



Oris™ Pro Collagen I Cell Invasion Assay

Product No.: PROCIACC1 & PROCIACC2

96-well, 3-D Assay for Investigating Cell Invasion
of Adherent Cell Lines on Collagen I

PROTOCOL & INSTRUCTIONS

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Bringing Science to the Surface™

ORIS™ PRO COLLAGEN I CELL INVASION ASSAY

I. INTRODUCTION

The Oris™ Pro Collagen I Cell Invasion Assay is a reproducible, sensitive, and flexible assay that can be used to monitor cell invasion. The Oris™ Pro Cell Invasion Assay uses a non-toxic biocompatible gel (BCG) to form a centrally located and temporary cell-free zone on culture surfaces. Cells are seeded into the 96-well plate and attach in a monolayer around the BCG. The BCG dissolves to reveal a Detection Zone and a Collagen I Overlay is added to create a 3-D ECM environment for cell invasion. Cell invasion above the plate surface can be assessed by performing a Z-stack analysis. The Oris™ Pro Collagen I Cell Invasion 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 Collagen I Cell Invasion Assay is designed to be used with any commercially available stain or labeling technique. Researchers can capture and quantify real-time cell invasion data using inverted microscopes, high content screening (HCS) and high content imaging (HCI) instruments.

The Oris™ Pro Collagen I Cell Invasion 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.

Using the Oris™ Pro Collagen I Cell Invasion 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 $\leq 12\%$ and generate robust Z' factors suitable for compound library screening.
- **Real-Time Analysis** - Experience unlimited access to cells, cell morphology and cell movement throughout your experiment.

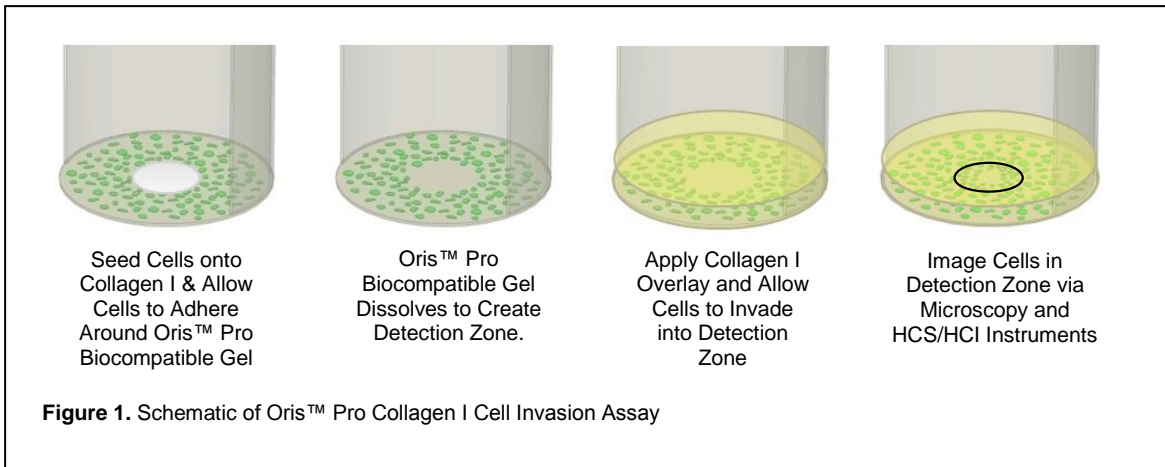


Figure 1. Schematic of Oris™ Pro Collagen I Cell Invasion Assay



II. ORIS™ PRO PLATE DIMENSIONS

Diameter of Well - Bottom	6.58 mm
Diameter of Well - Top	6.96 mm
Well Volume	340 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	0.19 mm
Well Coating Material	Collagen I, rat-tail

NOTE: For Research Use Only.

