

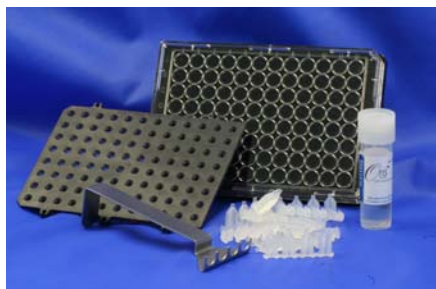
Oris™ Cell Invasion & Detection Assay

Product No.: CIA101DE & CIA200DE

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

PROTOCOL & INSTRUCTIONS

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Oris™ CELL INVASION & DETECTION ASSAY

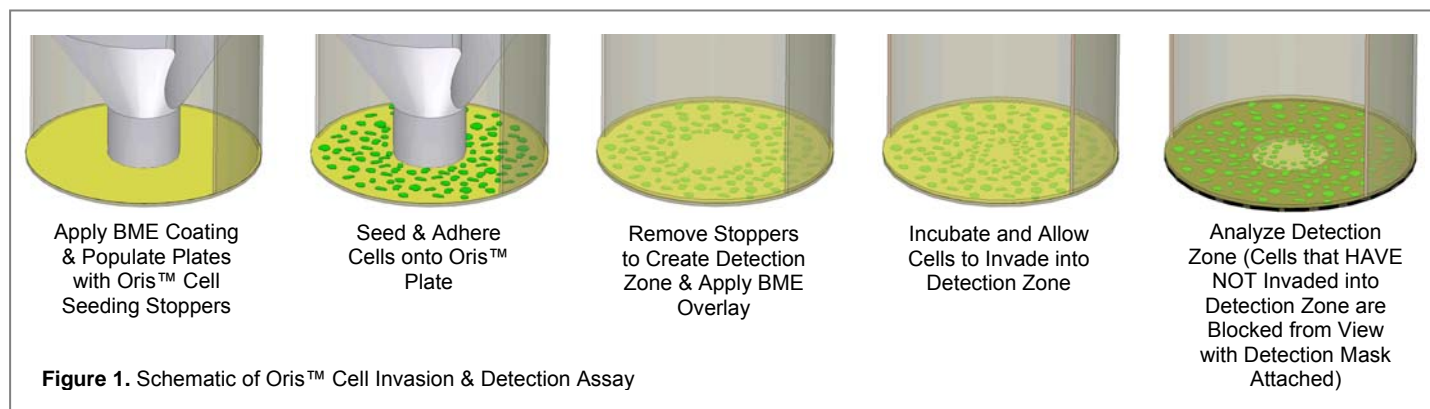
I. INTRODUCTION

The Oris™ Cell Invasion & Detection Assay is a reproducible, sensitive, and flexible assay that can be used to monitor cell invasion. Formatted for a 96-well plate, the assay utilizes Oris™ Cell Seeding Stoppers made from a medical-grade silicone to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2 mm diameter unseeded region in the center of each well, i.e., the detection zone, into which the seeded cells may then invade. The Oris™ Detection Mask is applied to the plate bottom and restricts visualization to the detection zone, thus allowing only invading cells to be detected (see Figure 1). The Oris™ Cell Invasion & Detection Assay is designed to be used with any commercially available stain or labeling technique, but comes complete with a Calcein AM Staining Reagent. Calcein AM is a fluorescent dye that passes through the membrane of live cells and is useful for short-term labeling of cells at the end of the invasion period. Calcein AM is processed by esterases inside the cell allowing the dye to bind calcium and be trapped inside the cell, and has an excitation/emission wavelength of 495/515 nm. Readout can be performed by microscopy or use of a microplate reader.

The Oris™ Cell Invasion & Detection Assay kit has been uniquely designed to detect cellular invasion *in vitro* within a 3-dimensional extracellular matrix comprised of a basement membrane extract (BME) of the Murine Engelbreth-Holm-Swarm tumor. The Oris™ Cell Invasion & Detection Assay system has been designed for use with adherent cell cultures. Performance of the Oris™ Cell Invasion & Detection Assay was optimized using the invasive HT-1080 fibrosarcoma and the non-invasive 3T3-Swiss albino fibroblast cell lines.

Using the Oris™ Cell Invasion & Detection Assay offers the following features & benefits:

- **Membrane-free Invasion** - perform studies without manipulating transmembrane inserts; no membrane to restrict the ability to image cells.
- **Reproducible Results** – obtain low well-to-well CV's due to the unique assay design.
- **Preserve Cell Morphology** – realize a more native 3-D invasion environment since cells are embedded in an extracellular matrix.
- **Versatile** - analyze data using multiple probes in a single well by using a microscope, digital imager, or fluorescence microplate reader.
- **Flexible** - perform kinetic or endpoint cell invasion assays without the use of special instrumentation.



