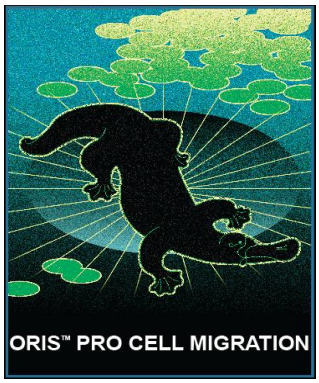
PLATYPUS TECHNOLOGIES



Oris™ Pro Cell Migration Assay Tissue Culture Treated

Product No.: PROCMA1 & PROCMA5

96-well, 2-D Assay for Investigating
Cell Migration of Adherent Cell Lines

PROTOCOL & INSTRUCTIONS

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ORIS™ PRO CELL MIGRATION ASSAY

I. INTRODUCTION

The Oris™ Pro Cell Migration Assay is a reproducible, sensitive, and flexible assay that can be used to monitor cell migration. Formatted for a 96-well plate, the assay uses a non-toxic biocompatible gel (BCG) to form a cell-free zone on cell culture surfaces. After seeding cells into the 96-well plate, the BCG dissolves permitting cells to migrate into the well centers (see Figure 1). The Oris™ Pro Cell Migration Assay enables the use of automated liquid handling equipment for cell seeding and allows for unlimited access to wells from cell seeding through data readout. The Oris™ Pro Cell Migration Assay is designed to be used with any commercially available stain or labeling technique. Researchers can capture and quantify real-time cell migration data using inverted microscopes, High Content Screening (HCS) and High Content Imaging (HCI) instruments.

The Oris™ Pro Cell Migration Assay system has been designed for use with adherent cell cultures. This assay has been successfully used with HT-1080 and MDA-MB-231 cell lines, and Human Umbilical Vein Endothelial Cells (HUVECs).

Using the Oris™ Pro Cell Migration Assay offers the following features & benefits:

- **Increased Productivity -** Treat cells with multiple fluorescent probes, labels, or colorimetric stains for multi-parametric measurements with inverted microscopes, High Content Screening (HCS) and High Content Imaging (HCI) instruments.
- **Less Handling -** Realize reduced assay handling time with an assay format in which a centrally placed biocompatible gel automatically dissolves to reveal a detection zone.
- **Automation-Friendly Design -** Utilize automated liquid handling equipment for fast set-up of high throughput, 96-well assays.
- **Reproducible Results -** Achieve well-to-well CV's ≤12% and generate robust Z' factors suitable for compound screening.
- **Real-Time Analysis -** Experience unlimited access to cells, cell morphology and cell movement throughout your experiment.

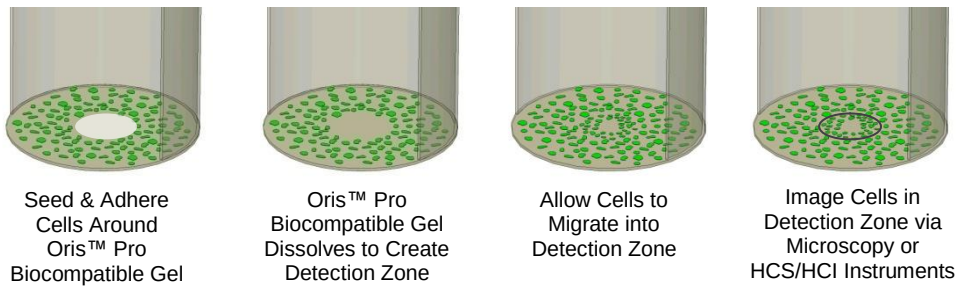


Figure 1. Schematic of Oris™ Pro Cell Migration Assay

II. ORIS™ PRO PLATE DIMENSIONS

Diameter of Well - Bottom	6.58 mm
Diameter of Well - Top	6.96 mm
Well Volume	392 µL
Suggested Media Volume per Well	100 µL
Plate Height	14.4 mm
Plate Height with Lid	17 mm
Offset of Wells (A-1 location, X)	14.38 mm
Offset of Wells (A-1 location, Y)	11.24 mm
Distance between Wells	9.0 mm
Well Depth	10.9 mm
Thickness of Well Bottom	190 µm +/- 10 µm
Storage Conditions	15 – 30°C

NOTE: For Research Use Only.