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

III. MATERIALS PROVIDED

Product No.: PROCIACC1

Component	Quantity	Storage
Oris™ Pro Collagen I Coated, 96-well Plate	1	Room Temperature
Oris™ Collagen I Stock Reagent *	4 mL	Refrigerate (4°C)
Oris™ 10X PBS Buffer	1 mL	Refrigerate (4°C) / Room Temperature

Product No.: PROCIACC2

Component	Quantity	Storage
Oris™ Pro Collagen I Coated, 96-well Plates	2	Room Temperature
Oris™ Collagen I Stock Reagent *	2 x 4 mL	Refrigerate (4°C)
Oris™ 10X PBS Buffer	2 x 1 mL	Refrigerate (4°C) / Room Temperature

* Oris™ Collagen I Stock Reagent must be stored at 4°C for use within 6 months of receipt. **Do not freeze.**



IV. MATERIALS REQUIRED

- Biological Cells
- 7.5% Sodium Bicarbonate
- Complete Cell Culture Growth Medium (containing serum)
- Serum-Free Cell Culture Medium
- Sterile PBS (containing both Ca⁺⁺ and Mg⁺⁺)
- 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. PRECAUTIONS AND RECOMMENDATIONS

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

Handling and Use of the *Oris™ Collagen I Stock Reagent*:

- Please note that it is crucial that the Collagen I Overlay concentration be optimized for cell line and experimental conditions, since different cell lines and different experimental conditions can result in varied amounts of cell invasion.
- A suggested starting concentration for the *Oris™ Pro Collagen I Overlay* is 3 mg/mL.

Experimental Conditions

- Please note that cell movement along the X-Y axes will likely occur in addition to invasion in the Z-axis. 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™ Cell-based Assays are accessible from our website at: <http://www.platypustech.com/applicationnotes.html>.

Oris™ is a trademark of Platypus Technologies, LLC.
CellTracker™ Green is a trademark of Invitrogen Corporation.



VI. COLLAGEN I CELL INVASION 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 invasion, 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 invasion assay. Please refer to Appendix I for a discussion of this process.

3. Pipette 100 μ L 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.



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).

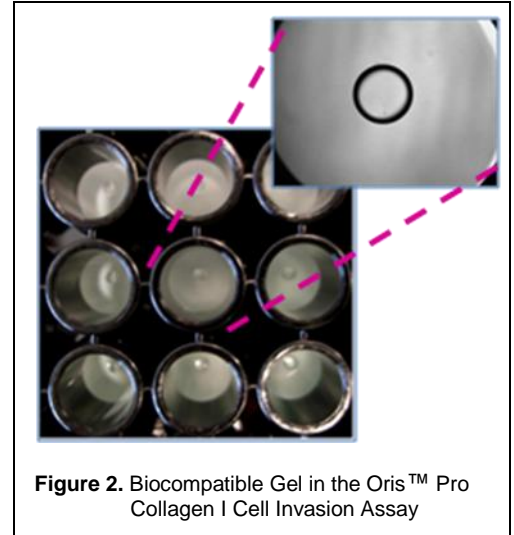


Figure 2. Biocompatible Gel in the Oris™ Pro Collagen I Cell Invasion Assay

4. Incubate the seeded plate(s) containing the Oris™ Pro Biocompatible Gel (see Figure 2) in a humidified chamber (37°C, 5% CO₂) for 30 minutes to 2 hours (cell line dependent) to permit cell attachment.

5. Remove plate(s) from incubator.

6. Remove media with a pipette and **gently** wash wells with 100 μ L of serum-free media (or sterile PBS) to remove any unattached cells.



IMPORTANT: Prior to/during use, keep the **Oris™ Collagen I Stock Reagent** and the **Oris™ Pro Collagen I Overlay Solution** on ice. In addition, the use of chilled pipette tips/reservoirs might be beneficial.