II. ORIS™ PLATE DIMENSIONS (per well)

Diameter of Well	6.5 mm
Diameter of Stopper Space (Detection Zone)	2 mm
Suggested Media Volume per Well (populated with Stoppers)	100 µl
Effective Area of Outer Annular Region (seeding region) per Well	30.03 mm ²
Effective Area of Central Detection Zone per Well	3.14 mm ²

Important: Read Instructions Before Performing any Oris™ Assay.



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III. MATERIALS PROVIDED

Product No.: CIA101DE

Component	Quantity	Storage
Oris™ 96-well Plate	1	Room Temperature
Oris™ Cell Seeding Stoppers	96	Room Temperature
Oris™ Detection Mask	1	Room Temperature
Oris™ Stopper Tool	1	Room Temperature
Oris™ Basement Membrane Extract (BME) Stock Reagent	5 mL	≤ -20°C*
Calcein AM Reagent	20 µL	-20°C with dessicant**

Product No.: CIA200DE

Component	Quantity	Storage
Oris™ 96-well Plates	2	Room Temperature
Oris™ Cell Seeding Stoppers	2 x 96	Room Temperature
Oris™ Detection Mask	2	Room Temperature
Oris™ Stopper Tool	2	Room Temperature
Oris™ Basement Membrane Extract (BME) Stock Reagent	2 x 5 mL	≤ -20°C*
Calcein AM Reagent	2 x 20 µL	-20°C with dessicant**

* Oris™ BME Stock Reagent can be stored at -20°C in a manual defrost freezer if kit will be used within 3 months of receipt. For long-term storage, maintain Oris™ BME Stock Reagent at -80°C.

** Calcein AM can be stored at -20°C in a manual defrost freezer with a dessicant for use within 6 months of receipt.

IV. MATERIALS REQUIRED

- Biological Cells
- Complete Cell Culture Growth Medium (containing serum)
- Sterile PBS (containing both Calcium and Magnesium)
- Hanks Balanced Salt Solution (HBSS)
- Serum-Free Cell Culture Medium
- Sterile Pipette Tips/Pipette or Multi-Channel Pipette
- Trypsin or Cell Scraper
- Inverted Microscope (optional)
- Fluorescence Microplate Reader (optional)
- Cell Culture Labeling Medium (phenol red-free/serum-free media)
- Cell Labeling Fluorescent Agent (e.g., CellTracker™ Green) - *required if performing staining in addition to or in place of Calcein AM.*



V. PRECAUTIONS AND RECOMMENDATIONS

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

Handling and Use of the *Oris™ BME Stock Reagent*:

- Thaw on ice (2-8°C) overnight.
- Please note that there will be lot-to-lot variations in the concentration of the *Oris™ BME Stock Reagent*. Refer to the BME concentration listed on the bottle label when preparing dilutions. It is crucial that the BME concentration be optimized for cell line and experimental conditions, since different cell lines and different experimental conditions can result in a range of cell invasiveness.
- A suggested starting concentration for the *Oris™ BME Stock Reagent* is 10 – 12 mg/mL. However, do not dilute *Oris™ BME Stock Reagent* below 9 mg/mL, as this will preclude gel formation.
- Aliquot and freeze any remaining *Oris™ BME Stock Reagent*. Avoid repeated freeze-thaw cycles.

Recommendations for Preparation of Reference Wells:

- To establish t=0 pre-invasion reference wells by seeding both test and reference wells at the **same time**, it is necessary to seed cells at **different concentrations**. Seed test wells at a density determined optimal in Appendix I, but seed reference wells at sub-optimal density (50-75% confluency). Allow cells to adhere and remove stoppers from test wells (overlay test wells with the *Oris™ BME Stock Reagent* and then incubate to allow for gel formation). Reference wells will remain populated with *Oris™ Cell Seeding Stoppers* until the end of the assay. At that point, remove the *Oris™ Cell Seeding Stoppers* from the reference wells, overlay the cells with the *Oris™ BME Stock Reagent*, and incubate to allow for gel formation. At this point, the entire plate can be stained and analyzed.
- To establish t=0 pre-invasion reference wells by seeding test and reference wells at the **same concentration**, it is necessary to seed cells at **different times** during the assay. Seed test wells at density determined optimal in Appendix I, allow cells to adhere, remove stoppers, overlay with the *Oris™ BME Stock Reagent*, and incubate to allow for gel formation. Allow cells to invade for a set amount of time. At 4 - 18 hours prior to analyzing test wells, seed reference wells at a density determined optimal in Appendix I, and allow cells to adhere. At the end of the assay, remove stoppers from the reference wells, overlay them with the *Oris™ BME Stock Reagent*, and then incubate to allow for gel formation. At this point, the entire plate can be stained and analyzed.

