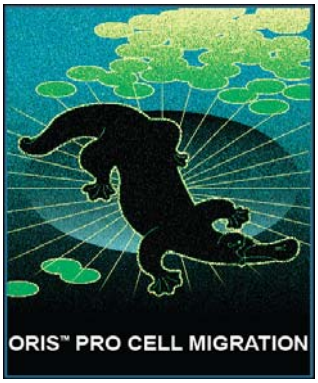

Oris™ Pro 384 Cell Migration Assay Collagen I Coated

Product No.: PRO384CMACC1 & PRO384CMACC5

384-well, 2-D Assay for Investigating
Cell Migration of Adherent Cell Lines on Collagen I

PROTOCOL & INSTRUCTIONS

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ORIS™ PRO 384 CELL MIGRATION ASSAY
COLLAGEN I COATED

I. INTRODUCTION

The Oris™ Pro 384 Cell Migration Assay – Collagen I Coated is a reproducible, sensitive, and flexible assay that can be used to monitor cell migration. Formatted in a 384-well plate, the assay uses a non-toxic biocompatible gel (BCG) to form a cell-free zone. After seeding cells into the 384-well plate, the BCG self-dissolves permitting cells to migrate into the well centers (see Figure 1). The Oris™ Pro 384 Cell Migration Assay – Collagen I Coated 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 384 Cell Migration Assay – Collagen I Coated is designed to be used with any commercially available stain or labeling technique. Researchers can capture and quantify real-time and endpoint cell migration data using inverted microscopes, High Content Screening (HCS) and High Content Imaging (HCI) instruments.

The Oris™ Pro 384 Cell Migration Assay – Collagen I Coated system has been designed for use with adherent cell cultures. This assay has been successfully used with HT-1080 and primary Human Umbilical Vein Endothelial Cells (HUVECs).

Using the Oris™ Pro 384 Cell Migration Assay offers the following benefits:

- **Enhance Efficiency –** Screen more compounds in a fully automatable 384-well format.
- **Increase Reliability –** Obtain robust and reproducible data from high content imaging / high content screening (HCI/HCS) instrumentation.
- **Generate More Useful Data –** Use multiplexed staining to simultaneously measure cell movement, morphology and phenotypic changes.

