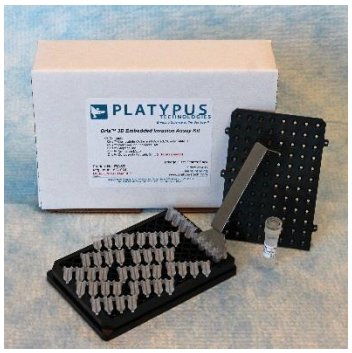
Oris™ 3D Embedded Invasion Assay

Product No.: EIAST

3D Assay for Investigating Embedded Cell Movement through Collagen I

PROTOCOL & INSTRUCTIONS



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Important: Read Instructions Before Performing any Oris™ Assay.

I. MATERIALS PROVIDED

Product No.: EIAST

Component	Quantity	Storage
Oris™ -compatible 96-well Collagen I Coated Plate	1	Refrigerate (4°C)
Oris™ Cell Seeding Stoppers	2 x 24	Refrigerate (4°C)
Oris™ Detection Mask	1	Room Temperature
Oris™ Stopper Tool	1	Room Temperature
Oris™ Collagen I (Rat tail) *	1 x 2 mL	Refrigerate (4°C)

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

II. MATERIALS REQUIRED

- Cells
- 7.5% Sodium Bicarbonate
- Cell Culture Growth Medium
- Sterile PBS
- 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)
- Cell Labeling Fluorescent Agent (e.g., Calcein AM) - *required if performing staining.*

III. PRECAUTIONS AND RECOMMENDATIONS

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

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

- Be sure to keep Oris™ Collagen I (Rat tail) on ice while being used.
- A suggested concentration for the ***Oris™ Collagen I Matrix*** is 3 mg/mL.
- If you prefer to use a different concentration of the Collagen I Matrix solution for your particular cell line, be sure to adjust the volumes of the other associated reagents in proportion to the adjustments made to the volume of the collagen.

Recommendations for Preparation of Reference Wells:

Establishing a time zero (t=0) reference can be accomplished by any of several methods: real-time analysis, treating reference wells with a migration inhibitor, or utilizing the stopper as a physical barrier to prevent cell movement into the detection zone.

- **Real-time analysis using microplate reader**: treat the cells with a live fluorescent stain at the start of the assay. Attach the Oris™ Detection Mask to the bottom of the plate (**see Section IV, Step 5**). Quantify fluorescence using a microplate reader. This data is the pre-invasion reference value.
- **Real-time analysis using microscopy** (with imaging capabilities): images collected at the start of the experiment can be used as the t=0 reference to be analyzed compared with subsequent images.
- **Inhibitor treatment**: treat control wells with a concentration of drug that fully inhibits cellular movement. Choice of drug and concentration will be cell line dependent. To ensure the correct drug concentration, you may need to test a range of drug concentrations in a separate experiment.
- **Oris™ Cell Seeding Stoppers as barrier** (**NOTE**: Stoppers come in strips of n=4): Set up the assay as normal following the protocol in Section IV. For the t=0 reference wells, skip steps 12-14. Continue with the rest of the experiment as normal. The stoppers can remain in place as a t=0 reference until the assay is complete. Upon completion, the stoppers may then be removed to allow for endpoint fixation and labeling.

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.

IV. Oris™ 3D EMBEDDED INVASION 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. Alternatively, 0.5% fetal bovine serum may be used if needed.

2. Remove the Oris™ -compatible 96-well Collagen I Coated Plate from refrigeration and allow to equilibrate to room temperature for one hour.

3. 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 1A).
   - Gently press down on the strip backbone to partially insert the stopper halfway into the well (Figure 1B).
   - When both stopper strips have been partially inserted in one column, ensure that the position of the stoppers is vertical with respect to the well wall, making any necessary adjustments (Figure 1C).
   - Using the Oris™ Stopper Tool, firmly press down on the strip backbone to fully insert the stoppers into each well (Figure 1D and 1E). Repeat for all remaining stoppers.

