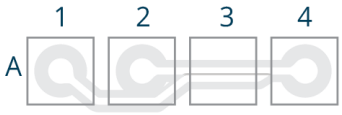
Immunostaining in the OrganoPlate®

1. Objective

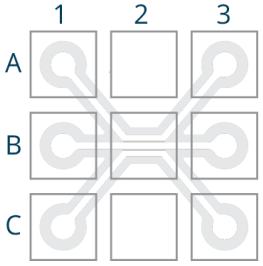
This protocol describes the immunostaining procedure of tissues grown in the OrganoPlate® 2-lane and 3-lane (see chip layout below)

OrganoPlate® 2-lane 96



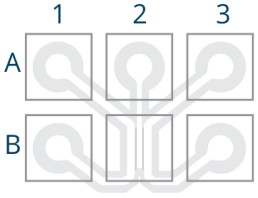
A1: ECM channel inlet
A2: Perfusion channel inlet
A3: Observation window
A4: Perfusion channel outlet

OrganoPlate® 3-lane 40



A1: Top perfusion channel inlet
A3: Top perfusion channel outlet
B1: ECM channel inlet
B3: ECM channel outlet
C1: Bottom perfusion channel inlet
C3: Bottom perfusion channel outlet

OrganoPlate® 3-lane 64



A1: Left perfusion channel inlet
B1: Left perfusion channel outlet
A2: ECM channel inlet
B2: Observation window
A3: Right perfusion channel inlet
B3: Right perfusion channel outlet

2. Background

Immunofluorescence staining is a technique that uses antibodies to target specific cellular biomolecules expressed on cells. Detection of the bound antibodies is done using fluorescence microscopy.

3. Materials

- Tween-20 (Sigma, cat# P9616)
- FBS (Gibco/ATCC, cat# A13450)
- Triton™ X-100 (Sigma, cat# T8787)
- HBSS (+Ca/Mg) (Sigma, cat# 55037C-1000ML)
- BSA (Sigma, cat# A2153)
- Crushed ice
- Repeating multichannel pipets and tips
- 1x PBS (Gibco, cat# 70013065)
- Rocker platform (optional)
- Fixative (see table 1)
    - Standard fixative is 3.7% formaldehyde (diluted 1:10 from stock, Sigma, cat# 252549-1L)

Fixative	Incubation time
3.7% formaldehyde in HBSS	10-15min RT
0.4% formaldehyde in HBSS	10-15min RT
-20°C 100% acetone	5min RT
-20°C 100% methanol	10-15min RT
-20°C 95% methanol, 5% acetic acid	5-10min RT

Table 1. *Fixation methods compatible with the OrganoPlate®*

- Permeabilization buffer: 0.3% Triton X-100
- Blocking solution: 2% FBS, 2% BSA, 0.1% Tween20 in PBS
- Washing solution: 4% FBS in PBS
- Antibodies and nuclear stain (Hoechst™/DraQ5™)