Important: Read Instructions Before Performing any Oris™ Pro Assay.

III. MATERIALS PROVIDED

Product No.: PROCMA1
Oris™ Pro 96-well, Tissue Culture Treated Plate (1)

Product No.: PROCMA5
Oris™ Pro 96-well, Tissue Culture Treated Plates (5)

IV. MATERIALS REQUIRED

- Biological Cells
- Sterile PBS (containing both Ca^{++} and Mg^{++})
- Complete Cell Culture Growth Medium (containing serum)
- Pipette or Multi-Channel Pipette with Sterile Pipette Tips
- Trypsin or Cell Scraper
- Inverted Microscope (optional)
- High Content Screening, High Content Imaging System (optional)
- Cell Culture Labeling Medium, eg., phenol red-free/serum-free media (optional)
- Cell Labeling Fluorescent Agent, eg., CellTracker™ Green, DAPI, TRITC-Phalloidin (optional) - *required if performing assay readout via fluorescence analysis.*

V. CELL MIGRATION ASSAY PROTOCOL

The following steps should be performed in a biological hood using aseptic technique to prevent contamination.

1. If performing a kinetic analysis of cell migration, pre-label cells with a fluorescent stain now. Please refer to Appendix II for a discussion of suggested staining techniques.

2. Collect cells and prepare a suspension that is at the optimal seeding concentration.

   First Time Users: The optimum seeding density of cells must be determined as an integral part of the design of the cell migration assay. Please refer to Appendix I for a discussion of this process.

3. Pipette 100 µL of suspended cells into each test well.

NOTE: If you plan to fix and label test cells at the conclusion of the cell migration, you will need additional wells (or an additional Oris™ Pro plate) to serve as pre-migration reference wells.

NOTE: Place your seeded plate(s) into the incubator as soon as possible after cells have been seeded. Take care not to jostle the plate(s).

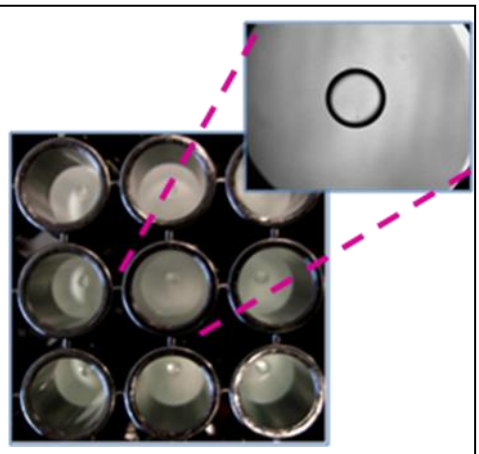


Figure 2. Biocompatible Gel in the Oris™ Pro Cell Migration Assay

4. Incubate the seeded plate(s) containing the Oris™ Pro Biocompatible Gel (see Figure 2) in a humidified chamber (37°C, 5% CO_2) for 1 to 4 hours (cell line dependent) to permit cell attachment.

5. Remove plate(s) from incubator.

NOTE: At this step, test compounds may be added directly to the well, or it may be preferable to first remove media and add fresh culture media containing test compounds to each well.

6. Capture pre-migration images of the Detection Zone (to be used as reference wells) according to the following options:

   Option I: If utilizing unlabeled cells or live, labeled cells (GFP-labeled, or a non-toxic fluorescent dye, such as CellTracker™ Green), use an inverted microscope or HCS/HCI instrument to capture pre-migration images of the Detection Zone formed in the wells.

   Option II: If utilizing fixed, labeled cells (TRITC-phalloidin, DAPI, etc), fix cells in the pre-migration reference wells. These cells can be labelled immediately or at the same time as the test cells. Use an inverted microscope or HCS/HCI instrument to capture pre-migration images of the Detection Zone formed in the wells.

7. Incubate plate in a humidified chamber (37°C, 5% CO_2) to permit cell migration. Cells may be examined by inverted microscope or other imaging instrument throughout the incubation period to monitor progression of migration, which will vary depending upon cell type and experimental design.