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

Oris™ 10X PBS (sterile)
7.5% sodium bicarbonate (sterile)
Deionized water (sterile)
Oris™ Collagen I Stock Reagent (5 mg/mL)

Calculate the volume of **Oris™ Collagen I Stock Reagent** needed to make the desired concentration of the **Oris™ Pro Collagen I Overlay** solution. Calculate the volume of sodium bicarbonate needed to neutralize the collagen where 0.0125ml of 7.5% sodium bicarbonate is required for every 1 mL of 5 mg/mL **Oris™ Collagen I Stock Reagent** used. Appropriate volumes of 10X PBS and deionized water are used to prepare the **Oris™ Pro Collagen I Overlay** in a final 1X PBS solution.

On ice, combine the water, Oris™ 10X PBS, and sodium bicarbonate. Next, add the **Oris™ Collagen I Stock Reagent** to achieve the desired concentration of the Collagen I Overlay solution. Mix by gently pipetting multiple times.

The following example protocol provides volumes for a 3.0 mg/mL Collagen I Overlay solution:

1.4625 mL deionized water
0.5 mL Oris™ 10X PBS buffer
0.0375 mL 7.5% sodium bicarbonate
3 mL Oris™ Collagen I Stock Reagent (5mg/mL)
5.0 mL total volume



NOTE: Supplements, such as growth factors, may also be mixed with the 3-D Collagen I Overlay.



8. Remove media from wells and add 40 μ L of the **Oris™ Pro Collagen I Overlay** to each well.

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

9. Incubate plate in a humidified chamber (37°C, 5% CO₂) for 1 hour to permit polymerization of the 3-D **Oris™ Pro Collagen I Overlay**.

10. 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, 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-invasion 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-invasion 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-invasion images of the Detection Zone formed in the wells.

11. Add 100 μ L of complete media (containing serum) on top of the 3-D **Oris™ Pro Collagen I Overlay**. **Optional:** Invasion inhibitors or stimulants may be added to the media.

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

12. Incubate plate in a humidified chamber (37°C, 5% CO₂) to permit cell invasion. Cells may be examined by inverted microscope or other imaging instrument throughout the incubation period to monitor progression of invasion, which will vary depending upon cell type and experimental design.

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

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



VI. DATA ACQUISITION

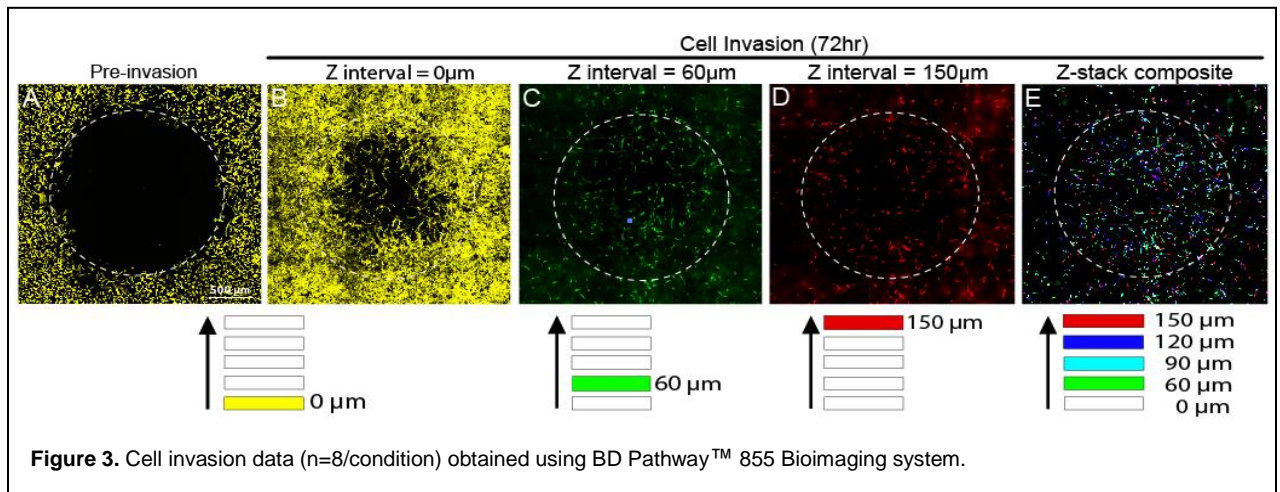
The readout the Oris™ Pro Collagen I Cell Invasion Assay can be conducted at any time, allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Pro Collagen I Cell Invasion Assay is designed to be used with any commercially available stain or labeling technique. Readout can be performed 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.