4. Assay

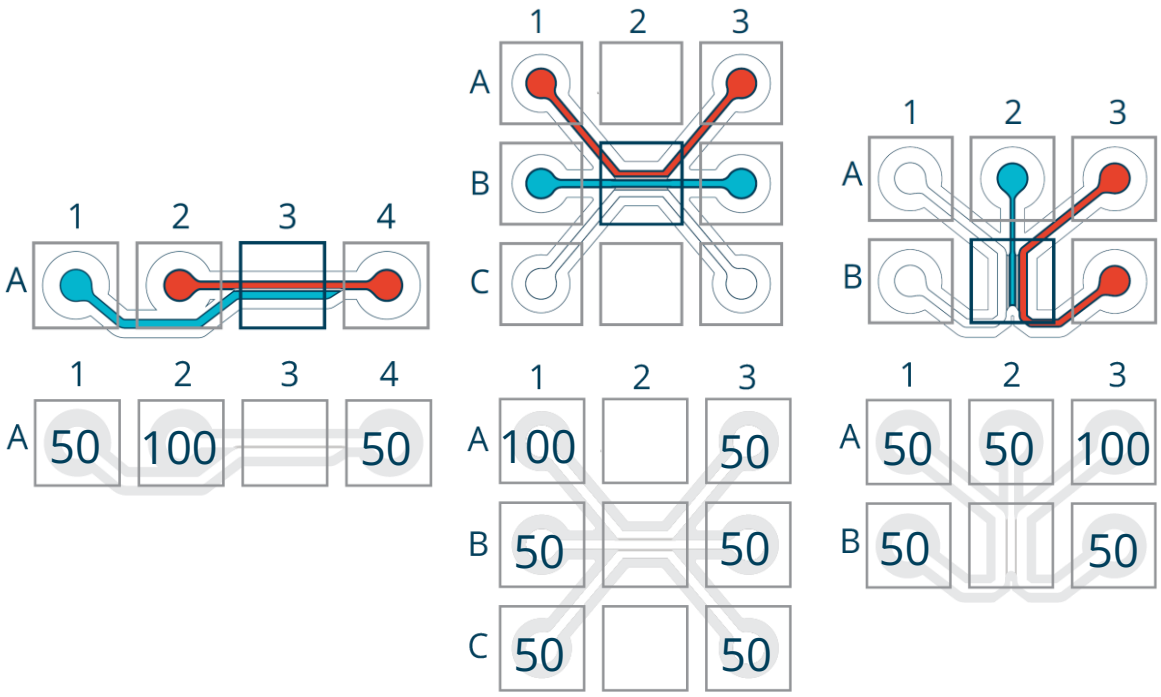
Fixation

Perform all steps at room temperature

1. Prepare fixative (see table 1)
    a. Standard fixative is 3.7% formaldehyde in HBSS (dilute 1:10 from 37% stock)
    b. Complete OrganoPlate® 2-lane: 21 mL
    c. Complete OrganoPlate® 3-lane 40: 17 mL
    d. Complete OrganoPlate® 3-lane 64: 25 mL

2. Aspirate medium from the chips and add fixative to the chips' inlets and outlets according to the volume scheme below:

- OrganoPlate® 2-lane: 50 µL to ECM inlet, 100 µL to the perfusion channel inlet, 50 µL to perfusion channel outlet
- OrganoPlate® 3-lane 40 and 64: 100 µL to tubule inlet, 50 µL to all other inlets and outlets NOTE: for tubular structures, it is important to have a difference in volumes from the inlet to the outlet to induce flow through the channel. The protocol details for tube on the top and right perfusion channel of the 3 lane-40 and 64 format as shown below (red for cell tubule, blue for ECM).

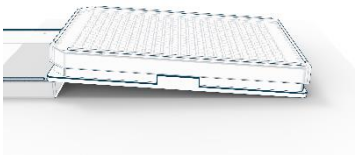


3. Incubate the fixative for the appropriate amount of time (see table 1)
4. Aspirate fixative and wash chips 2x (5 min each) with PBS using the volume scheme shown in step 2. Additionally, remove the HBSS from the observation window an replace with 1x PBS.

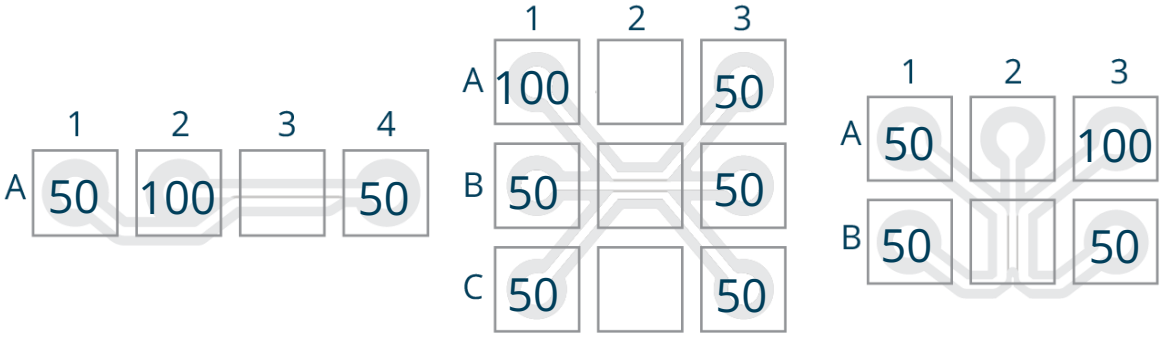
5. Proceed to immunostaining or seal the plate around the edges with Parafilm® and wrap the plate in aluminum foil. The fixed plate can be stored at RT up to 2 weeks. For optimal imaging quality, we recommend immediately proceeding with the antibody staining and image acquisition steps. Do not freeze the plate post fixation, as this will cause the glass bottom and the microfluidics to delaminate from the plate.

