere.

23. Use the protocol **Media port filling only (100ul) (idenTx 40)**.

24. 100ul of media will be added into 1 side of each media channel. The wetted media ports will allow the culture medium to flow through the channel and equilibrate. This can be observed by the medium build-up in the opposite port.

25. Keep the idenTx 40 in a 5 % CO_2 incubator at 37°C.

CHANGING MEDIUM

MATERIALS

Reagents

- Cell culture medium (Lonza, Cat. No. CC3202)

Others

- idenTx 40

Opentrons Equipment

- Opentrons 10 tube rack with nest 4 x 50 ml, 6 x 15ml conical
- Opentrons 96 tip rack 300ul
- Opentrons 96 tip rack 20ul

Opentrons protocols available

- Media removal and filling (x1) (idenTx 40)
- Media removal only (idenTx 40)
- Media port filling only (100ul) (idenTx 40)

Reminder:

1) Turn on the HEPA filter module to high and wipe down the deck's surface and sterilize each item before bringing placing it into the deck.
2) For each run, import the respective files onto https://designer.opentrons.com/.
3) The pipettes, module and equipment and reagent placement on the deck has been preselected. Arrange deck accordingly.
4) Finally, check the final deck state to check the wells that would have been filled.
5) Export the protocol and upload it onto the Opentrons App and start the run.

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26. Use the protocol **Media removal and filling (x1) (idenTx 40)**.

27. 70ul of medium will be removed from each port.

28. 100ul of culture medium will be added into one side of each media channel. Ensure that the medium builds up on the opposite media port to indicate a successful medium change.

29. Keep the idenTx 40 in a 5 % CO_2 incubator at 37°C. If the cells need to be kept longer in culture, change the medium daily.

STAINING CELLS

MATERIALS

Reagents

- 4% Formaldehyde (See REAGENT SET UP; Sigma Aldrich, Cat. No.158127)
- 0.1% Triton X-100 (See REAGENT SET UP; Sigma Aldrich, Cat. No. T8787)
- Blocking buffer (Life Technologies, Cat. No. B-10710)
- 1X PBS (Life Technologies, Cat. No. 70011044)
- Primary Antibody: VE-cadherin (Enzo Life Sciences, Cat. No. ALX-210-232-C100)
- Corresponding Secondary Antibody (Life Technologies, Cat. No. A11034)
- Rhodamine Phalloidin (Life Technologies, cat. No. R415)
- Hoechst (Life Technologies, Cat. No. H1399)

Others

- Parafilm

Opentrons Equipment

- Opentrons 10 tube rack with nest 4 x 50 ml, 6 x 15ml conical
- Opentrons 96 tip rack 300ul
- Opentrons 96 tip rack 20ul

Opentrons protocols available

- Staining cells (Cell fixation) (idenTx 40)
- Staining cells (Cell permeabilization) (idenTx 40)
- Staining cells (Blocking) (idenTx 40)
- Staining cells (Primary antibody) (idenTx 40)
- Staining cells (5x Wash + Secondary antibody) (idenTx 40)
- Staining cells (3x Wash + Fluorescent staining) (idenTx 40)
- Staining cells (3x Wash and store in PBS) (idenTx 40)

Reminder:

1) Turn on the HEPA filter module to high and wipe down the deck's surface and sterilize each item before bringing placing it into the deck.
2) For each run, import the respective files onto https://designer.opentrons.com/.
3) The pipettes, module and equipment and reagent placement on the deck has been preselected. Arrange deck accordingly.
4) Finally, check the final deck state to check the wells that would have been filled.
5) Export the protocol and upload it onto the Opentrons App and start the run.

Cell Fixation

30. Use the protocol **Staining cells (Cell fixation) (idenTx 40)**.

31. 70ul of medium will be removed from each port.

32. The channels will be washed with 100ul of 1X PBS once. Observe for fluid build-up in the opposite connected port to indicate a successful reagent exchange.

33. 100ul of 4% formaldehyde will be added into one side of each media channel.

34. The protocol will be paused for 13 min at RT for incubation.

Reminder *Incubation time has been changed to 13 min instead of 15 min to*

account for time taken to dispense the reagent into the wells.

35. 4% formaldehyde will be removed from all the ports.

36. Channels will be washed with 1x PBS twice.

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

Cell Permeabilization

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

37. Use the protocol **Staining cells (Cell permeabilization) (idenTx 40)**.

38. 1X PBS will be removed from all the ports.

39. 100ul of 0.1% Triton X-100 into one side of each media channel.