8. If performing an endpoint analysis of cell migration, stain cells with a fluorescent stain after sufficient migration has occurred. Refer to Appendices II and III for further information on fluorescence staining techniques of live and fixed cells, respectively.

9. Capture post-migration images of the Detection Zone using HCS/HCI instrumentation, or phase, bright-field, or fluorescence microscopy.

VI. DATA ACQUISITION

The readout of the Oris™ Pro Cell Migration Assay can be conducted at any time, thereby allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Pro Cell Migration Assay is designed to be used with any commercially available stain or labeling technique. Readout can be performed by using an inverted microscope or a High Content Screening (HCS) or High Content Imaging (HCI) instrument.

Microscope Analysis:

- Cell counting or image capture/analysis software, such as NIH ImageJ freeware, can be used.
- Note: Microscopy observations are possible using phase contrast, fluorescence or bright field microscopy.

Sample data using phase contrast microscopy (Figure 3). Wells were seeded with 30,000 HT-1080 cells (i.e., 100 µL of $3.0x10^5$ cells/mL) and incubated (37°C, 5% CO_2) for 2 hours. Upon removal of the plate from the incubator, phase contrast images were taken for pre-migration references. The plate was returned to the incubator for 24 hours to permit cell migration. At the end of the incubation, images of cell migration were captured using phase contrast microscopy. The images below illustrate representative data from pre-migration (t=0 hrs) and post-migration (t=24 hrs) wells (CV of 7% Δ migration and Z' = 0.5 for migration*). Scale bar = 500 µm.

* Reference:Zhang JH, Chung TD, Oldenburg KR, "*A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays*." *J Biomol Screen.* 1999; 4(2):67-73.

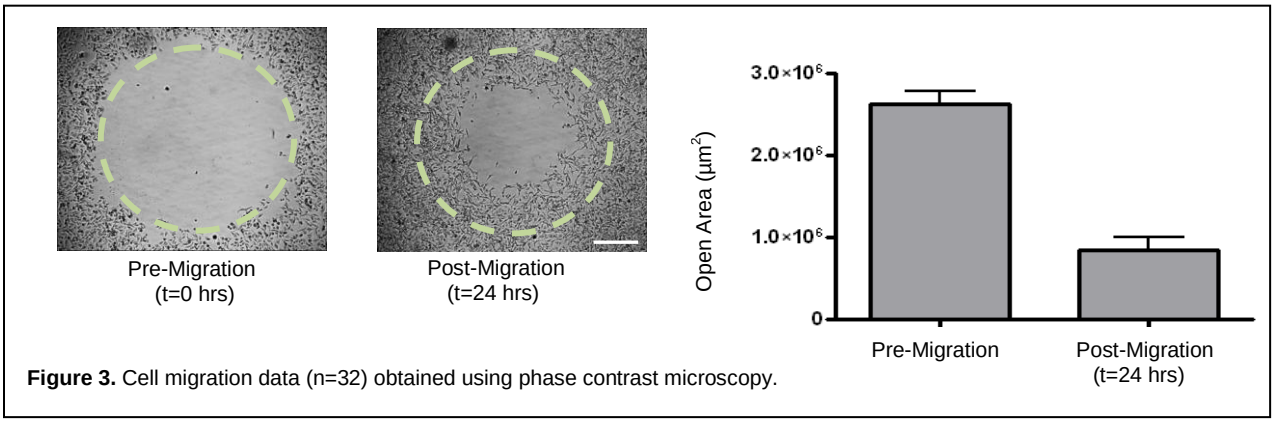


Figure 3. Cell migration data (n=32) obtained using phase contrast microscopy.

Sample data using fluorescence microscopy (Figure 4). Wells were seeded with 25,000 HUVECs (i.e., 100 µL of $2.5x10^5$ cells/mL) on two plates and incubated (37°C, 5% CO_2) for 2 hours. At the end of the incubation, one plate was removed from the incubator, fixed, and stained for F-actin (TRITC-phalloidin, pseudocolored green). The second plate was incubated for an additional 24 hours to permit cell migration. At the end of the migration, the second plate was fixed and stained for F-actin (TRITC-phalloidin, pseudocolored green). Images of cell migration were captured using fluorescence microscopy and the images below illustrate representative data from pre-migration (t=0 hrs) and post-migration (t=24 hrs) wells (CV of 5.7% Δ migration and Z' = 0.77 for migration). Scale bar = 500 µm.