Immunostaining

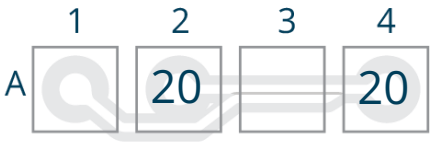
All steps are performed at room temperature unless specified otherwise.
To allow successful binding of primary and secondary antibodies, the antibody solution is perfused through the OrganoPlate® during antibody incubation steps. Perfusion can be created by placing the OrganoPlate® on a regular rocker platform and having it switch sides regularly. Use a small angle and a low switching interval (i.e. 5° angle, 2-5 min interval). Alternatively, flow can be induced by placing the OrganoPlate® under an angle by positioning one end on top of an object, e.g. a second 384 well plate (see figure on the right) and regularly switching sides manually.

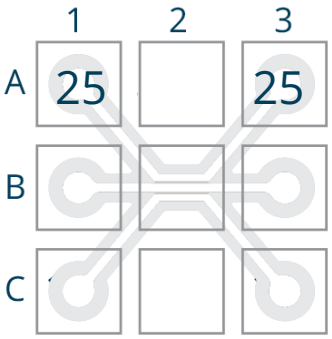


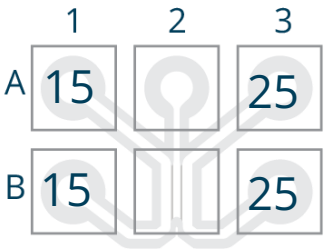
6. Prepare permeabilization buffer, blocking solution, and washing solution (see materials list)
7. Wash chips 1x 5 min with washing solution according to the volume scheme below.
    a. OrganoPlate® 2-lane: 50 µL to ECM inlet, 100 µL to the perfusion channel inlet, 50 µL to the perfusion channel outlet
    b. OrganoPlate® 3-lane 40: 100 µL to top perfusion channel inlet, 50 µL to all other inlets and outlets
    c. OrganoPlate® 3-lane 64: 100 µL to right perfusion channel inlet, 50 µL to all other inlets and outlets



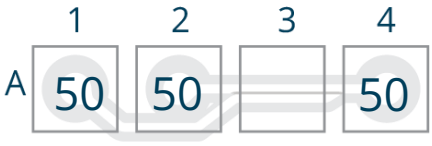
8. Aspirate the washing buffer and permeabilize cells for 10 minutes with permeabilization buffer according to the volume scheme shown in step 7
9. Aspirate permeabilization buffer and wash chips 1x 5 min with washing solution using the volume scheme shown in step 7
10. Aspirate washing buffer and block cells for 30-45 min with blocking solution according to the volume scheme shown in step 7
11. Meanwhile, prepare primary antibody in blocking solution in the appropriate dilutions
    a. OrganoPlate® 2-lane: 40 µL antibody per chip
    b. OrganoPlate® 3-lane 40/64: 80 µL antibody per chip
12. Aspirate blocking solution and add primary antibody solution according to the volume scheme below

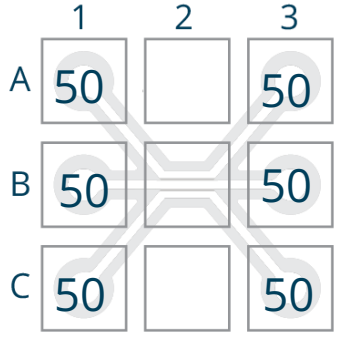


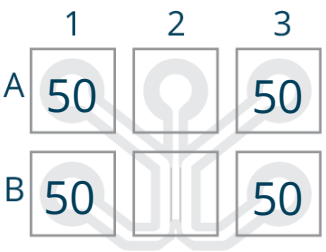




13. Incubate primary antibody on the rocker platform at RT (or at 4°C overnight if desired)
    a. For tubular cultures, 1-2 hours of incubation on the rocker platform at RT is sufficient
    b. For cultures of cells in gel, longer incubation times may be required (see page 4)
14. Meanwhile, prepare secondary antibody in blocking solution in the appropriate dilutions
    a. OrganoPlate® 2-lane: 40 µL antibody per chip
    b. OrganoPlate® 3-lane 40/64: 80 µL antibody per chip
15. Aspirate primary antibody solution and wash chips 2x (3 min each) with washing solution using the volume scheme shown in step 7
16. Aspirate washing solution and add secondary antibody solution according to the volume scheme shown in step 12
17. Incubate secondary antibody in the dark on the rocker platform at RT
    a. For tubular cultures, 30 minutes of incubation on the rocker platform at RT is sufficient
    b. For cultures of cells in gel, longer incubation times may be required (see page 4)
18. Aspirate secondary antibody solution and wash chips 2x (3 min each) with washing solution using the volume scheme described in step 7
19. If desired, stain cells with direct stains (e.g. Hoechst or ActinRed), using manufacturer's instructions
    a. Use stains for fixed cells
    b. Use the volume scheme shown in step 12
    c. Incubate stains on the rocker at RT at least 15 min for cells grown as tubes against the ECM gel and at least 30 min for cells embedded in ECM gel (see page 4)
20. Wash chips 1x (5 min) with PBS according to the volume scheme shown in step 7
21. Aspirate all wells and add 50 µL of PBS to all wells