40. The protocol will be paused for 8 min at RT for incubation.

Reminder *Incubation time has been changed to 8 min instead of 10 min to account for time taken to dispense the reagent into the wells.*

41. 0.1% Triton X-100 will be removed from all the ports.

42. Channels will be washed with 1x PBS once.

Blocking

(Optional: Blocking is only necessary for immunofluorescent staining)

43. Use the protocol **Staining cells (Blocking) (idenTx 40)**.

44. 1X PBS will be removed from all the ports.

45. 100ul of blocking buffer will be added into one side of each media channel.

46. Incubate for 2 h at RT.

Primary Antibody Staining

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

47. Prepare antibodies according to the manufacturer's recommendations. For example, dilute the anti-VE-cadherin antibody with 1X PBS in a 1:100 ratio.

48. Use the protocol **Staining cells (Primary antibody) (idenTx 40).**

49. Blocking buffer will be removed from all the ports.

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

50. The protocol has been designed for cells embedded within the 3D hydrogel. Differential volumes of antibodies of 110ul for top media channel and 70ul for bottom media channel will be added to one side for each media channel to generate interstitial flow that can transport the antibodies to the cells.

51. Seal idenTx 40 with parafilm. Incubate overnight at 4°C.

Secondary Antibody Staining

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

52. Prepare the secondary antibodies according to the manufacturer's recommendations. For example, dilute the Alexa Fluor-conjugated goat anti rabbit-secondary antibody with 1X PBS in a 1:100 ratio.

53. Use the protocol **Staining cells (5x Wash + Secondary antibody) (idenTx 40)**.

54. Primary antibodies will be removed from all the ports.

55. Channels will be washed with 1X PBS 3 times with 3 min incubation between each wash.

Reminder *Incubation time has been changed to 3 min instead of 5 min to account for time taken to dispense the reagent into the wells.*

56. The protocol has been designed for cells embedded within the 3D hydrogel. Differential volumes of antibodies of 110ul for top media channel and 70ul for bottom media channel will be added to one side for each media channel to generate interstitial flow that can transport the antibodies to the cells.

! Caution *Cover the idenTx 40 with aluminium foil to minimize photobleaching.*

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

Fluorescent Staining

58. Prepare the fluorescent staining solution. Eg. Hoechst (10 µg/ml)/Rhodamine Phalloidin (3 U/ml).

Reminder *The fluorescent staining of nuclei and actin by using Hoescht and Rhodamine Phalloidin can be carried out either concurrently or separately.*

! Caution *Hoechst (or other nucleus staining reagents) should always be handled using protective gloves and clothing.*

59. Use the protocol **Staining cells (3X Wash + Fluorescent staining) (idenTx 40)**.

60. Secondary antibodies will be removed from all the ports.

61. Channels will be washed with 1X PBS 3 times with 3 min incubation between each wash.

Reminder *Incubation time has been changed to 3 min instead of 5 min to account for time taken to dispense the reagent into the wells.*

62. 100ul of fluorescent staining solution will be added to one side of each media channel.

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

Reminder *Incubation time for actin staining can be increased if stronger fluorescent intensity is needed.*

64. Use the protocol **Staining cells (3X Wash + Store in PBS) (idenTx 40)**.

65. Channels will be washed with 1X PBS 3 times with 3 min incubation between each wash. The channels will be left in PBS without any removal after the last wash.

Reminder *Incubation time has been changed to 3 min instead of 5 min to account for time taken to dispense the reagent into the wells.*

66. Seal the idenTx 40 with parafilm and keep them in 4 °C and protect them from light until imaging.

Reminder *The staining protocol should be optimized for your specific application (with different cell types and different proteins of interest).*

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

TROUBLESHOOTING

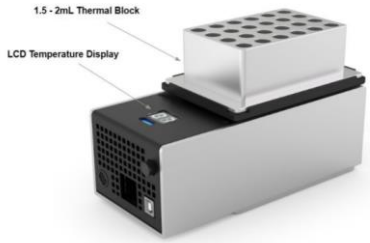
Table 2 Troubleshooting advice

Step	Problem	Possible Reason	Solution
5.	Collagen has not been mixed fully or NaOH not distributed fully.	Volume of collagen prepared is too low	Manually mix it without bubbles
		Number of transfer or mixing steps is less	Duplicate more transfer steps or increase the number of times solution is mixed
12.	Medium does not form spherical cap on the opposite inlet	Pippete tip is not a tight fit (The protocol has been optimized to the best with Opentrons P20 pippete. Medium will flow through all channels but some sites might not have the spherical cap with this tip so manual aspiration might be required.)	Wait 2 minutes to observe if medium flows through if not manually aspirate Or manually aspirate a small volume using a P200 tip from the opposite inlet. Medium should form a cap immediately. Or tilt the plate slightly to the opposite side of injection.
13.	Media forms a V-shape for first media addition of 75ul to right media port and 55ul to opposite left media port	The tip is pointing right on top of the media inlet now instead of the trough.	Tap the entire idenTx 40 plate slightly on a table a few times. The media should settle in the troughs.

