1. Objective

Seeding of Caco-2 cells against extracellular matrix (ECM) gel in an OrganoPlate® 3-lane 64

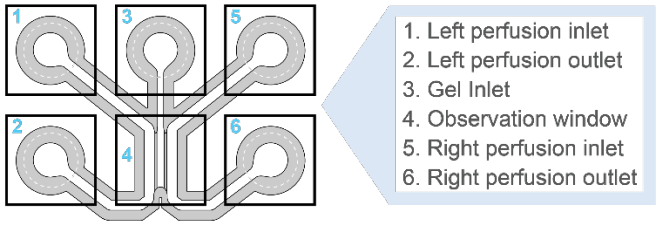


***Figure 1**: Schematic representation of an OrganoPlate® 3-lane 64 tissue chip*

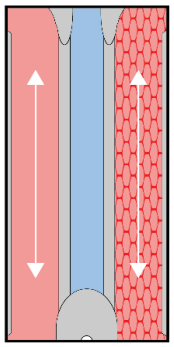


***Figure 2**: Illustration of a tubule of Caco-2 cells (right perfusion channel) grown against an ECM gel (middle channel) in an OrganoPlate® 3-lane 64 tissue chip*

2. Background

Tubular structures, such as endothelial or epithelial barrier tissues, are established in the OrganoPlate® by growing cells against an ECM gel. Morphology and function of the tubule can be assessed by microscopy, a barrier integrity assay, or other functional assays. This protocol describes the culturing of a tubule of Caco-2 cells against an ECM in the perfusion channel of an OrganoPlate® 3-lane 64 (see figure 1 and 2). The resulting tubular culture allows for access to apical and basal side of the barrier tissue.

3. Materials

- OrganoPlate® 3-lane 64 (MIMETAS, 6405-400-B)
- OrganoFlow® S or L (MIMETAS, MI-OFPR-S or MI-OFPR-L)
- Human colon adenocarcinoma cell line Caco-2 (Sigma-Aldrich, #86010202)
- Caco-2 medium components: EMEM (ATCC, 30-2003) + 10% FBS HI (ThermoFisher, 16140-071) + 1% Non-Essential Amino Acid (ThermoFisher, 11140050) + 1% Pen/Strep (Sigma, P4333)
- Collagen-I 5 mg/mL (AMSbio Cultrex® 3D collagen I rat tail, 5 mg/mL, 3447-020-01)
- 1 M HEPES (ThermoFisher, 15630-122, pH 7.2-7.5)
- 37 g/L $NaHCO_3$ (Sigma S5761-500G, dissolve in sterile MilliQ water, adjust pH to 9.5 using NaOH)
- Medium: 14 mL per OrganoPlate®
- Repeating pipette for gel loading and cell seeding, we recommend:
    - The Eppendorf® Multipette® M4 with the Eppendorf® Biopur® 0.1 mL tip (VWR #613-2067) for dispensation of 2 µL, or
    - The Sartorius eLINE® electronic pipette (Sartorius, #735021 (previously #730021)) for accurate dispensation of volumes ranging from 0.2 to 10 µL. Use with corresponding Sartorius tips or with Eppendorf® ep Dualfilter tips (Eppendorf, 022491211 / 0030077512)

- HBSS (Sigma H6648)
- Multichannel pipette (1200 µL and 300 µl) and multichannel tips
- Crushed ice

4. Tubule seeding in the OrganoPlate®

A collagen-I ECM gel is loaded in the gel inlet of the OrganoPlate® and fills the gel channel. After polymerization of the gel, a cell suspension is seeded in the perfusion inlet and fills the perfusion channel. After cell attachment, medium perfusion is started to aid the formation of a tubule (figure 3).