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



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VI. CELL INVASION & DETECTION ASSAY PROTOCOL

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

1. If desired, cells can be starved by incubating for 18 - 24 hours in serum-free medium prior to assay (0.5% fetal bovine serum may be used if needed).
2. Make 2 mL of a *BME Coating Solution* (**final concentration of 3.5 mg/mL**) by mixing thawed Oris™ BME Stock Reagent with cold Hanks Balanced Salt Solution (HBSS) or serum-free media. For example, if you have a 14 mg/mL Oris™ BME Stock Reagent, then mix 500 µL of the Oris BME Stock Reagent with 1.5 mL of HBSS or serum-free media.



NOTE: BME gels in 5-10 min above 15°C; therefore, keep the *BME Coating Solution* on ice while coating all wells. The use of chilled pipette tips/reservoirs may be beneficial.

Also, keep the remaining Oris™ BME Stock Reagent on ice until ready to use (Oris™ BME Stock Reagent will be used again in Step 17).

3. Pipette 100 µl of the *BME Coating Solution* into the wells of Column 1. Avoid bubble formation in the BME Coating by not fully expelling all contents of the pipette. Immediately remove the *BME Coating Solution* from the wells of Column 1 and return contents back into the *BME Coating Solution* reservoir. Repeat procedure for the remainder of plate.



NOTE: The *BME Coating Solution* is only used in Step 3.

4. After the *BME Coating Solution* has been applied to all wells of the plate, check each well for excess solution. If excess solution is apparent, remove via pipette.
5. Incubate the plate in a humidified chamber (37°C, 5% CO₂) for 15 - 30 minutes.
6. Under sterile conditions, populate the 96-well plate with Oris™ Cell Seeding Stoppers:
 - Vertically position the tip ends of two, 4-stopper strips into one full column of 8 wells at a time (Figure 2A).
 - Gently press down on the strip backbone to partially insert the stoppers halfway into the well (Figure 2B).
 - When both stopper strips have been partially inserted in 1 column, ensure that the position of the stoppers is vertical with respect to the well wall, making any necessary adjustments (Figure 2C).
 - Using the Oris™ Stopper Tool, firmly press down on the strip backbone to fully insert the stoppers into each well (Figure 2D and 2E). Repeat for all remaining columns.

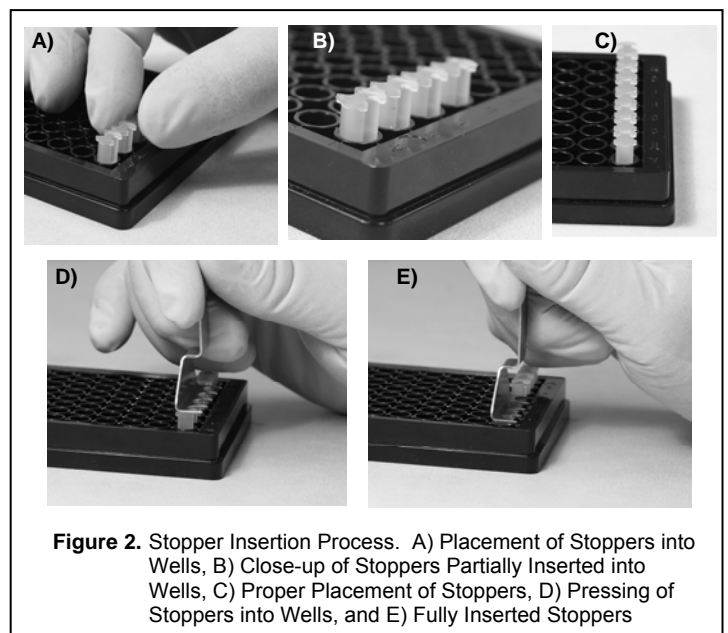


Figure 2. Stopper Insertion Process. A) Placement of Stoppers into Wells, B) Close-up of Stoppers Partially Inserted into Wells, C) Proper Placement of Stoppers, D) Pressing of Stoppers into Wells, and E) Fully Inserted Stoppers



NOTE: It is extremely important to ensure that the stoppers are inserted perpendicular to the well bottom and are fully engaged with the bottom of the well. Failure to do so will increase the CV of your data set.

7. Visually inspect the underside of the populated 96-well plate to ensure that the Oris™ Cell Seeding Stoppers are firmly sealed against the bottom of the plate. To inspect the stoppers, turn the plate over and examine the stoppers for sealing (see Figure 3). If incomplete sealing is observed, return the plate to the upright position and use a sterile instrument to gently push the stopper back into the well until sealing is observed.



NOTE: The sealing of the stoppers can be most easily observed if the plate is tipped at an angle and viewed under indirect light to reveal the “bullseye” pattern at the bottom of each well.

8. Apply the Oris™ Detection Mask to the bottom of the 96-well plate if microplate reader data is being collected. The Detection Mask is not necessary if collecting imaging data.