OPENTRONS LABWARE

Table 3 Labware used for the protocols

Opentrons 24 Well Aluminium Block with Nest 1.5ml Snapcap (1.5 – 2ml Thermal Block)



Temperature Module With 1.5 – 2ml Thermal Block

Opentrons 20ul tips

Opentrons 300ul tips

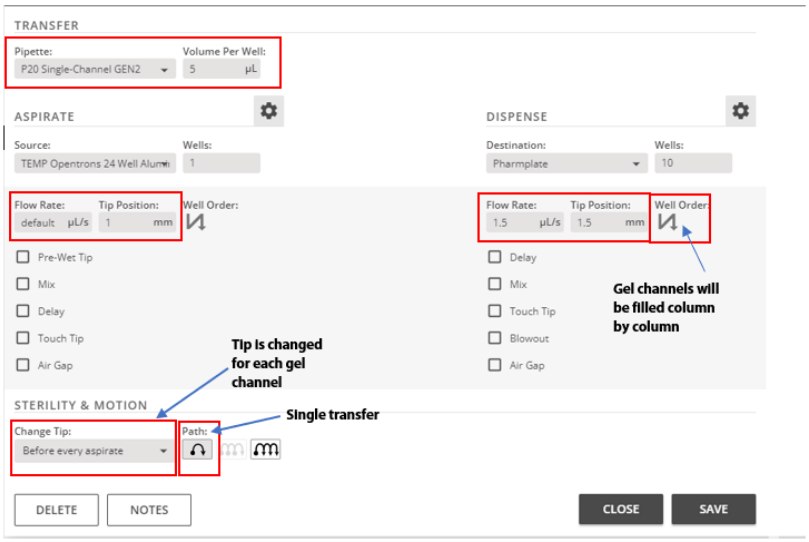
Opentrons 24 tube rack with Eppendorf 1.5ml safe-lock snapcap