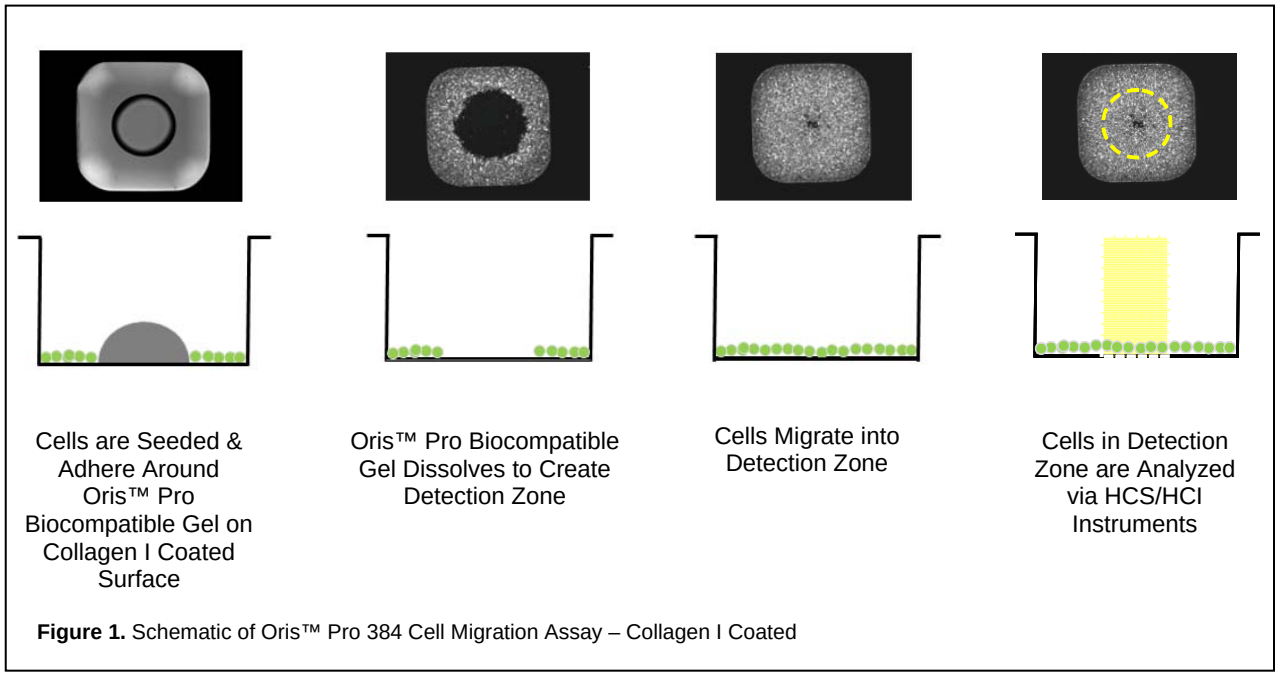

Figure 1. Schematic of Oris™ Pro 384 Cell Migration Assay – Collagen I Coated

II. ORIS™ PRO 384 PLATE DIMENSIONS

Well Diameter - Bottom	3.3 mm
Well Diameter - Top	3.7 mm
Well Volume	138 µL
Suggested Media Volume per Well	20 µL
Plate Height	14.4 mm
Well Offset (A-1 location, X)	12.13 mm
Well Offset (A-1 location, Y)	8.99 mm
Distance between Wells	4.5 mm
Well Depth	11.5 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.: PRO384CMACC1
Oris™ Pro 384-well, Collagen I Coated Plate (1)

Product No.: PRO384CMACC5
Oris™ Pro 384-well, Collagen I Coated 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 is critical and 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 20 µL of suspended cells into each test well.

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

   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 384 Collagen I Coated plate) to serve as pre-migration reference wells. If you are using multiple cell lines within a single assay plate, each cell line should have its own pre-migration reference wells in order to determine the initial size of the Detection Zone.

4. Incubate the seeded plate(s) 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 media may be removed and fresh culture media containing test compounds may be added 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 labeled 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.
   Option III: Using a drug known to block cell migration (such as Cytochalasin D), treat cells in the pre-migration reference wells at a concentration that will completely arrest cell migration, and obtain pre-migration images.

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 Appendix II for further information on fluorescence staining techniques of fixed cells.

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 384 Cell Migration Assay – Collagen I Coated can be conducted at any time, thereby allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Pro 384 Cell Migration Assay – Collagen I Coated 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.

Sample data using fluorescence microscopy (Figure 2). Human Umbilical Vein Endothelial Cells **(**HUVECs) were seeded at 4,500 cells/well into an Oris™ Pro 384 Collagen I Coated plate. A dose-response titration was performed using the RAF/MEK/ERK pathway inhibitor, Sorafenib. Cells were treated for 16 hours, fixed, and stained for F-actin using TRITC-phalloidin. Images were acquired using an inverted microscope and a dose-response curve was generated. The images below illustrate representative wells of BCG, a pre-migration control, and post-migration (t=16 hrs) in the presence of different concentrations of Sorafenib (Z'= 0.68 for migration*; n=4 wells/condition). The IC_{50} was calculated to be 21.5 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.