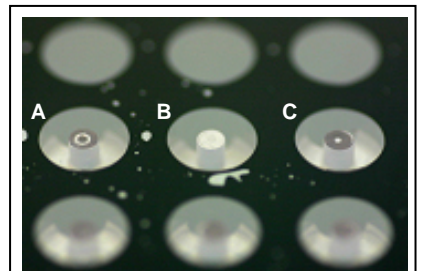


Figure 3. Stoppers that are:
A) Partially Sealed
B) Unsealed
C) Completely Sealed



VI. CELL INVASION & DETECTION ASSAY PROTOCOL, continued

First Time Users: In order to prevent splashing of well contents, familiarize yourself with the attachment and removal of the Detection Mask before any liquids are placed into the wells:

- Orient the chamfered corners of the mask with those of the 96-well plate, ensuring that the A1 corner of the mask is aligned with the A1 well of the plate (see Figure 4).
- Align the holes in the attachment lugs with the bosses on the bottom of the plate.
- Gently press the mask until it is flush with the bottom of the 96-well plate.



NOTE: It may be necessary to wash the mask with ethanol to remove dust and debris since the mask is **not** sterile. The mask may be applied at any point during the assay. For kinetic assays, it is often most convenient to apply the mask at the beginning of the assay before any liquids are placed in the well. For endpoint assays, using fixed and stained cells, it is often most convenient to apply the mask just before reading assay results.

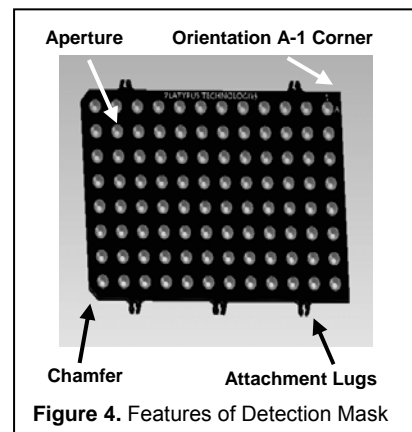


Figure 4. Features of Detection Mask

9. If performing a kinetic analysis of cell invasion, pre-label cells with a fluorescent stain now.
10. Collect cells and prepare a suspension that is 10-fold greater in density than the optimal seeding concentration using complete cell culture growth medium containing serum.

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.

IMPORTANT: For recommendations on designating 'reference' wells, please refer to Section V: Precautions and Recommendations.

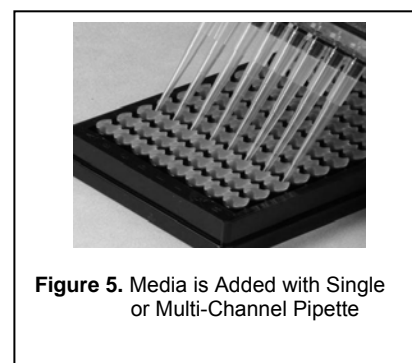


Figure 5. Media is Added with Single or Multi-Channel Pipette

11. Pipette 100 μ l of suspended cells into each test well through one of the side ports of the Oris™ Cell Seeding Stopper.



NOTE: For best results, add or extract media by placing the pipette tip along the wall of the well (see Figure 5). Care should be taken not to disturb the Oris™ Cell Seeding Stopper or the BME coating when introducing the pipette tip into the well. A slender/elongated tip or a gel loading tip may be useful.

IMPORTANT: Lightly tap the plate on your work surface to evenly distribute well contents (extreme tapping may result in splashing of well contents and lead to contamination).

12. Incubate the seeded plate containing the Oris™ Cell Seeding Stoppers in a humidified chamber (37°C, 5% CO₂) for 4 to 18 hours (cell line dependent) to permit cell attachment.
13. Remove plate from incubator.
14. Using the Oris™ Stopper Tool, remove stoppers (see Figure 6).



NOTE: It may be necessary to wash the Oris™ Stopper Tool with 70% ethanol as the Stopper Tool is not sterile.

- Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the Oris™ Stopper Tool under the backbone of the stopper strip, keeping the underside of the Oris™ Stopper Tool flush with the top surface of the plate.
- Lift the Oris™ Stopper Tool **vertically** to gently remove the stoppers.

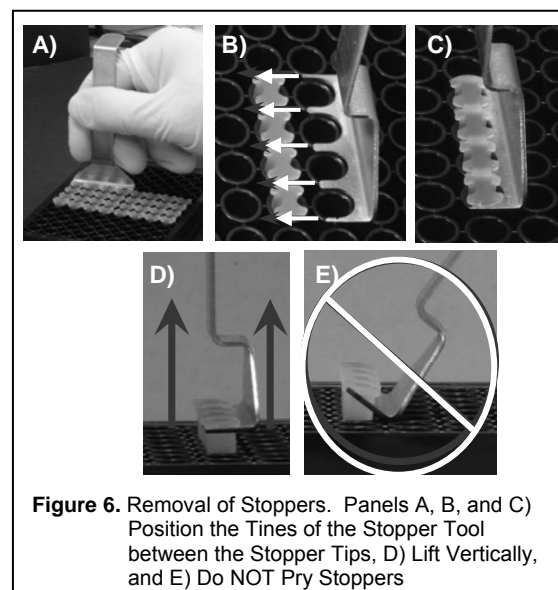


Figure 6. Removal of Stoppers. Panels A, B, and C) Position the Tines of the Stopper Tool between the Stopper Tips, D) Lift Vertically, and E) Do NOT Pry Stoppers