22. Proceed to microscopy or store the plate
    a. Perform microscopy within one week after staining for optimal results
    b. Imaging can be performed on all standard fluorescent microscopes
    c. Store plate by sealing edges with Parafilm® and wrapping it in aluminum foil. Store at RT for up to two weeks

5. Troubleshooting

Insufficient staining

Depending on the setup of the culture, some cultures may require longer staining procedures to obtain optimal results, for example neuronal networks grown in Matrigel® in the OrganoPlate® 2-lane. For these types of cultures, the following measures can be taken to improve antibody staining:

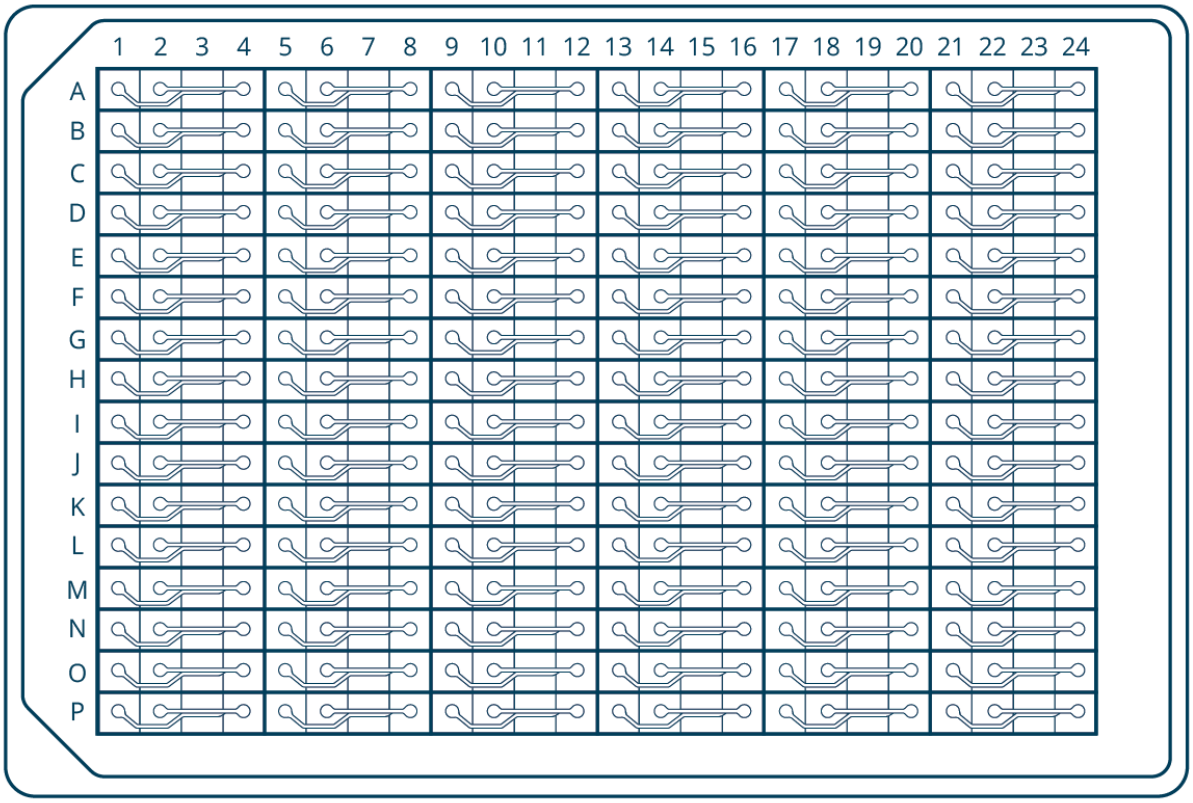
- Addition of 1% Triton to the primary and secondary antibody solution (steps 11 & 14)
- Prolongation of incubation times
    - Incubate primary antibody for 2 days, on the rocker, at RT (step 13b)
    - Incubate secondary antibody for 2 days, on the rocker, at RT (step 17b)
    - Incubate nuclear stains for 1 day, on the rocker, at RT (step 19c)