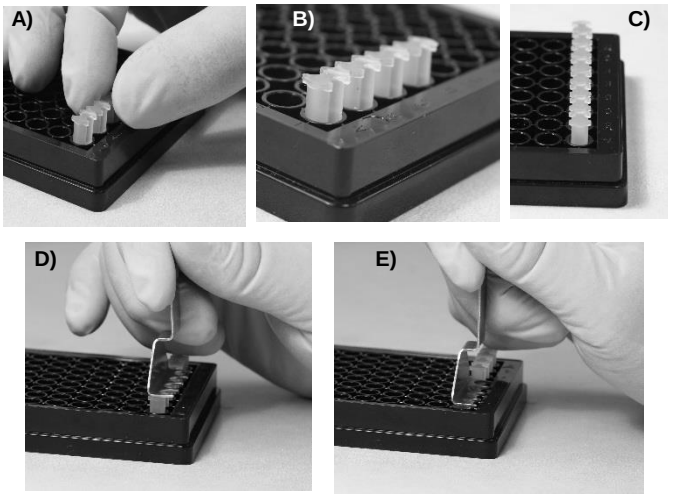

Figure 1. Stopper Insertion Process. A) Placement of Stoppers into Wells, B) Close-up of Stoppers Partially Inserted into Wells, C) Proper Placement of Stoppers, D) Pressing of Stoppers into Wells, and E) Fully Inserted Stoppers

NOTE: It is extremely important to ensure that the stopper are inserted perpendicular to the well bottom and are fully engaged with the well bottom. If you require the stoppers to be more consistently located, the pre-populated Oris™ 3D Embedded Invasion Assay Kit (Cat. No. EIA1) is recommended.

4. 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 2). 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.

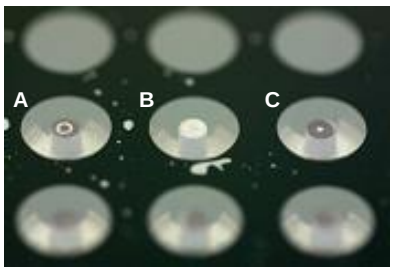

Figure 2. Stoppers that are:
A) Partially Sealed
B) Unsealed
C) Completely Sealed

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 (Figure 2).

5. If microplate reader data will be collected, apply the Oris™ Detection Mask to the bottom of the 96-well plate. The Detection Mask is not necessary if collecting imaging data.

   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 (Figure 3).
   - 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.

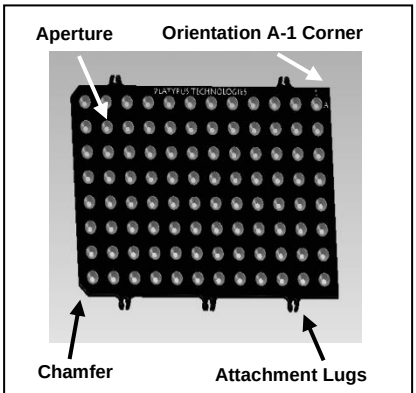

Figure 3. Features of Detection Mask

NOTE: You may wash the mask with ethanol as the mask is **not** sterile. The mask may be applied at any point during the assay. For real-time assays, it is 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 most convenient to apply the mask just before reading assay results.

6. If performing real-time analysis of cell invasion, pre-label cells with a fluorescent stain at this time. Refer to Section V and Appendix II for further suggestions on data acquisition and fluorescent labeling of live cells.

7. Collect cells and prepare a suspension in cell culture medium that is 125 times greater in cell density than the target seeding concentration.

   For example; for a final concentration of 30,000 cells/well, the suspension concentration should be 3.75 x 10^6 cells/mL.

   First Time Users: The optimum seeding density of cells should be determined for each cell type. Please refer to Appendix I for a discussion of this process.