NOTE: DO NOT use the Oris™ Stopper Tool as a lever to pry the stoppers from the well (see Figure 6E), as doing so may cause displacement of seeded cells and may distort the detection zone area.



VI. CELL INVASION & DETECTION ASSAY PROTOCOL, continued

15. Remove media with a pipette and **gently** wash wells with 100 µl of sterile PBS (or serum-free media) to remove any unattached cells. Do not aspirate using an in-house vacuum.
16. Add 40 µl of the thawed **Oris™ BME Stock Reagent** to each well (supplements, such as FBS or growth factors, may be mixed with BME) to create a 3-D BME overlay.



NOTE: Please note that there will be lot-to-lot variations in the concentration of the **Oris™ BME Stock Reagent**. Refer to the BME concentration listed on the bottle label when preparing dilutions. It is crucial that the BME concentration be optimized for cell line and experimental conditions, since different cell lines and different experimental conditions can result in a range of cell invasiveness.



NOTE: BME gels in 5-10 min above 15°C, therefore, you must keep the thawed **Oris™ BME Stock Reagent** on ice until ready to use. In addition, the use of chilled pipette tips/reservoirs might be beneficial.

17. Incubate plate in a humidified chamber (37°C, 5% CO₂) for 30 - 60 minutes to permit polymerization of the 3-D BME overlay.
18. Add 100 µl of serum-free cell culture medium on top of the 3-D BME overlay. **Optional:** Invasion inhibitors or stimulants may be added to the media.
19. Incubate plate in a humidified chamber (37°C, 5% CO₂) to permit cell invasion (length of incubation is cell line dependent). The 3-D BME overlay will remain gelled for up to 14 days. Refresh media or supplements, every 48 - 72 hours, as needed, for the duration of the invasion experiment.
20. If performing an endpoint analysis of cell invasion, stain cells with a fluorescent stain after sufficient invasion has occurred. Refer to Section VI and Appendix II for further information on data acquisition and fluorescence staining technique.



NOTE: Oris™ Cell Seeding Stoppers are for single use only; Platypus cannot guarantee the integrity of the stopper material after a second sterilization procedure.



VII. DATA ACQUISITION

The readout of the Oris™ Cell Invasion & Detection Assay can be conducted at any time, allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Cell Invasion & Detection Assay is designed to be used with any commercially available stain or labeling technique, but is supplied complete with the Calcein AM Reagent. The readout can be performed by using a microscope, a microplate reader, or a High Content Screening or High Content Imaging Analysis platform.

Microscope Analysis

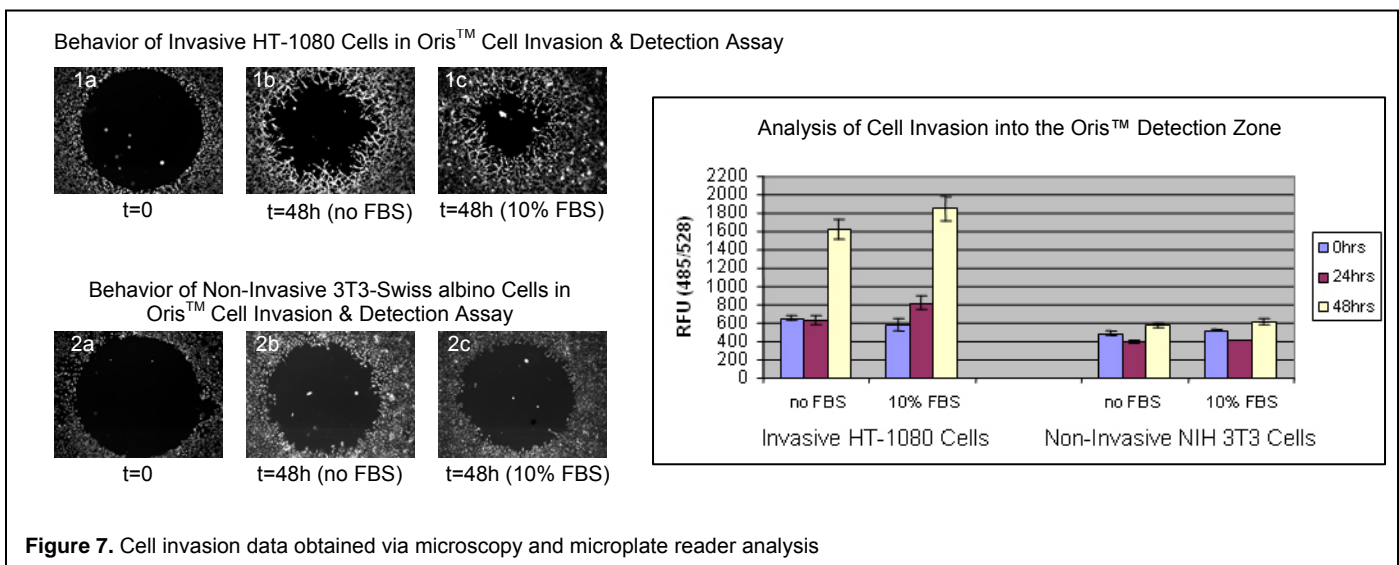
- Cell counting or image capture / analysis software, such as NIH ImageJ freeware, can be used.
- Note: Microscopic observations are possible using phase contrast or bright field microscopy with colorimetric stains.
- No need to attach the Oris™ Detection Mask to the Oris™ microplate.
- To set up reference controls, refer to Section V: Precautions and Recommendations.

