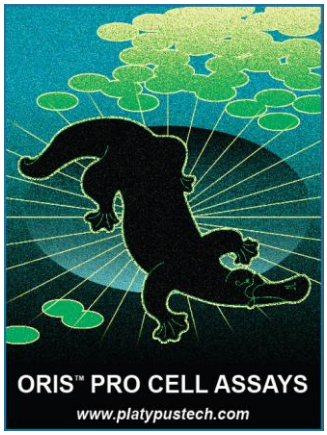

Oris™ Pro 96-well Invasion Assay

Product No.: PROIA1, PROIA3, PROIAPLUS1 & PROIAPLUS3

96-well, Assay for Investigating Cell Invasion of Adherent Cell Lines through Collagen I

With newly formulated Oris™ Pro Collagen I Overlay solution to enhance gelling over a larger concentration range.

PROTOCOL & INSTRUCTIONS

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PLATYPUS TECHNOLOGIES

ORIS™ PRO 96-WELL INVASION ASSAY

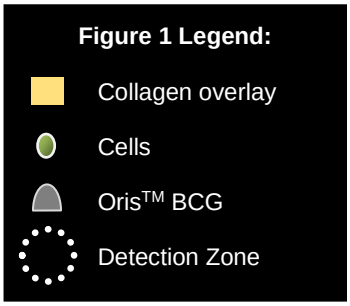
I. INTRODUCTION

The Oris™ Pro 96-well Invasion Assay is a reproducible, sensitive, and flexible assay that can be used to monitor cell invasion. The assay uses a non-toxic biocompatible gel (BCG) to form a centrally located and temporary cell-free zone on culture surfaces. Cells seeded into the 96-well plate attach in a monolayer around the BCG. The BCG dissolves to reveal a Detection Zone. A Collagen I Overlay is then added to create a 3-D extracellular matrix (ECM) for cell invasion. Cell invasion into the Detection Zone can be assessed by performing a Z-stack analysis. The Oris™ Pro 96-well Invasion Assay enables the use of automated liquid handling equipment for cell seeding and unrestricted access to wells from cell seeding through data readout. The Oris™ Pro 96-well Invasion Assay is designed to be used with commercially available stains and labelling techniques. Researchers can capture and quantify cell invasion in real time using inverted microscopes, high content screening (HCS) and high content imaging (HCI) instruments.

The Oris™ Pro 96-well Invasion Assay has been designed for use with adherent cell cultures. This assay has been validated with HT-1080 and MDA-MB-231 cell lines.

Features & benefits:

- **Versatile -** Treat cells with multiple fluorescent probes, labels, or colorimetric stains for multi-parametric measurements.
- **Decreased Handling -** Reduce assay handling time with an assay format where cells are seeded around a centrally placed BCG that dissolves to reveal a cell-free detection zone.
- **Reproducible Results -** Achieve high well-to-well consistency and robust Z' factors suitable for compound library screening.
- **Real-Time Analysis -** Unlimited access to wells and the ability to monitor cell morphology and movement throughout your experiment.