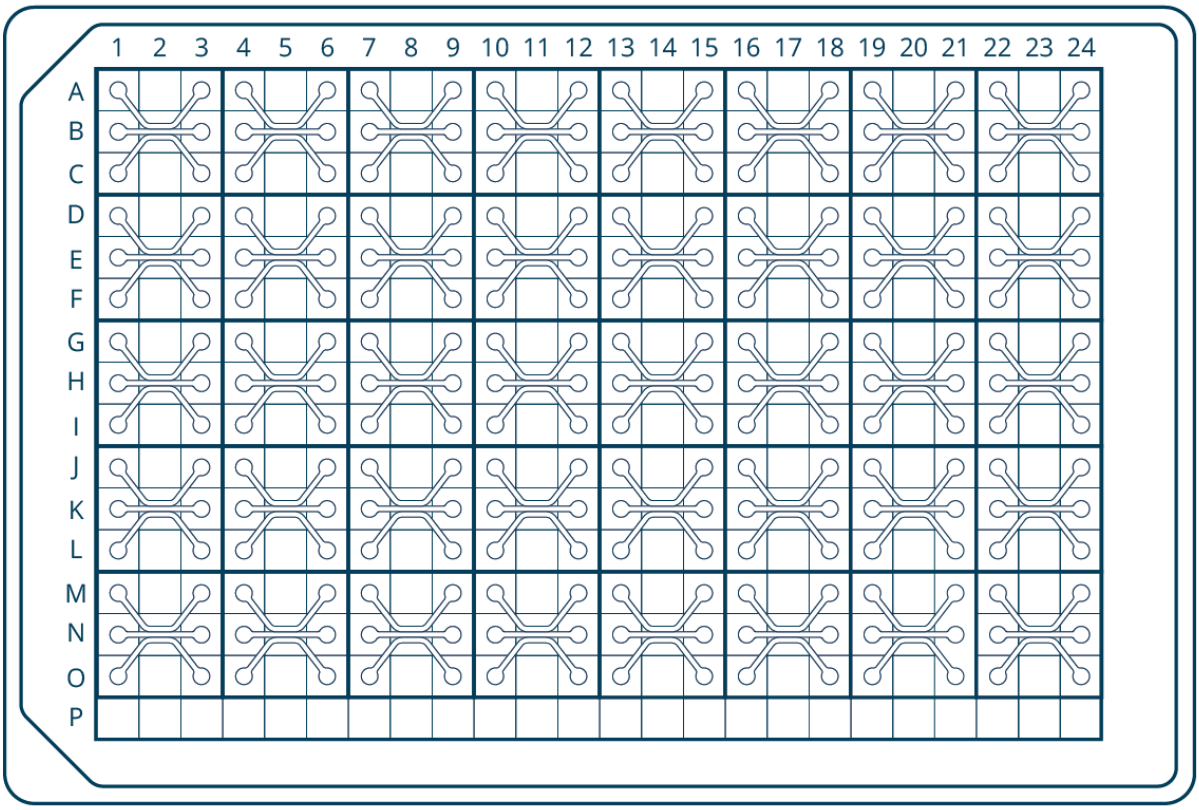
Note: because these measures prolong the staining procedure significantly, use of aseptic technique is recommended to avoid growth of bacteria in the cultures. After fixation, work in a sterile cabinet and use sterile solutions (prepare solutions using only sterile components or filter buffers after preparation).

Plate storage

If the plate is not immediately used after fixation for antibody incubation or for imaging after the immunostaining is finished, store the plate either at room temperature or at 4°C in the fridge. Never store the OrganoPlate® in the freezer or freeze existing cultures, as this will cause the glass bottom and the microfluidics to delaminate from the plate and liquid will leak out from the channels.