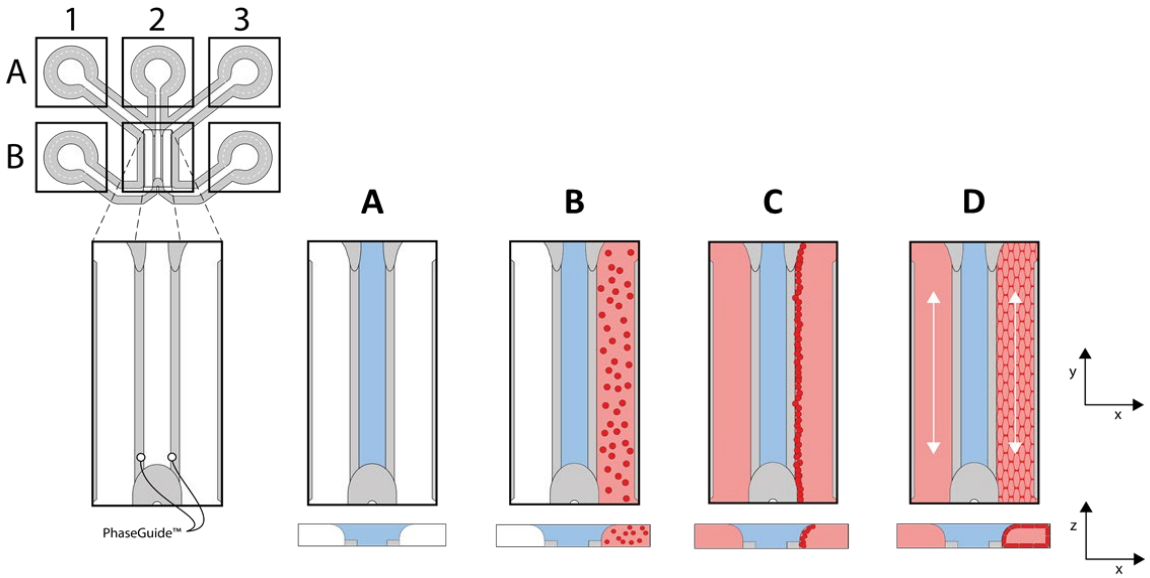


***Figure 3**: Schematic representation of Caco-2 culture in the OrganoPlate® 3-lane 64. A) ECM gel is loaded in the middle channel of OrganoPlate® 3-lane 64 tissue chips. B) A suspension of Caco-2 cells is seeded in a perfusion channel, adjacent to the ECM channel. C) The OrganoPlate® is placed under an angle that allows Caco-2 cells to settle against the gel and attach. D) The OrganoPlate® is placed on a rocker platform to initiate medium perfusion and allow tubule formation.*

Load ECM gel in the OrganoPlate®

Note: avoid touching the bottom glass plate of the OrganoPlate®

1. Take the OrganoPlate® from the packaging
2. Add 50 µL of PBS to all observation windows (columns 2, 5, 8, 11, 14, 17, 20, 23; rows B, D, F, H, J, L, N, P) using a multichannel repeating pipette.
3. Prepare the required amount of ECM gel (e.g. 2 µL gel per chip + 40% extra)
    a. Collagen-I 4 mg/mL preparation
        i. Place an Eppendorf tube on ice
        ii. The collagen-I 4 mg/mL gel is prepared by mixing 1 M HEPES, 37 g/L $NaHCO_3$, and 5 mg/mL collagen-I in a 1:1:8 ratio. For example, to prepare 100 µL of gel:
            - Place an Eppendorf tube on ice

- Mix 10 µL of 1 M HEPES with 10 µL of 37 g/L $NaHCO_3$
- Add 80 µL of collagen-I 5 mg/mL to the HEPES/$NaHCO_3$ mixture

iii. Prepare at least 100 µL of total gel volume to ensure proper mixing of all components
iv. Mix well by pipetting the mixture up and down >20 times, while keeping it on ice
v. If bubbles are formed, quickly spin the tube down (~5 seconds)
vi. Use gel immediately after preparation (within 10 minutes)

4. Dispense the gel into the gel inlets (columns 2, 5, 8, 11, 14, 17, 20, 23; rows A, C, E, G, I, K, M, O) using the Eppendorf® Multipette® M4 or the Sartorius eLINE electronic pipette.
   a. Gently place your pipette tip on top of the hole in the bottom of the well and dispense the gel. Contact between the pipette tip and the hole is essential for gel loading. Correct positioning of the gel on top of hole allows capillary forces to pull the gel into the microfluidic gel channel (see figure 4).
   b. The optimal loading volume depends on several factors, such as the viscosity of the gel and the temperature in the lab
   c. Start by loading 2 µL gel per gel inlet
   d. In case of incomplete gel filling, increase the loading volume (e.g. to 2.3 µL)
   e. In case the gel overflows from the gel channel into the adjacent medium channel, reduce the loading volume (e.g. to 1.7 µL)
5. For examples of correct gel filling in the OrganoPlate® 3-lane 64, see figure 5.