Figure 4. Media is added with Single or Multi-Channel Pipette

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

8. Prepare a 5.0 mL of the **Collagen I Matrix** solution with embedded cells using the following components:
   - 10X PBS (sterile), [to achieve a 1X PBS final concentration]
   - 7.5% Sodium Bicarbonate (sterile), [0.0125 mL / mL of Oris™ Collagen I (Rat tail) stock reagent]
   - Deionized water (sterile)
   - Oris™ Collagen I (Rat tail)
   - Cell suspension, [20% of total solution volume]

   IMPORTANT: Prior to/during use, keep the ***Oris™ Collagen I (Rat tail)*** and the resulting ***Collagen I Matrix*** solution on ice. In addition, the use of chilled pipette tips/reservoirs might be beneficial.

   The following are volumes for preparing 5.0 mL of 3.0 mg/mL **Collagen I Matrix** solution:

   0.5 mL 10X PBS buffer
   0.0375 mL 7.5% sodium bicarbonate
   0.4625 mL deionized water
   3 mL Oris™ Collagen I (Rat tail)
   1 mL cell suspension
   5.0 mL total volume

   On ice, combine the 10X PBS, 7.5% Sodium Bicarbonate, and deionized water. Next add the **Oris™ Collagen I (Rat tail)**. Lastly, add the prepared cell suspension. Gently pipette up and down to completely mix the **Collagen I Matrix** solution with the cells.

NOTE: Supplements, such as growth factors, may be mixed with the **Collagen I Matrix** solution.

9. Pipette a total 40 µL of the **Collagen I Matrix** into each test well through the side ports of the Oris™ Cell Seeding Stopper. It is recommended to pipette 20 µL down each side port to ensure even collagen distribution throughout the well.

NOTE: Do not disturb the Oris™ Cell Seeding Stopper or the Collagen I plate surface coating when introducing the pipette tip into the well. Keep the pipette tips vertical while dispensing to avoid the Collagen I Matrix solution getting caught on the well wall instead of the well bottom. An elongated tip may be useful.

10. Incubate the plate containing the Oris™ Cell Seeding Stoppers and Collagen I Matrix in a humidified chamber (37°C, 5% CO_2) for 1 hour to promote collagen polymerization.

11. Remove plate from incubator.

12. Using the Oris™ Stopper Tool, remove stoppers (see Figure 5). Leave any stoppers in place that have been designated as pre-invasion t=0 control until completion of the assay.
    - 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*** and remove stoppers gently.

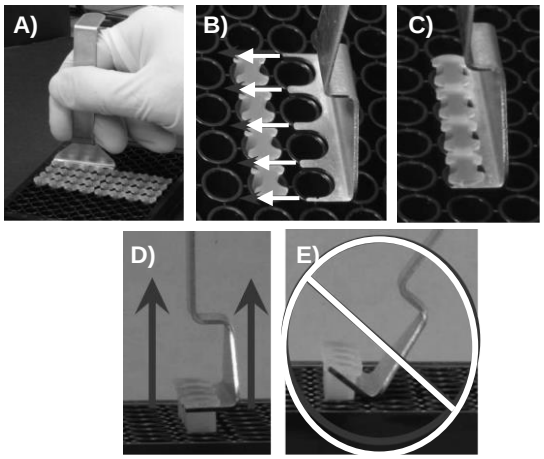

Figure 5. Removal of Stoppers. Panels A, B, and C) Position the Tines of the Stopper Tool between the Stopper Tips, D) Lift Vertically, and E) Do NOT Pry Stoppers

Do not use the Oris™ Stopper Tool as a lever to pry the stoppers from the well (see Figure 5E), as doing so may cause displacement of the collagen and may distort the detection zone area.

NOTE: You may wash the Oris™ Stopper Tool with 70% ethanol as the Stopper Tool is not sterile.

13. Prepare 1.5 mL of the ***Oris™ Collagen I Matrix*** solution in a similar way as Step 8, to fill the newly created detection zone. (This preparation process can be started prior to step 9 immediately before the plate is removed from incubation).