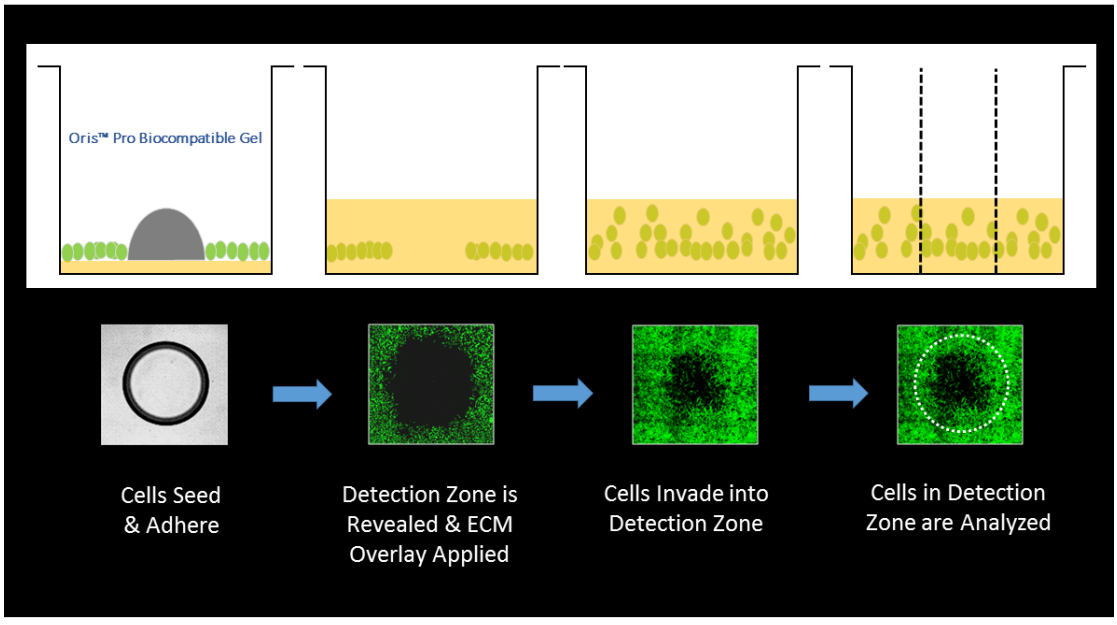


Figure 1: Assay Overview

Cells are seeded and allowed to adhere in an annular monolayer surrounding the BCG. Culture medium is removed and a collagen I overlay matrix is added. After collagen polymerizes an overlay of culture medium ± treatment is applied and cells invade into the Detection Zone. Invasion is imaged and analyzed using a microscope or High Content Imager.

II. ORIS™ PRO PLATE SPECIFICATIONS

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 %
Well Coating Material	Collagen I, Rat Tail
Storage Conditions	15 – 30°C

NOTE: For Research Use Only.

Important: Read Instructions before Performing an Oris™ Pro 96-well Invasion Assay.

III. MATERIALS PROVIDED

NOTE: Oris™ Pro Collagen I Overlay *solution has been newly formulated to increase stability under storage conditions and to enhance gelling over a larger concentration range.*

Product No.: PROIA1

Component	Quantity	Storage
Oris™ Pro Collagen I Coated, 96-well Plate	1	(15 – 30°C)
Oris™ Collagen I (Rat Tail), 5 mg/mL *	2 mL	Refrigerate (4°C)

Product No.: PROIA3

Component	Quantity	Storage
Oris™ Pro Collagen I Coated, 96-well Plates	3	(15 – 30°C)
Oris™ Collagen I (Rat Tail), 5 mg/mL *	3 x 2 mL	Refrigerate (4°C)

Product No.: PROIAPLUS1

Component	Quantity	Storage
Oris™ Pro Collagen I Coated, 96-well Plate	1	(15 – 30°C)
Oris™ Collagen I (Rat Tail), 5 mg/mL *	2 x 2 mL	Refrigerate (4°C)

Product No.: PROIAPLUS3

Component	Quantity	Storage
Oris™ Pro Collagen I Coated, 96-well Plates	3	(15 – 30°C)
Oris™ Collagen I (Rat Tail), 5 mg/mL *	6 x 2 mL	Refrigerate (4°C)

* Oris™ Collagen I (Rat Tail) must be stored at 4°C and used within 6 months of receipt. ***Do not freeze.***

IV.

MATERIALS REQUIRED

- Cells (Adherent)
- 1N Sodium Hydroxide
- Sterile 10X PBS
- Deionized Sterile Water
- Complete Cell Culture Growth Medium (containing serum)
- Serum-Free Cell Culture Medium
- 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 medium (optional)
- Fluorescent Stain, eg., CellTracker™ Green, DAPI, TRITC-Phalloidin, Calcein AM (optional)
  - required if performing assay readout via fluorescence analysis.

V. PRECAUTIONS AND RECOMMENDATIONS

For Research Use Only. Not for use in diagnostic procedures.