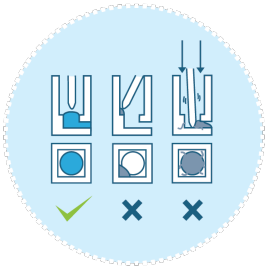


***Figure 4**: Gel loading*

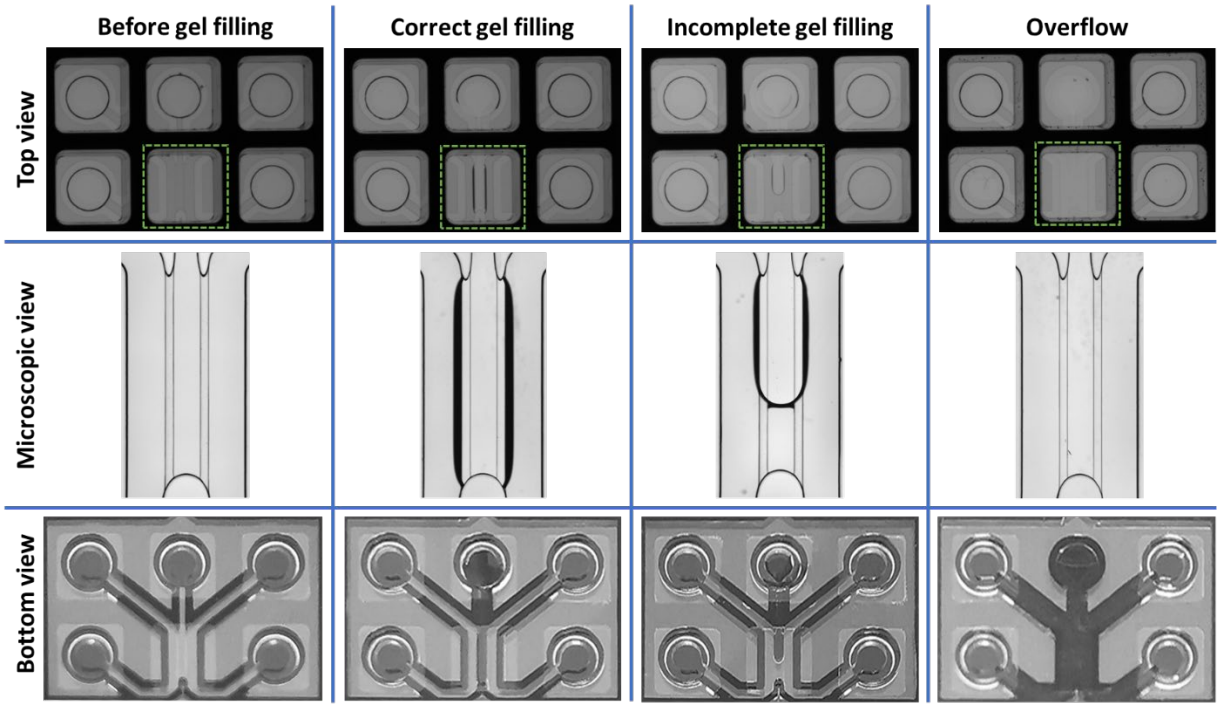


***Figure 5**: Overview of correct gel filling, incomplete gel filling, and overflow in the OrganoPlate® 3-lane 64*

Both the Eppendorf® Multipette® M4 and the Sartorius eLINE electronic pipette can successfully be used to load gel in the OrganoPlate®. Table 1 shows an overview of each pipette's advantages and disadvantages for gel loading.

Table 1

Pipette for gel loading	Advantage	Disadvantage
Eppendorf® Multipette® M4	Allows user to load many chips in one go without having to reload the pipette tip*	Only allows whole-microliter volumes (1 µl, 2 µL, etc.), making it more difficult to correct incomplete gel filling or overflow
Sartorius eLINE® electronic pipette	Allows user to select the loading volume with 10 nL steps, such as 1.75 µL, 1.80 µL, 1.85 µL, etc.	Total volume of pipette is 10 µL, allowing user to load approximately 5 chips at a time before having to reload the pipette

*We recommend loading a maximum of 20 chips at once before emptying and reloading the pipette tip with cold gel. This will avoid gelation of the gel while it is in the pipette tip.