Microplate Reader Analysis

- Attach the Oris™ Detection Mask to the bottom of the Oris™ microplate (see Step 8 of Protocol).
- Optimal settings will vary according to the microplate reader make and model. Consult Appendix II and the equipment user manual for your particular instrument.
- The microplate reader MUST be set to read from the bottom of the plate.
- To set up reference controls, refer to Section V: Precautions and Recommendations.

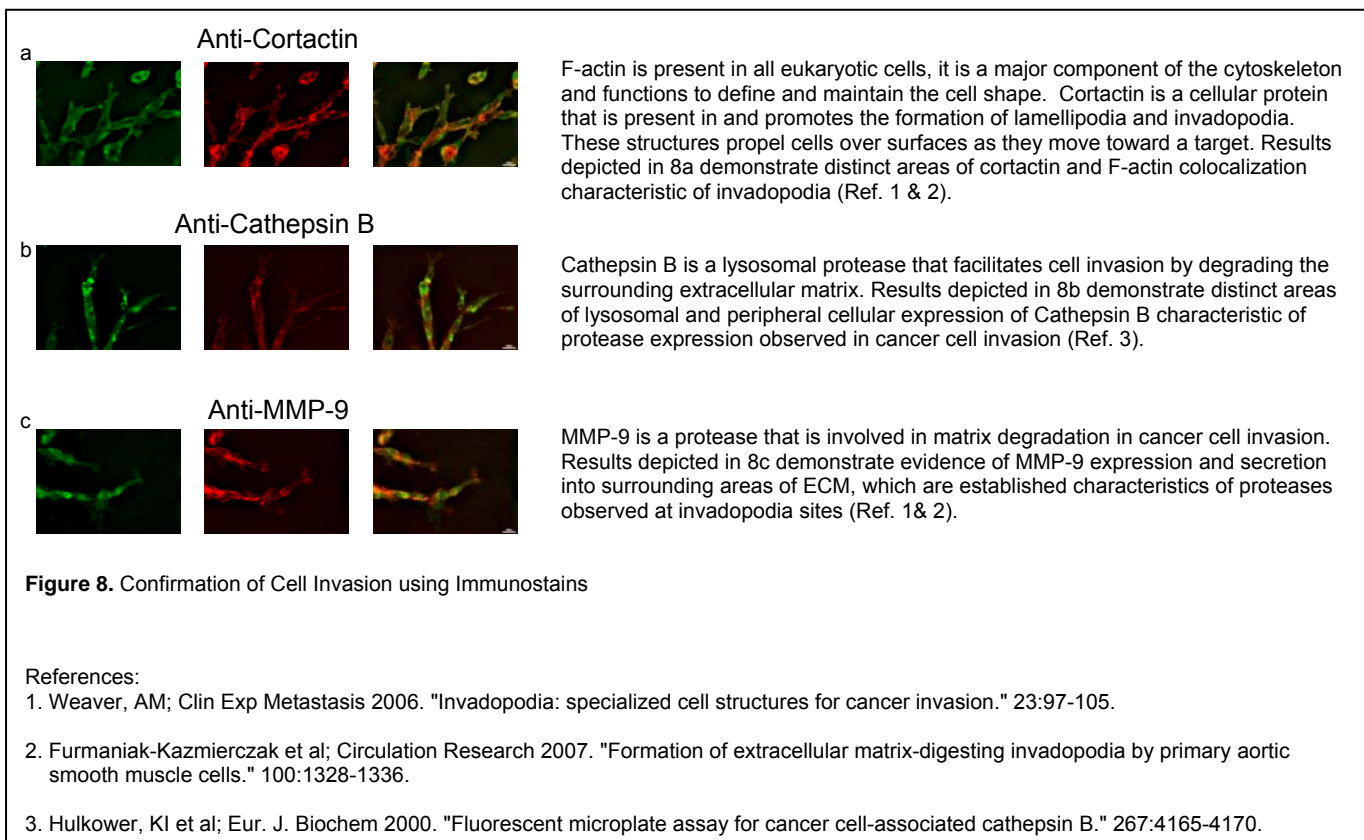
Sample Data Obtained via Microscopy and Microplate Reader are shown in Figure 7.

