PLATYPUS TECHNOLOGIES

Bringing Science to the Surface™

Oris™ Universal Cell Migration Assembly Kit

Product No.: CMAU101 & CMAU505

96-well Assay for Investigating
Cell Migration and Cell Invasion of Adherent Cell Lines

PROTOCOL & INSTRUCTIONS

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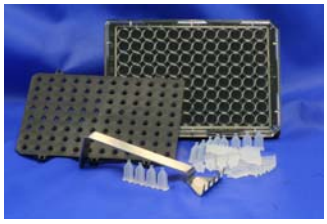


AMSBIO| www.amsbio.com | info@amsbio.com

UK & Rest of the World
AMS Biotechnology (Europe) Ltd
184 Park Drive, Milton Park
Abingdon, UK
T: +44 (0)1235 828 200
F: +44 (0) 1235 820 482

North America
amsbio LLC
1035 Cambridge Street,
Cambridge, MA 02141
T: +1 617 945 5033 or
T: +1 800 987 0985
F: +1 617 945 8218

Germany
AMS Biotechnology (Europe) Ltd
Bockenheimer Landstr. 17/19
60325 Frankfurt/Main
T: +49 (0) 69 779099
F: +49 (0) 69 13376880

Switzerland
AMS Biotechnology (Europe) Ltd
Centro Nord-Sud 2E
CH-6934 Bioggio-Lugano
T: +41(0) 91 604 55 22
F: +41(0) 91 605 17 85

ORIS™ UNIVERSAL CELL MIGRATION ASSEMBLY KIT

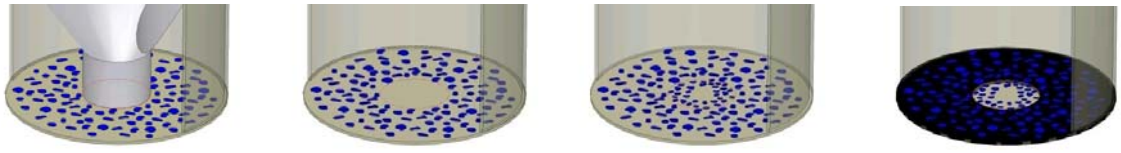
I. INTRODUCTION

The Oris™ Universal Cell Migration Assembly Kit is a reproducible, sensitive, and flexible assay that can be used to monitor cell migration or cell invasion. The Oris™ Universal Cell Migration Assembly Kit now gives researchers more control over designing a cell migration assay. Each Oris™ Cell Migration Assembly kit contains the Oris™ Cell Seeding Stoppers for creating a detection zone at the center of each well in a 96-well plate. Since the stoppers are not pre-inserted into the wells, researchers can coat the plate with an extracellular matrix (ECM) component to design their assay. Researchers may also apply 3-dimensional overlays in each well to watch how cells invade and respond to various compounds (chemokinesis). Each kit is supplied with a 96-well, black, clear bottom plate, an Oris™ Detection Mask, an Oris™ Stopper Tool, and Oris™ Cell Seeding Stoppers. The Oris™ Universal Cell Migration Assembly Kit is designed to be used with any commercially available stain or labeling technique. Readout can be performed by microscopy or use of a microplate reader.

The Oris™ Universal Cell Migration Assembly Kit has been designed for use with adherent cell cultures. This assay has been successfully used with 3T3-Swiss albino, HT-1080, HCEC, HUVEC, and MCF10A cell lines.

Using the Oris™ Universal Cell Migration Assembly Kit offers the following features & benefits:

- **Membrane-free, Cell Migration or Invasion** - perform studies without manipulating transmembrane inserts: observe live images of cell movement.
- **Creative Assay Design** - coat any ECM or BME on the plate to create a 2-D environment for cell migration or cell invasion assays.
- **Preserve Cell Morphology** - eliminate need for cells to penetrate through a polycarbonate membrane; cells can move across a treated surface or within a user-applied ECM
- **Real-time Monitoring** - monitor changes in cell structure in real-time throughout the experiment.
- **Versatile Detection** - analyze cells treated with multiple fluorescent probes, labels or stains by using a microscope, digital imaging system or fluorescence microplate reader.
- **Reproducible Results** - obtain greater reproducibility using the Oris™ Cell Seeding Stoppers compared to wound healing or scratch assays.
- **Flexible** - perform kinetic or endpoint assays without the use of special instrumentation.