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 3.0×10^5 cells/mL) and incubated (37°C, 5% CO₂) for 1 hour. At the end of the 1 hour incubation, a 3.0 mg/mL **Oris™ Pro Collagen I 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 media 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 **Oris™ Pro Collagen I Overlay** are also presented. The Z-stack composite (E) represents color-coded cells that correspond to invading cells at Z-intervals > 60 μm . Scale bar = 500 μm .



BD Pathway™ 855 is a trademark of BD Biosciences.



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VII. ORDERING INFORMATION

Product Name	Coating	Size	Detection Zone Format
Oris™ Pro Cell Invasion Assays	Collagen I Coated	1-pack (PROCIACC1) 2-pack (PROCIACC2)	Biocompatible Gel
Oris™ Pro Cell Migration Assays	Tissue Culture Treated	1-pack (PROCMA1) 5-pack (PROCMA5)	Biocompatible Gel
	Collagen I Coated	1-pack (PROCMA1) 5-pack (PROCMA5)	
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 (CMA1.101) 5-pack (CMA5.101)	
	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 Assays	Collagen I	1-pack (CIA101CC) 2-pack (CIA200CC)	Oris™ Cell Seeding Stoppers (pre-populated)
	BME	1-pack (CIA101DE) 2-pack (CIA200DE)	Oris™ Cell Seeding Stoppers (not pre-populated)

For a complete list of assays, visit Platypus Technologies at www.platypustech.com/order_main.html. For technical assistance, contact Technical Support at (866) 296-4455 or techsupport@platypustech.com.

VIII. TERMS & CONDITIONS

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This warranty shall not be effective if PLATYPUS determines, in its sole discretion that PURCHASER has altered or misused the goods or has failed to use or store them in accordance with instructions furnished by PLATYPUS. PLATYPUS's sole and exclusive liability and PURCHASER's exclusive remedy with respect to goods proved to PLATYPUS's satisfaction (applying analytical methods reasonably selected by PLATYPUS) to be defective or nonconforming shall be the replacement of such goods free of charge, upon the return of such goods in accordance with our instructions, although at its discretion, PLATYPUS may provide a credit or refund. If PLATYPUS manufactures custom goods for PURCHASER based on instructions, specifications, or other directions provided by PURCHASER, PLATYPUS shall not be liable for the lack of sufficiency, fitness for purpose or quality of the goods to the extent attributable to such instructions, specifications, or other directions. PLATYPUS shall not be liable for any loss, damage or penalty as a result of any delay in or failure to manufacture, deliver or otherwise perform hereunder due to any cause beyond PLATYPUS's reasonable control. 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 Collagen I Cell Invasion 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 cm². 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 4.0x10⁵, 3.0x10⁵, and 2.0x10⁵ 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% CO₂) 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 visually inspect each well to determine the minimum cell seeding concentration that yields a confluent monolayer at the perimeter of the Detection Zone (See Figure 3 for representative pre-invasion Detection Zone images).

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

APPENDIX II: Fluorescent Labeling Live Cell Options

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

The Oris™ Pro Collagen I Cell 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. 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 invasion. 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 x 10⁴ cells per well in 100 µL volume, adjust the concentration of the labeled cell suspension to 3.5 x 10⁵ 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 invasion.

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



APPENDIX III: Fluorescent Labeling Fixed Cell Options

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

The Oris™ Pro Collagen I Cell 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. 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 motility. 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 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 (ThermoScientific; prepared from 1 mg/mL stock) 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 cyto-staining 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 14 of the Cell Invasion Assay Protocol.