6. Place the OrganoPlate® in a humidified incubator (e.g. 37°C, 5% CO_2) for 15 minutes to allow polymerization of the collagen-I gel
7. Add 30 µL of HBSS to the gel inlet (columns 2, 5, 8, 11, 14, 17, 20, 23; rows A, C, E, G, I, K, M, O) to prevent the gel from drying out
    a. For examples of dried out gel, see section 5 "Trouble Shooting"
8. Place the OrganoPlate® back in the incubator and proceed to cell seeding.
    a. You can choose to proceed to cell seeding immediately or to wait until the next day. While cells generally form tubules with either option, Caco-2 cells show optimal results when seeded one day after gel loading.

Seed Caco-2 cells against the ECM gel

1. Harvest the Caco-2 cells according to their dissociation protocol
2. Count the number of live cells in the cell suspension
3. Calculate the required number of cells for seeding in the OrganoPlate® and pellet them
    a. For example:
        i. Number of chips to seed: 64
        ii. Volume of cell solution to seed per chip: ≈2 µL
        iii. Seeding density: 10,000 cells/µL
        iv. You need: 64 x 2 x 10,000 = $1.28*10^6$ cells
        v. Prepare 25% extra: pellet $1.6*10^6$ cells
4. Resuspend pellet in [$1.6*10^6$ / 10,000 =] 160 µL medium to obtain a 10,000 cells/µL cell suspension
5. Remove HBSS from the gel inlets
6. Seed 2 µL of cell suspension in the right perfusion inlet (columns 3, 6, 9, 12, 15, 18, 21, 24; rows A, C, E, G, I, K, M, O) using the same pipetting procedure as previously used for gel loading (see figure 3)
    a. Regularly resuspend the cell suspension during seeding to ensure homogenous cell density.
    b. In case you want to include cell-free controls, seed 2 µL of medium without cells in the top medium inlet of these chips (instead of the cell suspension)

7. Add 50 µL of medium to the right perfusion inlet (columns 3, 6, 9, 12, 15, 18, 21, 24; rows A, C, E, G, I, K, M, O)
8. Place the OrganoPlate® on its side in the MIMETAS plate stand for 3-4 hours in the incubator to allow the cells in the perfusion channel to settle onto the ECM gel and attach (see figure 6).

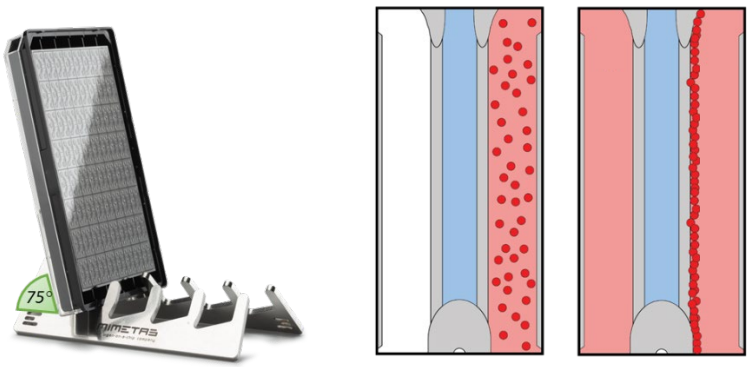


***Figure 6**: Incubate OrganoPlate® on the side to allow cells to attach to the ECM gel.*

9. After cells have attached, add 50 µL of medium to the right perfusion outlet (columns 3, 6, 9, 12, 15, 18, 21, 24; rows B, D, F, H, J, L, N, P)
10. Add 50 µL of medium to the left perfusion inlet (columns 1, 4, 7, 10, 13, 16, 19, 22; rows A, C, E, G, I, K, M, O)
    a. Ensure that the medium has filled the channel completely.
    b. Ensure that no air bubbles are trapped on medium inlet and outlet. If bubbles are trapped, remove the bubbles gently with a pipette tip.
11. Add 50 µL of medium to the left perfusion outlet (columns 1, 4, 7, 10, 13, 16, 19, 22; rows B, D, F, H, J, L, N, P)
12. Place the plate on the OrganoFlow® in a humidified incubator to start cell culture (see figure 7 for the orientation of the OrganoPlate®)
    a. An inclination of 14° and an interval of 8 minutes is compatible with Caco-2 cultures

***Figure 7**: Place the OrganoPlate® on the OrganoFlow® in the correct orientation.*

13. Refresh medium every 2-3 days by aspirating and replacing the medium from perfusion inlets and outlets (50 µL in each) using a repeating multichannel pipette.
    a. An example of a tubule culture against ECM in the OrganoPlate® 3-lane is shown in figure 8.