Insert Stoppers, Seed Cells onto Oris™ Plate & Allow to Adhere

Remove Stoppers to Create Detection Zone

Allow Cells to Migrate into Detection Zone

Analyze Cells in Detection Zone: Microplate Reader Analysis - Detection Mask Attached; Image Analysis - No Mask Required

Figure 1. Schematic of Oris™ Universal Cell Migration Assembly Kit

II. ORIS™ PLATE DIMENSIONS

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
Plate Height	14.9 mm
Plate Height with Lid (with Oris™ Cell Seeding Stoppers)	17.9 mm
Offset of Wells (A-1 location, X)	14.4 mm
Offset of Wells (A-1 location, Y)	11.2 mm
Distance between Wells	9 mm (on center)
Well Depth	12.2 mm
Thickness of Well Bottom	0.25 mm
Storage Conditions	Refrigerate (4°C)

Important: Read Instructions Before Performing any Oris™ Assay.

III. MATERIALS PROVIDED

Product No.: CMAU101
Oris™-compatible, 96-well (black, clear bottom) Plate, 1
Oris™ Cell Seeding Stoppers, 96
Oris™ Detection Mask, 1
Oris™ Stopper Tool, 1

Product No.: CMAU505
Oris™-compatible, 96-well (black, clear bottom) Plates, 5
Oris™ Cell Seeding Stoppers, 5 x 96
Oris™ Detection Mask, 1
Oris™ Stopper Tool, 1

IV. MATERIALS REQUIRED

- Biological Cells
- Sterile PBS (containing both Calcium and Magnesium)
- Complete Cell Culture Growth Medium (containing serum)
- 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 (eg., CellTracker™ Green, Calcein AM)
  - *required if performing assay readout via microplate reader.*
- Extracellular Matrix (ECM) or Basement membrane Extract (BME) for creating a 2-D coating or a 3-D assay (optional)

V. UNIVERSAL CELL MIGRATION ASSEMBLY PROTOCOL

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

1. Remove the Oris™-compatible 96-well plate from refrigeration and place on lab bench for ~1 hour to allow it to equilibrate to room temperature.

   Optional: If desired, coat the bottom of the wells with an ECM component (collagen, fibronectin, laminin, etc.) and allow the ECM to dry prior to populating the plate with the Oris™ Cell Seeding Stoppers.

2. 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.

NOTE: It is extremely important to ensure that the stoppers are inserted perpendicular to the well bottom and are fully engaged with the well bottom. Failure to do so will increase the CV of your data set. If you require data sets with low CVs [potential for ≤12 %], the pre-populated Oris™ Cell Migration Assay kit (#CMA1.101) is recommended.

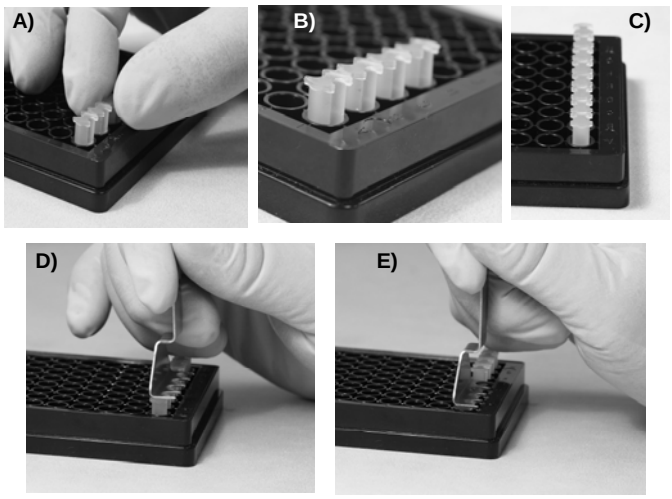


Figure 2. Stopper Insertion Process. A) Placement of Stoppers into Wells, B) Close-up of Stoppers Partially Inserted into Wells, C) Proper Placement of Stoppers, D) Pressing of Stoppers into Wells, and E) Fully Inserted Stoppers

3. 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.