Opentrons 10 tube rack with nest 4 x 50ml, 6 x 15ml conical

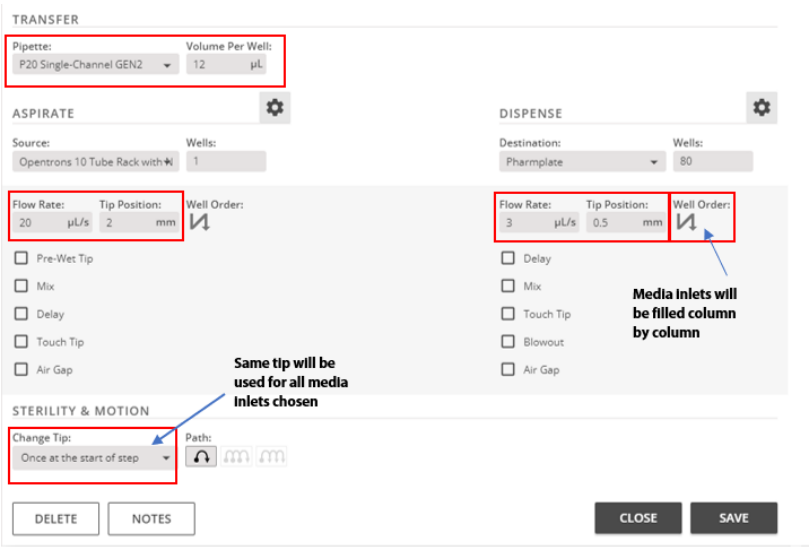
PROTOCOLS' DETAILS

Table 4 Details of steps used in protocol

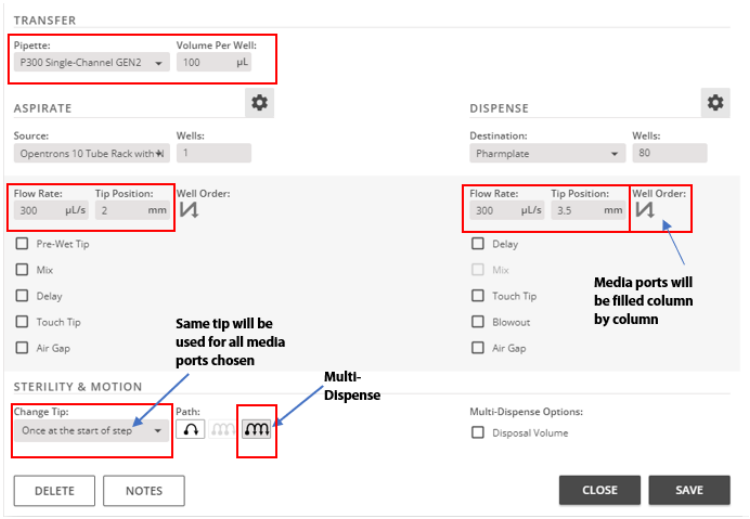
Boxes in red highlight the various details present in the steps of each protocol. The values seen below have been optimized through trial to ensure optimal success when used on idenTx 40.