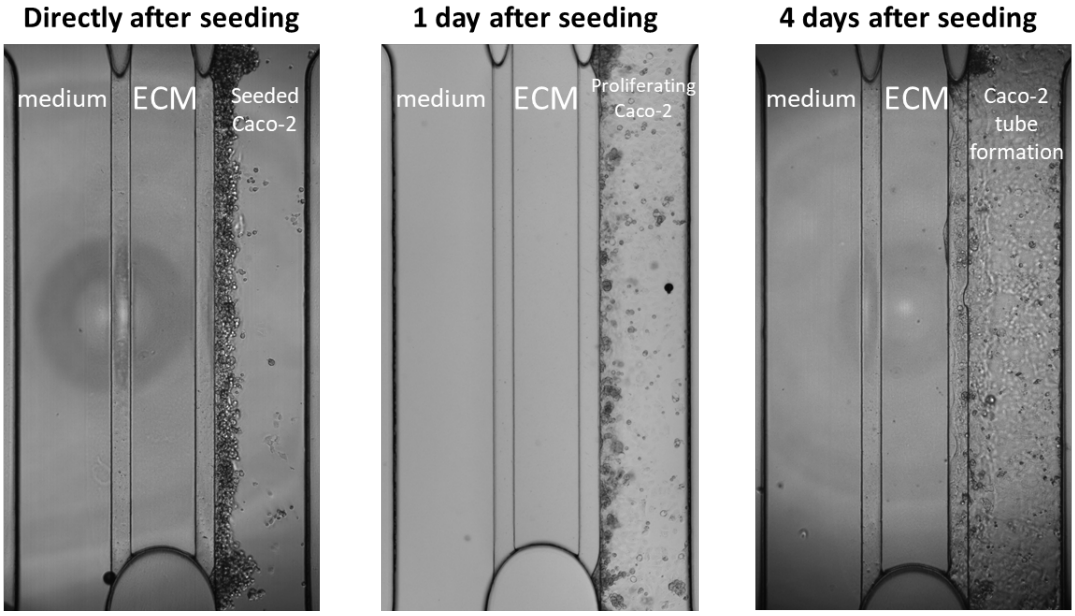


***Figure 8**: Caco-2 cells form a tubule against an ECM gel in the OrganoPlate® 3-lane 64*

5. Troubleshooting

ECM drying

In some cases, the ECM gel can dry out during the gel loading and polymerization process (figure 9). This generally happens when the gel loading process takes longer than expected and the gel in the chips that were loaded first has been incubated much longer than the gel in the chips that were loaded last. When loading goes smoothly, this problem does not occur. However, if loading takes longer than expected (> 10 min), check regularly under the microscope to see if the gel starts to dry out and if you observe that it is, quickly add HBSS to the gel inlet of those chips to prevent further drying.