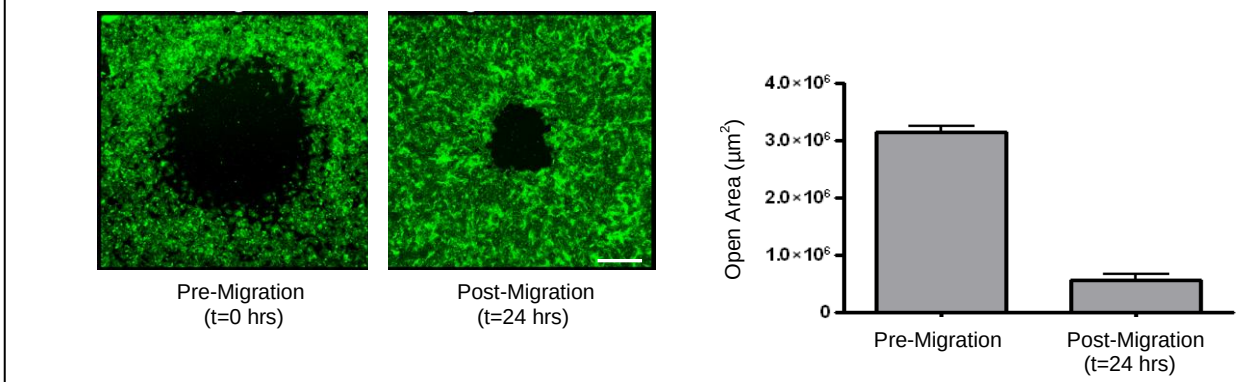
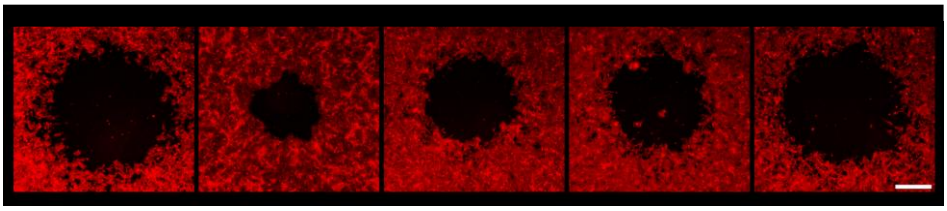


Figure 4. Cell migration data (n=32) obtained using fluorescence microscopy.

High Content Screening / High Content Imaging Analysis:

Sample data using BD Pathway™ 855 Bioimaging system (Figure 5). Wells were seeded with 25,000 HUVEC's (i.e., 100 µL of 2.5x10^5 cells/mL) on two plates and incubated (37°C, 5% CO_2) for 2 hours. At the end of the 2 hour incubation, one plate was removed from the incubator and cells were fixed with 0.25% glutaraldehyde. At this time, varying concentrations of the MEK inhibitor, UO126, were added to select wells of the second plate and incubated for an additional 24 hours to permit cell migration. At the end of the migration, the second plate was fixed with 0.25% glutaraldehyde. Both plates were stained using TRITC-phalloidin. The images below illustrate representative data from pre-migration (t=0 hrs) and post-migration (t=18 hrs) wells. Scale bar = 500 µm.



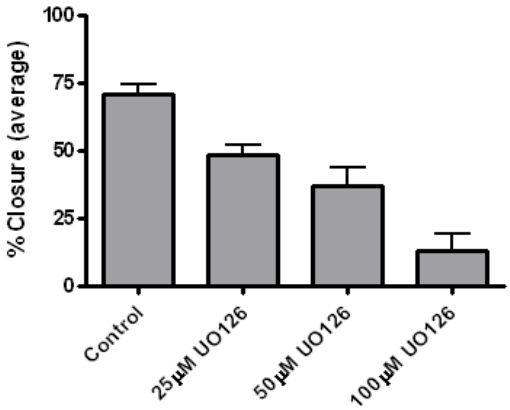


Figure 5. Cell migration data (n=4) obtained using BD Pathway™ 855 Bioimaging system.