Plates layout

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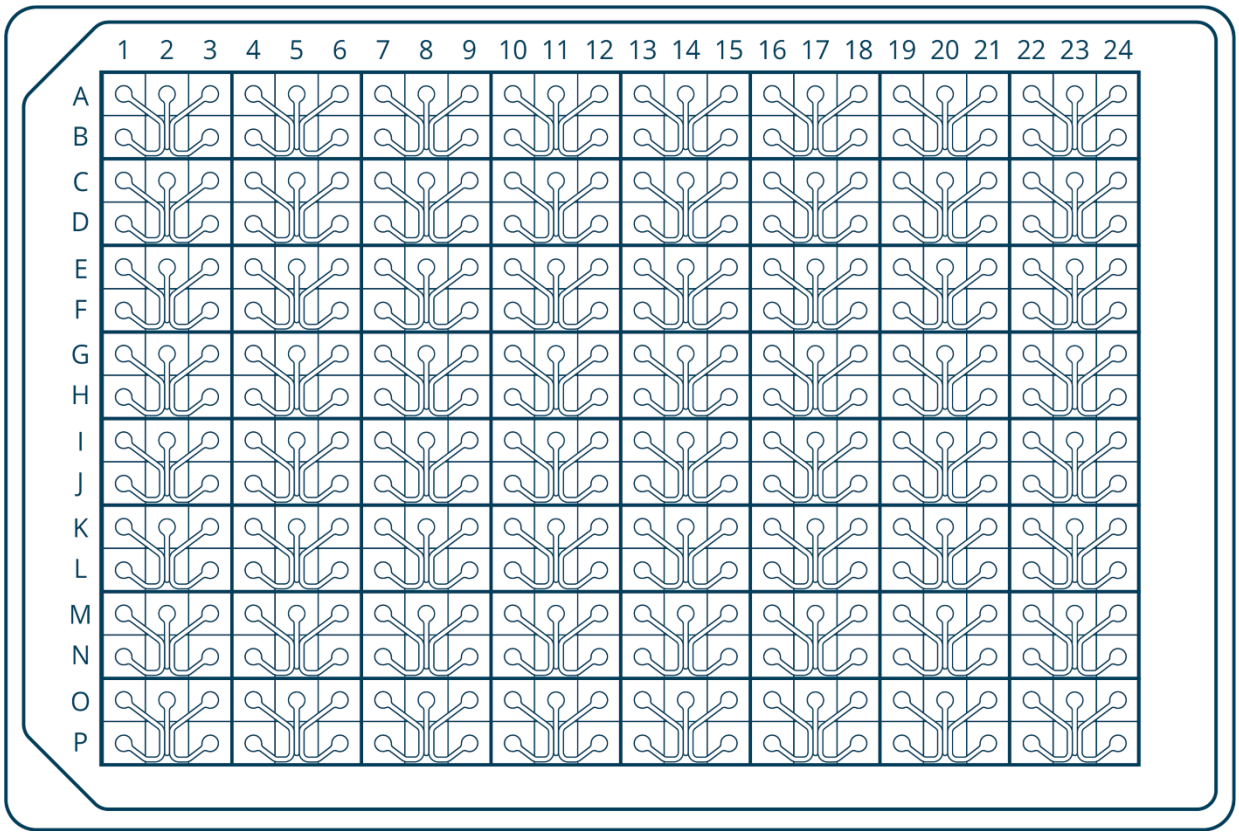
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MIMETAS Product List

Cat. No.	Product Name
MI-OR-CC-01	OrganoReady® Colon Caco-2 3-lane 40
MI-OR-CC-02	OrganoReady® Colon Caco-2 3-lane 64
MI-OR-BV-01	OrganoReady® Blood Vessel HUVEC 3-lane 40
MI-OR-BV-02	OrganoReady® Blood Vessel HUVEC 3-lane 64
MI-OR-AN-01	OrganoReady® Angiogenesis HUVEC 3-lane 64
MI-OR-HB-01	OrganoReady® BBB HBMEC 3-lane 40
MI-OR-HB-02	OrganoReady® BBB HBMEC 3-lane 64
MI-OR-VB-01	OrganoReady® Vascular Bed HUVEC
MI-OR-CO-CU -01	OrganoReady® Collagen 3-lane 40
MI-OR-CO-CU-02	OrganoReady® Collagen 3-lane 64
9605-400-B	OrganoPlate® 2-lane 96
4004-400-B	OrganoPlate® 3-lane 40
6405-400-B	OrganoPlate® 3-lane 64
6401-400-B	OrganoPlate® Graft
MI-OFPR-S	OrganoPlate® S
MI-OFPR-L	OrganoPlate® L
MI-OT-VP2	OrganoPlate® Standard package

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