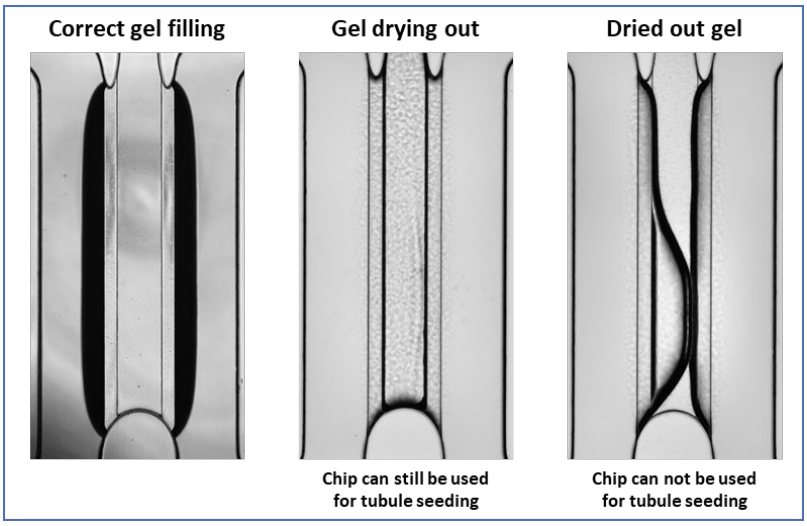
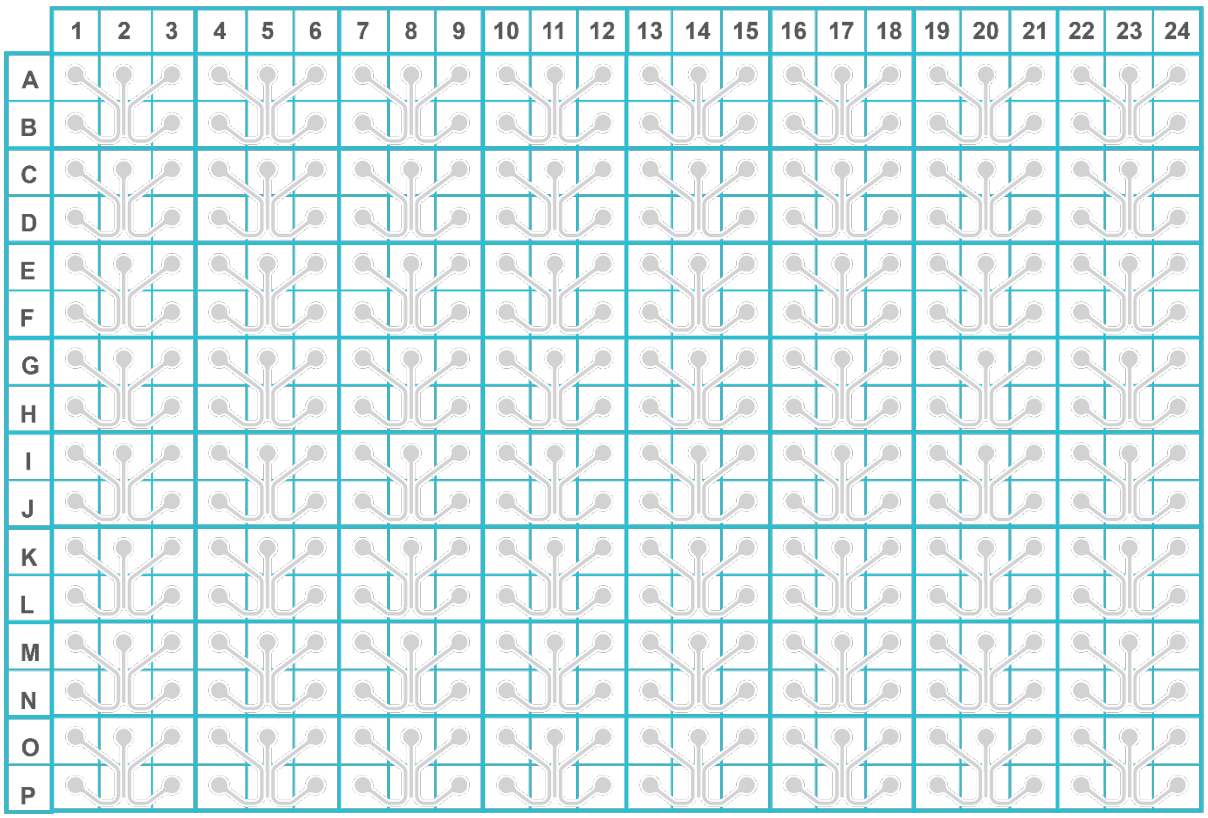


***Figure 9**: ECM gel drying out due to prolonged gel loading or polymerization.*

Cell invasion

In case of undesired cell invasion into the gel, the use of MMP inhibitors is recommended (e.g. addition of 10 µM of MMP-I inhibitor GM6001 (Abcam, ab120845) to the culture medium).

Plate layout



MIMETAS product list

Cat. No.	Product Name
MI-AR-CC-01	OrganoReady® Caco-2
9605-400-B	OrganoPlate® 2-lane
4004-400-B	OrganoPlate® 3-lane 40
6405-400-B	OrganoPlate® 3-lane 64
6401-400-B	OrganoPlate® Graft
MI-OFPR-S	OrganoFlow® S
MI-OFPR-L	OrganoFlow® L
MI-OT-1	OrganoTEER®

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