    The following are volumes for preparing 1.5 mL of 3.0 mg/mL **Collagen I Matrix** solution:

    0.150 mL 10X PBS buffer
    0.01125 mL 7.5% sodium bicarbonate
    0.13875 mL deionized water
    0.9 mL Oris™ Collagen I (Rat tail)
    0.3 mL media
    1.5 mL total volume

    NOTE: Supplements, such as growth factors, may be mixed with the **Collagen I Matrix** solution.

14. Pipette 10 µL of the **Collagen I Matrix** solution into the detection zone in the center of each well. Try to get the **Collagen I Matrix** solution all the way to the bottom of the well of the detection zone so that the material fills the cylindrical detection zone completely from bottom to top.

    NOTE: Due to the clarity of the collagen, it may be difficult to see the detection zone. The Oris™ Cell Seeding Stoppers position the detection zone in the center of every well. It may be helpful to use a slender pipette tip when pipetting the Collagen I Matrix solution to avoid damaging to the outer collagen ring.

15. Incubate plate in a humidified chamber (37°C, 5% CO_2) for 1 hour to permit polymerization of the **Collagen I Matrix** in the detection zone.

16. Remove plate from the incubator.

17. Add 100 µL of cell culture growth medium on top of the 3D Embedded Collagen I Matrix. **Optional**: Invasion inhibitors or stimulants may be added to the medium.

    IMPORTANT: Use caution when adding media to not disturb **Collagen I Matrix** solution.

18. Incubate plate in a humidified chamber (37°C, 5% CO_2) to permit cell movement (the period of incubation is cell line dependent). Monitor the cells periodically to check for extent of migration. Refresh media or supplements, every 48 - 72 hours, as needed, for the duration of the experiment.

19. If performing an endpoint analysis of cell invasion, fix and stain cells with a fluorescent stain after sufficient invasion has occurred. Refer to Section V and Appendices II & III for further information on data acquisition and fluorescence staining technique.

NOTE: Oris™ Cell Seeding Stoppers are for single use only.

V. DATA ACQUISITION

The readout of the Oris™ 3D Embedded Invasion Assay can be conducted at any time throughout, as well as, at the end of the assay. You may use any commercially available stain or labeling technique. The readout can be performed using a microscope, a microplate reader, or a High Content Screening or High Content Imaging instrument.

Microscope Analysis

- Visual cell counting or image analysis software, such as NIH ImageJ freeware, can be used.
- Microscopic observations are possible using phase contrast or fluorescence microscopy.
- No need to attach the Oris™ Detection Mask to the Oris™ microplate.
- To set up reference controls, refer to Section III: Precautions and Recommendations.

Microplate Reader Analysis

- Attach the Oris™ Detection Mask to the bottom of the Oris™ microplate (see Section IV, Step 5).
- 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 III: Precautions and Recommendations.

Sample Data Obtained via Microscopy are shown in Figure 6.

- Oris™ Collagen I Matrix solution at 3 mg/mL with 30,000 HT-1080 cells/well (i.e. 40 µL with $3.75x10^6$ cells/mL) was added to the plate and incubated (37°C/5%CO_2) for 1 hour. The stoppers were removed from the wells and the detection zone was filled with 3 mg/mL Collagen I Matrix. Collagen in the detection zone was allowed to polymerize for 1 hour. Complete medium (10% FBS) with either GM6001 (MMP inhibitor) or DMSO (Control) was added on top of the Collagen I Matrix. The plate was then incubated for seven days to permit cell invasion. Cells were labeled with TRITC-phalloidin (F-actin) and DAPI (nuclei) fluorescent stains. Images were captured using a Zeiss Axio Observer.Z1 inverted microscope. Cells in the detection zone were quantified using ImageJ software (NIH). The images below illustrate representative data from full drug inhibition with 250 µM GM6001 (image 1a) and post-invasion, 0.25% DMSO (image 1b) wells. The histogram depicts the average cell count (mean +/- SD of seven wells) for number of cells invaded into the detection zones for each condition.