BD Pathway™ 855 is a trademark of BD Biosciences.

VII. ORDERING INFORMATION

Product Name	Coating	Size	Detection Zone Format
Oris™ Pro Cell Migration Assays	Tissue Culture Treated	1-pack (PROCMA1) 5-pack (PROCMA5)	Biocompatible Gel
	Collagen I Coated	1-pack (PROCMACC1) 5-pack (PROCMACC5)	
Oris™ Cell Migration Assays	Tissue Culture Treated	1-pack (CMA1.101) 5-pack (CMA5.101)	Oris™ Cell Seeding Stoppers (pre-populated)
	Collagen I Coated	1-pack (CMACC1.101) 5-pack (CMACC5.101)	
	Fibronectin Coated	1-pack (CMAFN1.101) 5-pack (CMAFN5.101)	
	TriCoated	1-pack (CMATR1.101) 5-pack (CMATR5.101)	
Oris™ Cell Migration Assembly Kits	Universal (Tissue Culture Treated)	1-pack (CMAU101) 5-pack (CMAU505)	Oris™ Cell Seeding Stoppers (not pre-populated)
	Collagen I Coated	1-pack (CMAUCC1) 5-pack (CMAUCC5)	
	FLEX (Tissue Culture Treated)	4-pack (CMAUFL4)	
Oris™ Cell Invasion & Detection Assays	BME	1-pack (CIA101DE) 2-pack (CIA200DE)	Oris™ Cell Seeding Stoppers (not pre-populated)

VIII. TERMS & CONDITIONS

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PLATYPUS shall not be liable for injury or damages resulting from the use or misuse of any of its products.

APPENDIX I: Determining Optimal Cell Seeding Concentration

This procedure is intended to assist in determining the cell seeding density needed to achieve confluency of your cell line when using the Oris™ Pro Cell Migration Assay. The intended goal is to achieve 90-95% confluency of the monolayer surrounding the Oris™ Pro Biocompatible Gel without overgrowth.

1. A suggested starting point is to evaluate a range of three cell densities as shown below. The cell seeding area of the well with the Oris™ Pro Biocompatible Gel is ~0.3 cm^2. Based on the typical seeding density of your particular cell line, you can infer a different cell number for your first serial dilution and adjust the numbers below accordingly.
2. Prepare a log-phase culture of the cell line to be tested. Collect cells and determine the total number of cells present.
3. Pellet cells by centrifugation (1,000 x g). Prepare final concentrations of $4.0x10^5$, $3.0x10^5$, and $2.0x10^5$ cells/mL.
4. Dispense 100 µL of cell suspension per well into the 96-well plate to result in the following plate layout:

Column	1	2	3
Cells / well	40,000	30,000	20,000
Number of wells	8	8	8

5. Incubate the plate in a humidified chamber (37°C, 5% CO_2) for 1 - 4 hours (cell line dependent) to allow the cells to firmly attach and spread on the well surface.
6. Following cell attachment, use an inverted microscope to visually inspect each well to determine the minimum cell seeding concentration that yields a confluent monolayer at the perimeter of the Detection Zone (See Figures 3 and 4 for representative pre-migration Detection Zone images).

At this point, you have successfully determined the optimal cell seeding concentration to be used in Step 3 of the Cell Migration Assay Protocol.

APPENDIX II: Fluorescent Labeling Live Cell Options

This procedure is intended to assist in obtaining data from the Oris™ Pro Cell Migration Assay using various fluorescent labels.

The Oris™ Pro Cell Migration Assay has been designed to work with all types of fluorescent stains and staining techniques. The precise method for staining cells with fluorescent stains varies according to the nature of the individual stain. It is important to stain cells using a fluorescent reagent that uniformly stains cells. Probes affected by experimental conditions will increase variability of results and reduce correlation between fluorescence signal and cell migration. Please consult the manufacturer of your fluorescent stain for specific considerations.