Handling and Use of the *Oris™ Collagen I (Rat Tail) Reagent*:

- You many need to adjust the Collagen I concentration for your specific cell line and experimental conditions.
- A suggested starting concentration for the ***Oris™ Pro Collagen I Overlay*** is 2 mg/mL.

Experimental Conditions

- Please note that cell movement will likely occur in X, Y, and Z-axes. The degree of X, Y, and Z movement will vary for different cell lines.

Recommendations for 10X PBS Buffer:

- When 10X PBS is refrigerated, sedimentation may occur due to the high salt concentration. If sediment forms, warm the PBS in a water bath (37°C) to completely dissolve any sediment prior to use.

Technical Resources:

- Application Notes, Technical Memos, and Literature References for Oris™ and Oris™ Pro Cell-based Assays are accessible from our website at: http://www.platypustech.com/applicationnotes.html

VI. INVASION ASSAY PROTOCOL

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

Pre-label cells: If performing a kinetic analysis of cell invasion, pre-label cells with a fluorescent live stain now (e.g. Calcein AM, Cell Tracker™ Green). Appendix II discusses suggested staining techniques.

1. 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 invasion assay. Appendix I discusses this process.

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

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

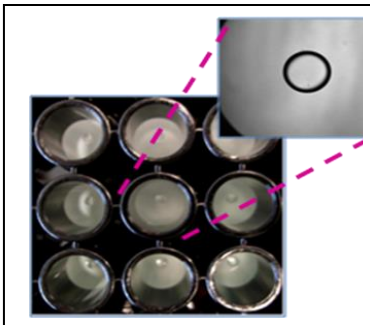


Figure 2. Biocompatible Gel in the Oris™ Pro 96-well Invasion Assay

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

3. Incubate the seeded plate(s) containing the Oris™ Pro Biocompatible Gel (see Figure 2) in a humidified chamber (37°C, 5% CO_2) for 30 minutes to 2 hours (cell line dependent) to permit cell attachment.

4. Remove plate(s) from incubator.

5. Hold the plate at an angle (~30°) and remove all cell culture medium with a pipette.

   - **Optional:** *gently* wash wells with 100 µL of serum-free medium (or sterile PBS) to remove any unattached cells.

6. Prepare 5.0 mL of an appropriate concentration of the **Oris™ Pro Collagen I Overlay** solution, using the following components:

NOTE: Oris™ Pro Collagen I Overlay solution has been formulated to increase stability under storage conditions and to enhance gelling over a larger concentration range.

10X PBS (sterile)
1N NaOH (sterile)
Deionized water (sterile)
Oris™ Collagen I (Rat Tail) (5 mg/mL)

Calculate the volume of Oris™ Collagen I (Rat Tail) needed to make the desired concentration of the **Oris™ Pro Collagen I Overlay** solution. Calculate the volume of sodium hydroxide needed to neutralize the collagen where 0.018 mL of 1N NaOH is required for every 1 mL of 5 mg/mL Oris™ Collagen I (Rat Tail) used. Appropriate volumes of 10X PBS and deionized water are used to prepare the **Oris™ Pro Collagen I Overlay** solution to a final 1X PBS solution.

IMPORTANT: Prior to/during use, keep the *supplied* Oris™ Collagen I (Rat Tail) and the *prepared* **Oris™ Pro Collagen I Overlay** solution on ice. In addition, the use of chilled pipette tips/reservoirs can be beneficial.

On ice, combine the water, 10X PBS, and 1N NaOH. Next, add the Oris™ Collagen I (Rat Tail) to achieve the desired concentration of the **Oris™ Pro Collagen I Overlay** solution. Mix by gently pipetting multiple times.

The following example provides volumes for 5 mL of a 2.0 mg/mL **Oris™ Pro Collagen I Overlay** solution:

2.464 mL deionized water
0.5 mL 10X PBS buffer
0.036 mL 1N NaOH
2 mL Oris™ Collagen I (Rat Tail) (5mg/mL)
5.0 mL total volume

NOTE: It is important to check the pH of the **Oris™ Pro Collagen I Overlay** solution prior to adding it to the wells. The pH should be between 6.5 – 7.5 to ensure that the **Oris™ Pro Collagen I Overlay** polymerizes.