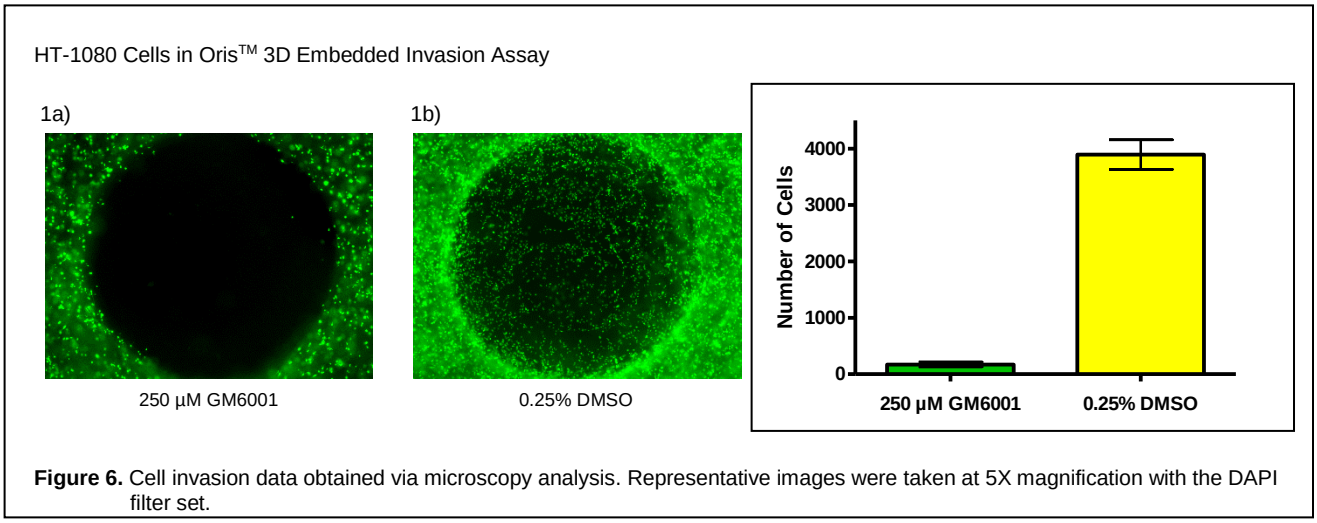

Figure 6. Cell invasion data obtained via microscopy analysis. Representative images were taken at 5X magnification with the DAPI filter set.

VI. Oris™ PLATE DIMENSIONS

Diameter of Well – Bottom	6.3 mm
Diameter of Well – Top	6.45 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	28.03 mm^2
Effective Area of Central Detection Zone per Well	3.14 mm^2
Plate Height	14.95 mm
Plate Height with Lid (with Oris™ Cell Seeding Stoppers)	17.9 mm
Offset of Wells (A-1 location, X)	14.32 mm
Offset of Wells (A-1 location, Y)	11.25 mm
Distance between Wells	9 mm (on center)
Well Depth	12.1 mm
Thickness of Well Bottom	0.25 mm
Well Coating Material	Collagen I, rat-tail
Storage Conditions	Refrigerate (4°C)

VII. ORDERING INFORMATION

Product Name	Coating	Size	Detection Zone Format
Oris™ 3D Embedded Invasion Assays	Collagen I Coated	1-pack (EIA1) 3-pack (EIA3)	Oris™ Cell Seeding Stoppers (pre-populated)
		Starter pack (EIAST)	Oris™ Cell Seeding Stoppers (not pre-populated)
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 (TC, Col, HFn)	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 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	1-pack (PRO384CMA1) 5-pack (PRO384CMA5)	Biocompatible Gel
	Collagen I Coated	1-pack (PRO384CMACC1) 5-pack (PRO384CMACC5)	
Oris™ Pro Invasion Assays	Collagen I Coated	1-pack (PROIA1) 3-pack (PROIA3) 1-pack (PROIAPLUS1) 3-pack (PROIAPLUS3)	Biocompatible Gel

For a complete list of assays, visit Platypus Technologies at 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