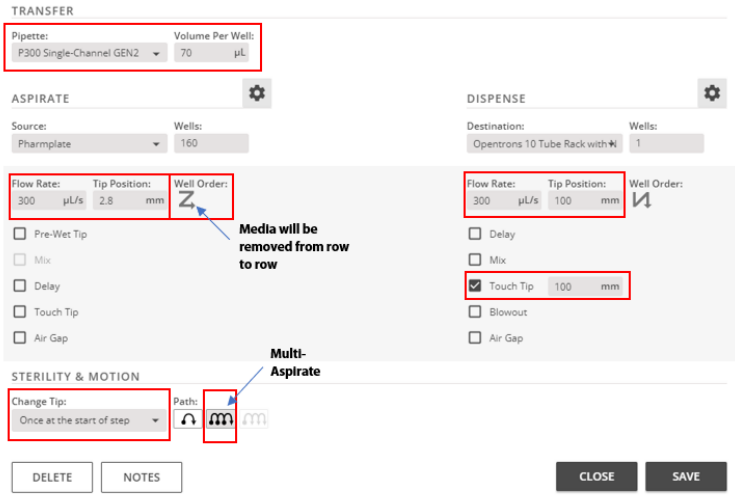
1) Details for collagen filling



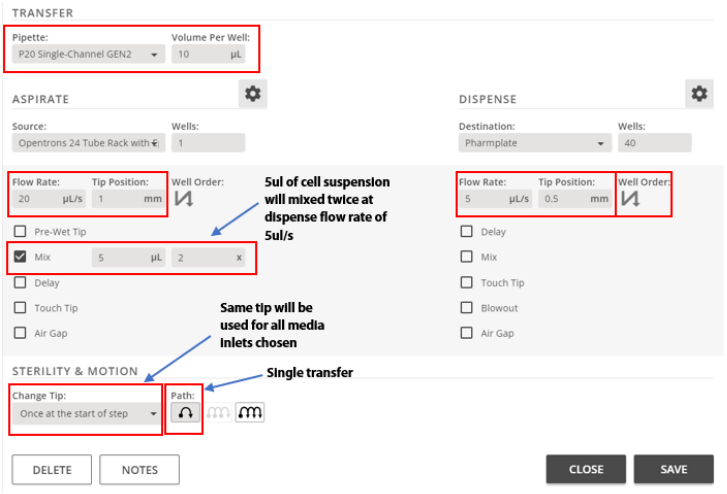
2) Details for media inlet filling



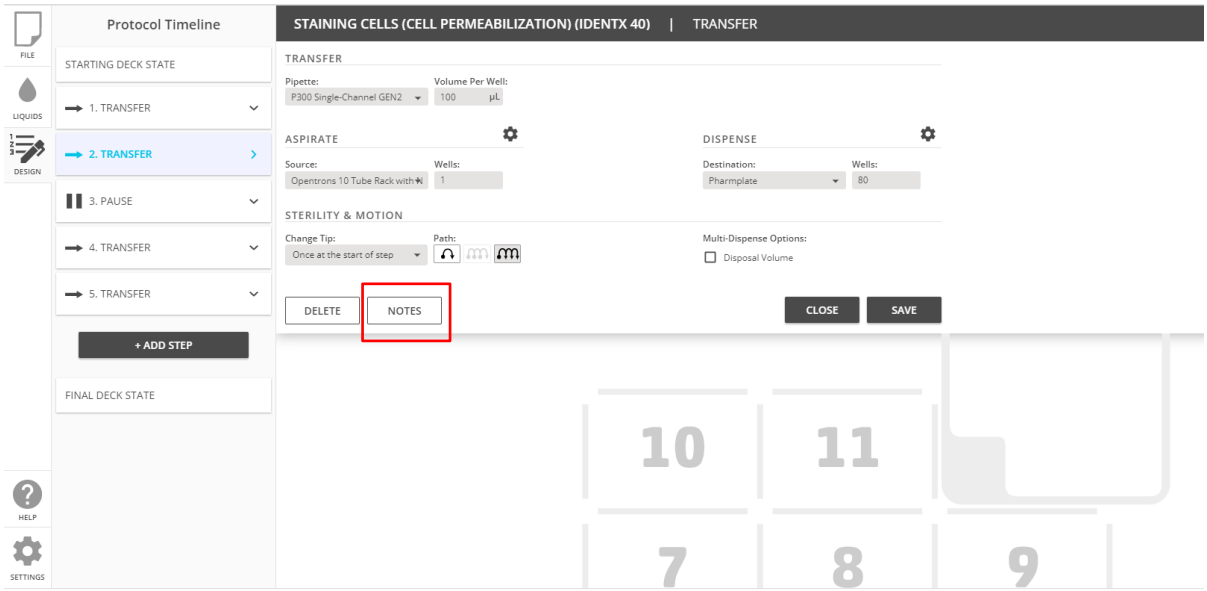
3) Details for media port filling



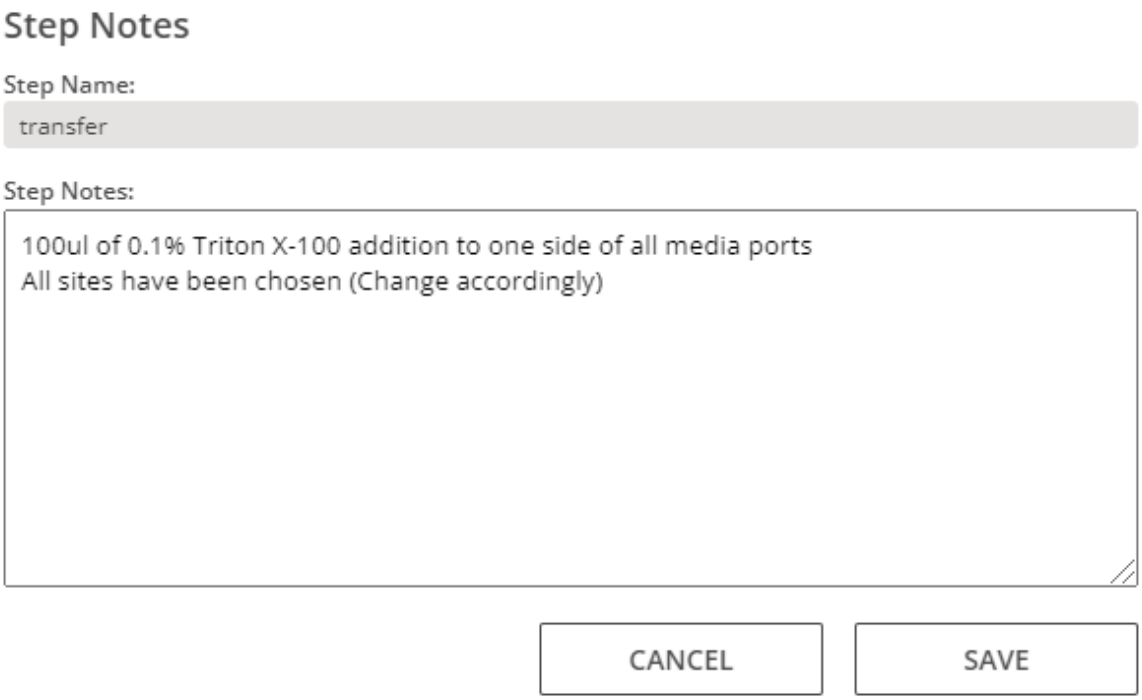
4) Details for media removal from ports



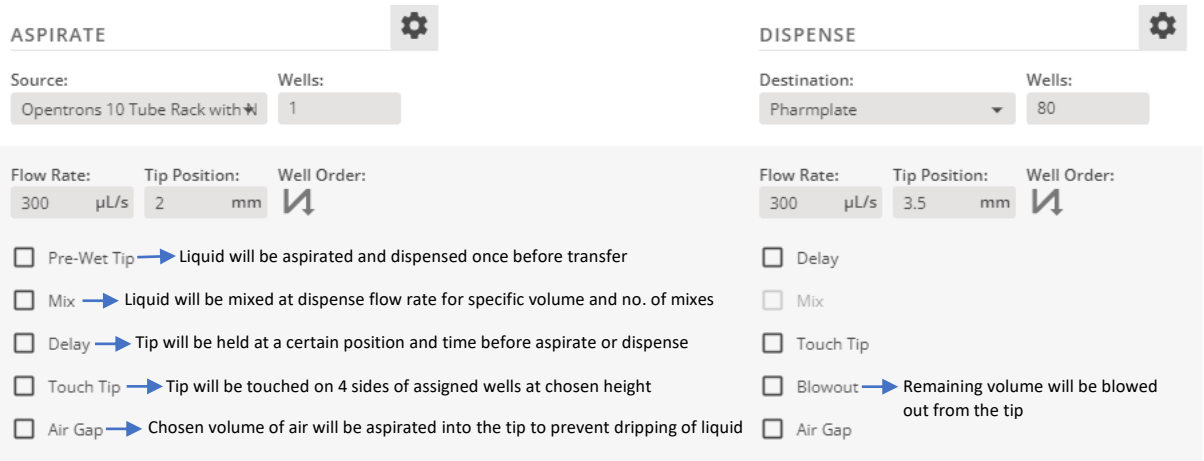
5) Details for seeding cells in media channels



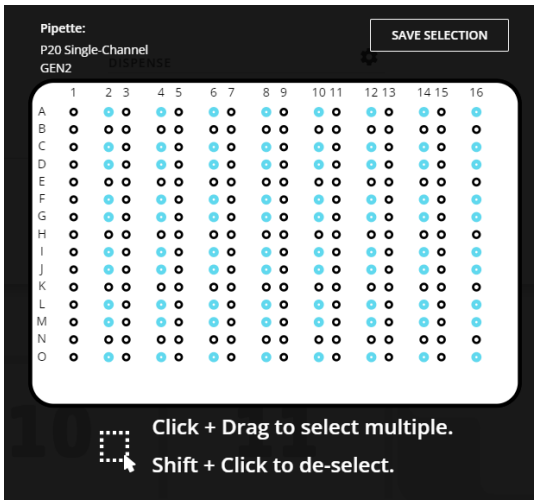
Each step of all protocols has notes detailing what is being done for that step



Description of the step in the notes



Description of various advanced settings found under steps in protocol designer. They have been preselected in the protocols for optimal aspiration and dispense of reagents.



Well selection for media inlet filling of the entire idenTx 40 plate. The wells needed for the respective protocols have already been preselected. Unselect the respective wells if all 40 sites are not going to be filled.

OPENTRONS CALIBRATION DATA

Table 5 Calibration data of each labware