IMPORTANT: Place plate on ice during addition of the **Oris™ Pro Collagen I Overlay** solution to reduce premature polymerization of the Collagen I.

7. Add 40 µL of the **Oris™ Pro Collagen I Overlay** solution to each well.

8. Incubate plate in a humidified chamber (37°C, 5% CO_2) for 1 hour to permit polymerization of the **Oris™ Pro Collagen I Overlay**. We recommend that you also incubate any remaining **Oris™ Pro Collagen I Overlay** solution to confirm that the solution polymerizes.

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

   Option I: If utilizing unlabeled cells or live-stain labeled cells (GFP-labeled, cell marker proteins, or a non-toxic fluorescent dye), use an inverted microscope or HCS/HCI instrument to capture pre-invasion images of the Detection Zone formed in the wells.

   Option II: If utilizing fixed, labeled cells (e.g. tetramethylrhodamine (TRITC), 4',6-diamidino-2-phenylindole (DAPI), etc), fix cells in the pre-invasion reference wells of a separate plate. These cells can be labeled immediately or at the same time as the cell of the separate test plate. Use an inverted microscope or HCS/HCI instrument to capture pre-invasion images of the Detection Zone formed in the wells.

10. Add 100 µL of complete medium (containing serum) on top of the **Oris™ Pro Collagen I Overlay**.

    - **Optional**: Invasion inhibitors or modulators may be added to the medium as pre-invasion control wells.

IMPORTANT: Use caution when adding medium so as not to disrupt the **Oris™ Pro Collagen I Overlay**.

11. Incubate plate in a humidified chamber (37°C, 5% CO_2) to permit cell invasion for 24 – 72 hours. Cells may be examined by inverted microscope or other imaging instruments throughout the incubation period to monitor the progression of invasion. The rate of invasion will vary depending upon cell type and experimental design.

12. If performing an endpoint analysis of cell invasion, stain cells with a fluorescent stain after sufficient invasion has occurred. Appendix II provides further information on staining techniques for fixed cells.

13. Capture post-invasion 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 96-well Invasion Assay can be conducted at any time, facilitating kinetic or endpoint assays. The Oris™ Pro 96-well Invasion Assay is designed to be used with any commercially available stain or labeling technique. Readout can be performed using an inverted microscope, a High Content Screening (HCS) or High Content Imaging (HCI) instruments.

Microscope Analysis:

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

High Content Screening / High Content Imaging Analysis:

Sample data obtained using BD Pathway™ 855 Bioimaging system (Figure 3). Two Oris™ Pro Collagen I coated plates 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 1 hour. After incubation, a 1.8 mg/mL collagen overlay was placed over the cells and both plates were incubated for an additional 1 hour to permit polymerization of the overlay. One plate was removed from the incubator and cells were fixed with 0.25% glutaraldehyde. Wells in the second plate received 100 µL medium containing 10% FBS and were incubated an additional 72 hours to permit cell invasion. At the end of the invasion, the second plate was fixed with 0.25% glutaraldehyde. Both plates were stained using TRITC-phalloidin and DAPI. The images below illustrate representative data from pre-invasion wells at t=0 hrs (A) and invasion wells at t=72hrs (B-E). Images captured at Z-intervals of 60 µm (C) and 150 µm (D) into the collagen overlay are also presented. The Z-stack composite (E) represents color-coded cells that correspond to invading cells at Z-intervals ≤ 60 µm. The diameter of the cell-free zone (Detection Zone) is ~2 mm.