Optimal cell seeding density can vary depending on the properties of each cell line being tested. A suggested final concentration range is between 5.0 x 10^5 – 9.0 x 10^5 cell/mL (i.e. 20,000 – 36,000 cell/well). It may be helpful to evaluate various cell densities. The following is an example of an experimental set up to determine optimal seeding concentration.

a) Prepare a log-phase culture of the cell line to be tested. Collect cells and determine the total number of cells present.
b) Pellet cells by centrifugation. Prepare the cell suspensions in a similar manner as described in Section IV, Step 7.
c) Continue with the assay in accordance with the remaining steps of the protocol in Section IV.
d) Dispense the collagen/cell suspension into the test wells of the 96-well plate to result in the following plate layout:

Column	1	2	3
Cells / well	80,000	40,000	20,000
Number of wells	8	8	8

e) Incubate the plate in a humidified chamber (37°C, 5% CO_2) for 1 hour to polymerize collagen/cell solution.
f) Remove the Oris™ Cell Seeding Stoppers from each well (see Figure 5). Add the Collagen I Matrix solution to fill the detection zone column and incubate for 1 hour to allow for collagen polymerization.
g) Add 100 µL of culture medium to each well and place the plate back in the incubator.
h) Monitor periodically to assess cell movement.
i) Upon completion of the assay, use a microscope to examine each well to determine the optimal cell seeding concentration for your specific cell type.

APPENDIX II: Fluorescent Labeling Live Cell Options

It is important to label cells using a fluorescent reagent that uniformly stains cells. Please consult the manufacturer of your fluorescent stain for specific considerations.

NOTE: Use caution when adding/removing solutions so that the Collagen I Matrix is not dislodged from the bottom/sides of the well.

The following is an example Fluorescent Staining Protocol to label live cells with Calcein AM:

a) To stain one fully-seeded 96-well plate, combine 5 µL of Calcein AM (1 mg/mL in anhydrous DMSO) with 10 mL of phenol red-free and serum-free medium or 1x PBS (containing both Ca^{++} and Mg^{++}). Protect diluted Calcein AM solution from light until ready to use in step d.
b) Carefully remove the culture medium from wells with a pipette.
c) Wash wells with 100 µL of PBS (containing both Ca^{++} and Mg^{++}).
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.
    i. 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 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 guidance on instrument settings.
g) Additionally, the plate may be analyzed by microscopy using fluorescent filters with excitation and emission wavelengths of 495/515 nm, respectively.

APPENDIX III: Fluorescent Labeling Fixed Cell Options

This procedure is intended to assist in obtaining data from the **Oris™ 3D Embedded Invasion Assay** using various fluorescent labels.

It is important to use a fluorescent reagent that uniformly stains cells. Please consult the manufacturer of your fluorescent stain for specific considerations.

NOTE: Use caution when adding/removing solutions so that the Collagen I Matrix is not dislodged from the bottom/sides of the well.

The following is an example protocol to label fixed cells with TRITC-phalloidin (F-actin) and DAPI (nuclei) fluorescent stains:

a) To fix one fully-seeded 96-well plate, prepare 10 mL of fixative solution to have a final well concentration of 0.25% glutaraldehyde solution in 1X PBS prepared from stock 8% glutaraldehyde solution (Electron Microscopy Sciences)).
b) Remove medium and rinse wells with 100 µL of 1X PBS.
c) Remove PBS and add 100 µL of a fixative solution (final well concentration of 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 protected from light.
g) Remove the TRITC-phalloidin and add 100 µL of a 1:1000 dilution of DAPI (ThermoScientific) in PBS.
h) Incubate plate at room temperature for 1 hour (protect from light).
i) Periodically check progress of stain.
j) Remove DAPI stain and wash wells 2x for 5 minutes each with 200 µL of PBS.
k) 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 to use only one stain. Substitutions or additional cytostaining or immunostaining may be performed using non-overlapping fluorophores and by utilizing the appropriate filters with your imaging equipment.