The following is an example Fluorescent Staining Protocol to label live cells with CellTracker™ Green CMFDA:

a) To stain one fully-seeded 96-well plate, prepare a 10 mM stock solution of CellTracker™ Green (Molecular Probes/Invitrogen) by adding 10.8 µL of DMSO to a 50 µg vial. Prepare a 2.5 µM working solution of CellTracker™ Green by combining 4 µL of the 10mM stock of CellTracker™ Green with 16 mL of serum-free, phenol red-free cell culture medium.
b) Harvest cells via trypsinization and resuspend in FBS containing growth medium. Determine the total number of cells using a hemacytometer.
c) Centrifuge cells (1,000 x g) and resuspend pellet in 16 mL of pre-warmed, serum-free, phenol red-free medium containing 2.5 µM CellTracker™ Green in a sterile 50 ml conical tube and incubate for 30 minutes at 37 °C; gently swirl the tube every 10 minutes.
d) Centrifuge cells (1,000 x g) and resuspend pellet in 16 mL of pre-warmed, serum-free, phenol red-free medium without CellTracker™ Green and incubate for 30 minutes at 37 °C; gently swirl the tube every 10 minutes.
e) Centrifuge cells (1,000 x g) and resuspend them at the desired concentration in an appropriate amount of FBS containing complete growth medium. For example, in order to dispense 3.5×10^4 cells per well in 100 µl volume, adjust the concentration of the labeled cell suspension to 3.5×10^5 cells/mL.

NOTE: The final concentration of CellTracker™ Green may need to be adjusted to be optimal for your cell line (i.e., 1 – 10 µM CellTracker™ Green). It is suggested to run parallel assay wells using unlabeled cells in order to ascertain whether staining affects cell migration.

At this point, you have successfully labeled your cells to be used in Step 3 of the Cell Migration Assay Protocol.

APPENDIX III: Fluorescent Labeling Fixed Cell Options

This procedure is intended to assist in obtaining data from the Oris™ Pro Cell Migration Assay using various fluorescent labels.

The Oris™ Pro Cell Migration Assay has been designed to work with all types of fluorescent stains and staining techniques. The precise method for staining cells with fluorescent stains varies according to the nature of the individual stain. It is important to stain cells using a fluorescent reagent that uniformly stains cells. Probes affected by experimental conditions will increase variability of results and reduce correlation between fluorescence signal and cell migration. Please consult the manufacturer of your fluorescent stain for specific considerations.

The following is an example Fluorescent Staining Protocol to label fixed cells with TRITC-phalloidin (F-actin) and DAPI (nuclei):

a) To fix one fully-seeded 96-well plate, prepare 10 mL of fixative solution (e.g., 0.25% glutaraldehyde solution in PBS prepared from 8% glutaraldehyde solution (Electron Microscopy Sciences)).
b) Remove media and rinse wells with 100 µL of PBS.

NOTE: Take care not to disrupt cell monolayer or to wash cells into Detection Zone.

c) Remove PBS and add 100 µL of a fixative solution (0.25% glutaraldehyde solution in PBS) to each well and incubate at room temperature for 15 minutes.
d) Remove fixative solution and rinse wells with 100 µL of PBS.
e) Remove PBS and replace with 100 µL of a 1:50-1:100 dilution of TRITC-phalloidin (Sigma; prepared as 10 µM stock in methanol) in PBS containing 0.1% Triton X-100.
f) Incubate plate at room temperature for 45 minutes (protect from light).
g) Remove the TRITC-phalloidin and add 100 µL of a 1:4000 dilution of DAPI (ThermoScientific) in PBS.
h) Incubate plate at room temperature for 2-10 minutes (protect from light).
i) Remove DAPI stain and wash wells 2x for 5 minutes each with 200 µL of PBS.
j) Replace final wash with 200 µL of fresh PBS.

NOTE: This protocol outlines double-labeling of cells with a cytoskeletal and a nuclear stain. The protocol can be simplified if only one stain is used. Substitutions or additional cytostaining or immunostaining may be performed using non-overlapping fluorophores and by utilizing the appropriate filters with your imaging equipment.

At this point, you have successfully fixed and labeled your cells and can proceed with Step 9 of the Cell Migration Assay Protocol.