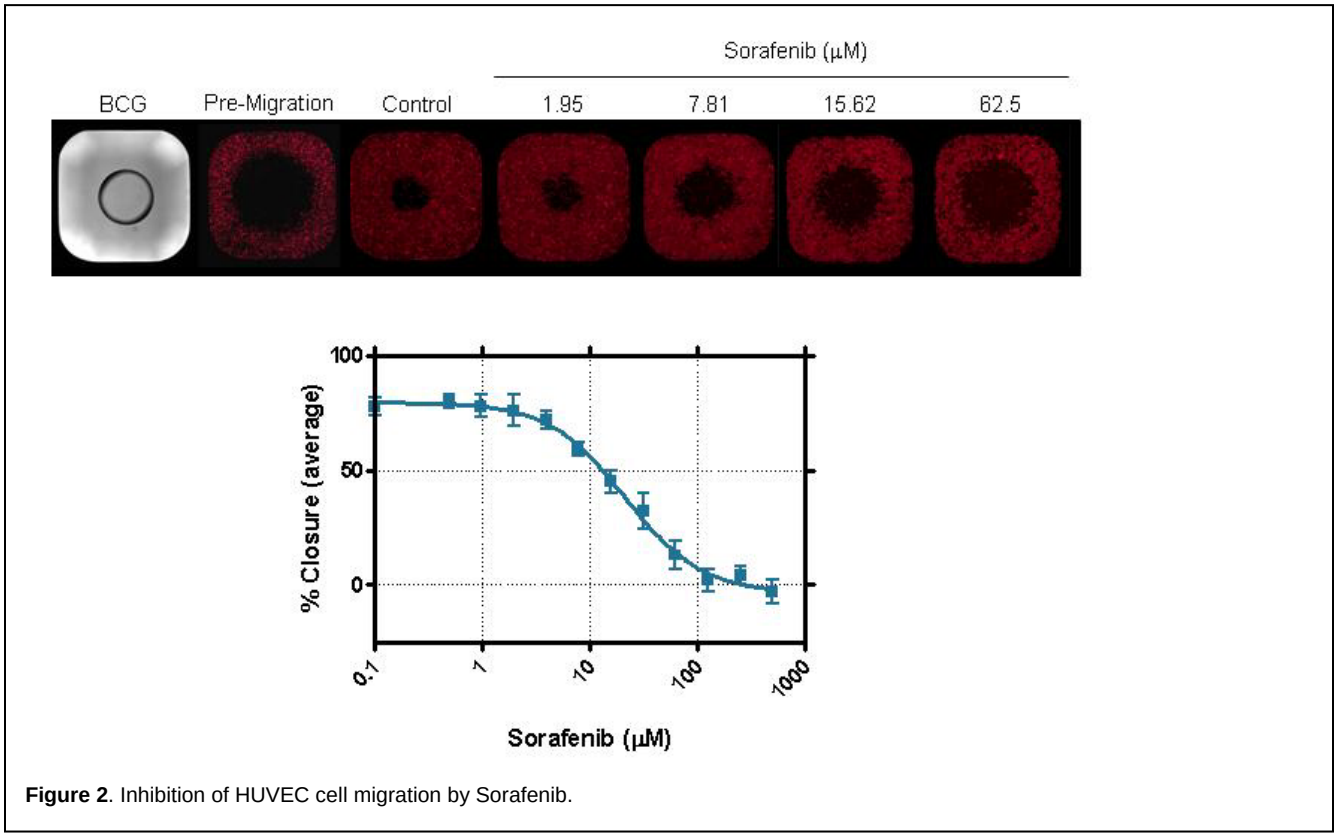

Figure 2. Inhibition of HUVEC cell migration by Sorafenib.

VII. ORDERING INFORMATION

The Oris™ Pro 384 Cell Migration Assay – Collagen I Coated is available in 1-packs as Product No. PRO384CMACC1 and in 5-packs as Product No. PRO384CMACC5. Please contact Platypus Technologies to discuss bulk orders.

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 384 Cell Migration Assay – Collagen I Coated. The intended goal is to achieve 95-100% 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 ~5 mm^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 $3.25x10^5$, $2.5x10^5$, and $1.75x10^5$ cells/mL.
4. Dispense 20 µL of cell suspension per well into the 384-well plate to result in the following plate layout:

Column	1	2	3
Cells / Well	6,500	5,000	3,500
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, while still providing a cell-free Detection Zone.

At this point, you have successfully determined the optimal cell seeding concentration to be used in Step 2 of the Cell Migration Assay Protocol.

APPENDIX II: Fixation and Fluorescent Labeling of Cells

This procedure is intended to assist in obtaining data from the Oris™ Pro 384 Cell Migration Assay using various fluorescent labels.

The Oris™ Pro 384 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 384-well plate, prepare fixative solution (e.g., 0.5% glutaraldehyde solution in PBS prepared from 8% glutaraldehyde solution (Electron Microscopy Sciences)).
b) Add a volume of fixative solution (0.5% glutaraldehyde in PBS) equivalent to volume of existing media to each well (without media removal) and incubate at room temperature for 15 minutes.
c) Remove all liquid from wells and rinse wells with 100 µL of PBS.
d) Remove PBS and replace with 25 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.
e) Incubate plate at room temperature for 45 minutes (protect from light).
f) Remove the TRITC-phalloidin and add 25 µL of a 1:4000 dilution of DAPI (ThermoScientific) in PBS.
g) Incubate plate at room temperature for 2-10 minutes (protect from light).
h) Remove DAPI stain and wash wells 2x for 5 minutes each with 100 µL of PBS.
i) Replace final wash with 100 µL of fresh PBS.

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

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.