Disclaimer: These data can be used as a guide to perform calibration for the deck and idenTx 40. However, calibration may vary each individual and the machine. Optimizations might have to be performed for optimal run of protocols.

Opentrons 96 Tip Rack 20ul	X: 0.1 Y: 0.5 Z: 0.3
Opentrons 96 Tip Rack 300ul	X: 0.6 Y: 0.9 Z: 0.0
Temperature module 1.5ml 24 well Snapcap	X: 0.3 Y: 0.8 Z: -0.2
idenTx 9	X: 0.1 Y: 1.2 Z: -4.4
idenTx 40 (Pharmaplate)	X: 0.1 Y: 0.6 Z: 0.0
Opentrons 10 tube rack with nest 4x50ml, 6x15ml conical	X: 0.0 Y: 0.0 Z: -1.0
Pipette calibration P20	X: -1.80 Y: -7.40 Z: 0.10
Pipette calibration P300	X: 0.30 Y: -5.40 Z: 0.20
15ml centrifuge tube (P300)	Tip position: 2mm Minimum volume to be filled: 100ul Max volume to be filled: 4ml
50ml centrifuge tube (P300)	Tip position: 2mm Minimum volume to be filled: 100ul Max volume to be filled: 20ml
50ml centrifuge tube (P20)	Tip position: 2mm Minimum volume to be filled: 100ul Max volume to be filled: 10ml

REAGENT SETUP

Please refer to the idenTx 40 Handling Protocol to prepare 0.5M Sodium Hydroxide (NaOH) Solution, 10X PBS with Phenol Red, 4% Formaldehyde, 0.1% Triton-X.