- Wells were seeded with 50,000 HT-1080 cells (i.e., 100 μ l of 5×10^5 cells/mL) that had been serum starved for 18 hours and the plate was then incubated for 4 hours. The stoppers were removed from the wells and the Oris™ BME (final concentration of [12 mg/mL]) with 10% FBS or without FBS was overlaid on the cells (four (4) wells were left stoppered to represent t=0 reference). The plate was incubated in a humidified chamber for 48 hours to permit cell invasion. Cells were labeled with Calcein AM and images were captured using a Zeiss Axiovert microscope (5X magnification). Fluorescence in the detection zone was quantified by using a microplate reader. Each column represents the mean \pm SD of at least 4 wells. A non-invasive cell line, 3T3-Swiss albino, served as the negative control. The images below, captured without a detection mask in place, illustrate representative data from pre-invasion (t=0 hrs) and post-invasion (t=48 hrs) wells. The graph depicts the average Relative Fluorescent Units (RFU's) in the detection zones for each condition, confirming the invasion augmenting effect of FBS on serum starved HT-1080 cells and the lack of invasion by non-invasive 3T3-Swiss albino cells.
- As observed in Figure 7, HT-1080 cells formed invadopodial structures that projected into the central detection zone (panel 1b) as compared to a time zero control (panel 1a).
- Invasion by serum-starved HT-1080 cells was augmented by including 10% v/v fetal bovine serum (FBS) in the BME overlay (panel 1c).
- In contrast, as observed in images 2b and 2c, the non-invasive 3T3-Swiss albino cells did not form any invadopodial structures and showed a nominal amount of FBS-independent migration into the central detection zone as compared to a time zero control (image 2a).



Immunostaining Analysis

- As verified by immunostaining (shown in Figure 8), the structures projecting into the BME formed by the invasive HT-1080 cells exhibited classic hallmarks of invadopodia, namely colocalization of F-actin with cortactin (panel a), and the expression of proteases such as MMP-9 and cathepsin B (panels b and c). These invadopodia could also be observed in 3-dimensions at different focal points along the z-axis. This is in contrast to any migratory activity observed by non-invasive cells that occurs only within the 2-dimensional space of the x and y axes (data not shown).
- Cells from the Oris™ Cell Invasion & Detection Assay were fixed, permeabilized, and pre-treated by sequential incubations in 3.7% formaldehyde, PBS, 0.5% Triton X-100, PBS, Image-iT™ FX signal enhancer, and PBS. Immunostaining was performed by incubating with primary antibodies (2 hours, 37°C) and Alexa Fluor® 488 conjugated secondary antibodies at 1:200 dilution (1 hour, 37°C) followed by an Alexa Fluor® 555 phalloidin counterstain (1 hour). Images were collected using a Nikon TE300 inverted microscope equipped with a Photometrics Coolsnap fx CCD camera, deconvolved using Slidebook™ v4.2 (Intelligent Imaging Innovations) and processed using Adobe® Photoshop® CS2 (Adobe Systems).



VIII. ORDERING INFORMATION

Product No.	Product Description	Package Size
CIA101DE	Oris™ Cell Invasion & Detection Assay, 1-pack: Oris™ 96-well plate, 1 Oris™ BME Stock Reagent, 5 mL Oris™ Cell Seeding Stoppers, 96 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1 Calcein AM Reagent, 20 µL	1-pack
CIA200DE	Oris™ Cell Invasion & Detection Assay, 2-pack: Oris™ 96-well plates, 2 Oris™ BME Stock Reagent, 2 x 5 mL Oris™ Cell Seeding Stoppers, 2 x 96 Oris™ Detection Mask, 2 & Oris™ Stopper Tool, 2 Calcein AM Reagent, 2 x 20 µL	2-pack
CMA1.101	Oris™ Cell Migration Assay, 1-pack: Oris™ 96-well, Tissue Culture Treated Plate (black, clear bottom) with Oris™ Cell Seeding Stoppers, 1 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	1-pack
CMA5.101	Oris™ Cell Migration Assay, 5-pack: Oris™ 96-well, Tissue Culture Treated Plates (black, clear bottom) with Oris™ Cell Seeding Stoppers, 5 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	5-pack
CMATR1.101	Oris™ Cell Migration Assay - TriCoated, 1-pack: Oris™ TriCoated, 96-well Plate with Oris™ Cell Seeding Stoppers, 1 32 wells, Uncoated, Tissue Culture Treated 32 wells, Collagen I coated 32 wells, Fibronectin coated Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	1-pack
CMATR5.101	Oris™ Cell Migration Assay - TriCoated, 5-pack: Oris™ TriCoated, 96-well Plates with Oris™ Cell Seeding Stoppers, 5 32 wells, Uncoated, Tissue Culture Treated 32 wells, Collagen I coated 32 wells, Fibronectin coated Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	5-pack

To place an order, visit the Platypus Technologies website at: www.platypustech.com/order_main.html.
For technical assistance, contact Technical Support at (866) 296-4455 or techsupport@platypustech.com.