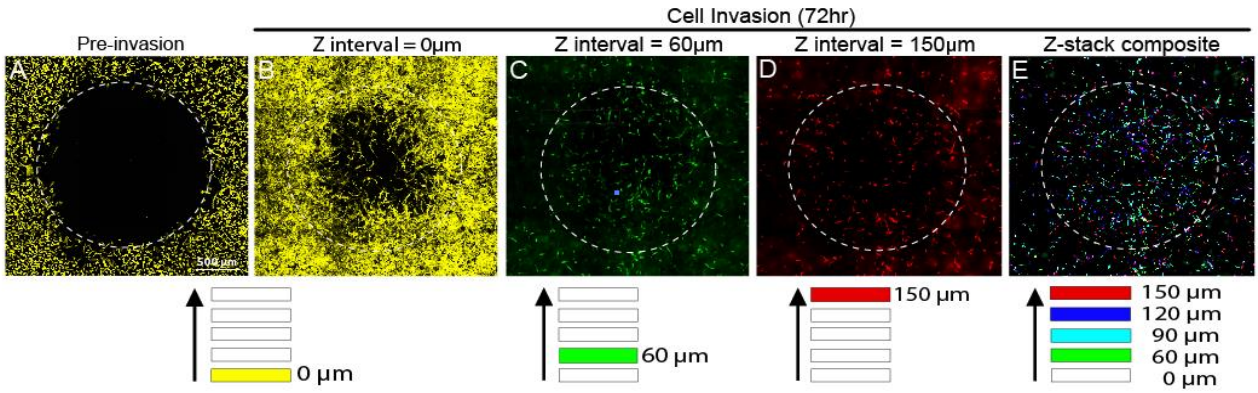


Figure 3. Cell invasion data (n=8/condition) obtained using BD Pathway™ 855 Bioimaging system.

BD Pathway™ 855 is a trademark of BD Biosciences.

VII. ORIS™ and ORIS™ PRO ASSAYS 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™ Pro 384 Cell Migration Assays	Tissue Culture Treated	5-pack (PRO384CMA5)	Biocompatible Gel
	Collagen I Coated	5-pack (PRO384CMACC5)	
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)
	FLEX (Tissue Culture Treated)	4-pack (CMAUFL4)	
Oris™ Pro 96-well Invasion Assays	Collagen I (low overlay conc.)	1-pack (PROIA1) 3-pack (PROIA3)	Biocompatible Gel
	Collagen I (high overlay conc.)	1-pack (PROIAPLUS1) 3-pack (PROIAPLUS3)	Biocompatible Gel

For a complete list of products, visit www.platypustech.com.
For technical assistance, contact Technical Support at 866.296.4455 or techsupport@platypustech.com.

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 for your cell line when using the Oris™ Pro 96-well Invasion Assay. The goal is to achieve 90 – 95% confluency of the monolayer surrounding the Oris™ Pro Biocompatible Gel.

1. A suggested starting point is to evaluate a range of three cell densities. 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 determine 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 30 minutes - 2 hours (cell line dependent) to allow the cells to firmly attach to the well surface.
6. Following cell attachment, use an inverted microscope to inspect each well to determine the minimum cell seeding concentration that yields a 90 – 95% confluent monolayer at the perimeter of the Detection Zone (See Figure 3 for representative pre-invasion Detection Zone images).

APPENDIX II: Fixation and Fluorescent Labeling of Cells

The Oris™ Pro 96-well Invasion 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. 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% EM-grade glutaraldehyde solution).
b) Remove medium and rinse wells with 100 µL of 1X PBS.

NOTE: Take care not to disrupt the **Oris™ Pro Collagen I Overlay.**

c) Remove PBS and add 100 µL of a fixative solution (final concentration of 0.25% glutaraldehyde solution in PBS) to each well on top of the collagen overlay gel. 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 from 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, rinse with PBS for 5 minutes, and add 100 µL of a 1:4000 dilution of DAPI (prepared from 1 mg/mL stock) in PBS.
h) Incubate plate at room temperature for 10-60 minutes (protect from light). Observe plate in 5 minute intervals and if needed allow additional time for complete staining.
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, additional cytostaining, or immunostaining may be performed using non-overlapping fluorophores and by utilizing the appropriate filters with your imaging equipment.