IX. TERMS & CONDITIONS

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PLATYPUS warrants that its products shall conform substantially to the description of such goods as provided in product catalogues and literature accompanying the goods until their respective expiration dates or, if no expiration date is provided, for 6 months from the date of receipt of such goods. PLATYPUS will replace, free of charge, any product that does not conform to the specifications. This warranty limits PLATYPUS's liability only to the replacement of the nonconforming product.

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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™ Cell Invasion & Detection Assay. The intended goal is to achieve 90-95% confluency of the monolayer surrounding the Oris™ Cell Seeding Stoppers without overgrowth.

1. A suggested starting point is to evaluate three serial dilutions at the cell densities shown below. The cell seeding area of the well with the stopper in place 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. Prepare three serial dilutions at final concentrations of 1.0 x 10⁶, 0.5 x 10⁶ and 0.25 x 10⁶ 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	100,000	50,000	25,000
Number of wells	8	8	8

5. Incubate the plate in a humidified chamber (37°C, 5% CO₂) for 4 - 18 hours (cell line dependent) with cell seeding stoppers in place to allow the cells to firmly attach to the well surface.
6. Following cell attachment, remove the Oris™ Cell Seeding Stoppers from each well (see Figure 6) and **gently** wash the wells with PBS to remove non-attached cells.
 - Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the Oris™ Stopper Tool under the backbone of the stopper strip, keeping the underside of the tool flush with the top surface of the plate.
 - Lift the Oris™ Stopper Tool **vertically** to gently remove the stopper. Do not use the Oris™ Stopper Tool as a lever to pry the stoppers from the well as doing so may cause displacement of the seeded cells.
7. Without a Detection Mask in place, use a microscope to visually inspect each well to determine the minimum cell seeding concentration that yielded a confluent monolayer at the perimeter of the detection zone.

At this point, if you plan to obtain the results of the Oris™ Cell Invasion & Detection Assay via colorimetric analysis or microscopy, you have successfully determined the optimal cell seeding concentration to be used in Step 11 of the Oris™ Cell Invasion & Detection Assay Protocol.

APPENDIX II: Determining Optimal Fluorescence Microplate Reader Settings

This procedure is intended to assist in optimizing your instrument settings when using a fluorescence microplate reader to capture data from the Oris™ Cell Invasion & Detection Assay.

1. Using the optimal cell seeding concentration determined in Appendix I, perform a cell invasion assay per Section VI, Oris™ Cell Invasion & Detection Assay Protocol using culture conditions expected to result in robust cell invasion. Be sure to include equal numbers of pre-invasion control wells (stoppers left in place until staining) and post-invasion test wells (stoppers removed after cell attachment period). A minimum of 8 wells per condition are recommended.
2. Perform the desired fluorescent staining technique.

The Oris™ Cell Invasion & Detection 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 for using Calcein AM:

- a. To stain one fully-seeded 96-well plate, combine 5 µl of Calcein AM (1 mg/mL in dry DMSO) with 10 mL of phenol red-free and serum-free media or 1x PBS. Protect diluted Calcein AM solution from light until ready to use in step d.
 - b. Carefully remove culture medium from wells.
 - c. Wash wells with 100 µl of PBS (containing both Calcium and Magnesium).
 - d. Add 100 µl of diluted Calcein AM solution to each well.
 - e. Incubate plate at 37°C for 30 - 60 minutes.
 - f. Attach mask and read promptly with microplate reader using appropriate filter set and sensitivity/gain settings (for a BioTek Synergy™ HT microplate reader, use 485/528 nm excitation/emission filters, sensitivity 55 nm).
3. If not already in place, apply the Oris™ Detection Mask to the plate. Using the bottom probe of a fluorescence microplate reader, obtain the fluorescence reading from each well. To achieve the optimal dynamic range, adjust the instrument settings (e.g., gain) to result in the greatest difference in fluorescence signal between pre-invasion and post-invasion wells. Refer to the instrument manual for your microplate reader for further guidance on instrument settings.

You have now successfully determined the optimal cell seeding concentration (to be used in Step 11 of the Oris™ Cell Invasion & Detection Assay Protocol) and microplate reader settings for analysis of cell invasion using a fluorescence microplate reader.