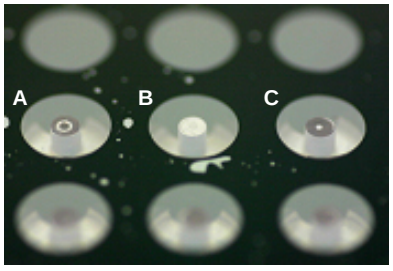


Figure 3. Stoppers that are:
A) Partially Sealed
B) Unsealed
C) Completely Sealed

4. 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.

   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 in 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 96-well 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,

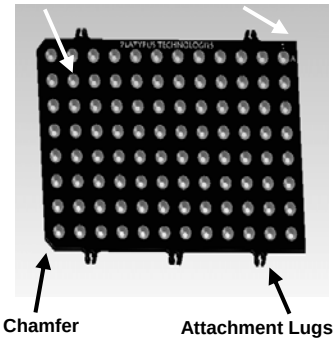


Figure 4. Features of Detection Mask

using fixed and stained cells, it is often most convenient to apply the mask just before reading assay results.

V. UNIVERSAL CELL MIGRATION ASSEMBLY PROTOCOL, continued

5. If performing a kinetic analysis of cell migration, pre-label cells to be seeded with a fluorescent stain now.

6. Collect cells and prepare a suspension that is 10-fold greater in density than 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 migration assay. Please refer to Appendix I for a discussion of this process.

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

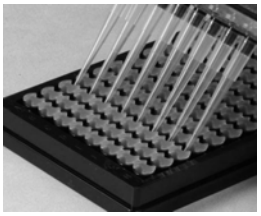


Figure 5. Media is Added with Pipette

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 when introducing the pipette tip into the well. A slender/elongated tip or a gel loading tip may be useful.

8. **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).

9. Incubate the seeded plate containing the Oris™ Cell Seeding Stoppers in a humidified chamber (37°C, 5% CO_2) for 4 to 18 hours (cell line dependent) to permit cell attachment.

10. Remove plate from incubator.

11. Designate several 'reference' wells in which the stoppers will remain in place until results are read (t=0 pre-migration controls).

12. Using the Oris™ Stopper Tool, remove all other 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.

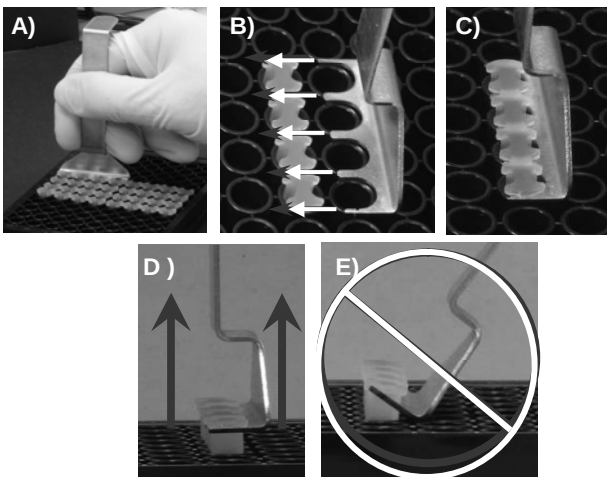


Figure 6. Removal of Stoppers. Panels A, B, and C) Position the Tines of the Stopper Tool between the Stopper Tips, D) Lift Vertically, and E) Do NOT Pry Stoppers

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.

13. Remove media with a pipette and ***gently*** wash wells with 100 µL of sterile PBS (or media) to remove any unattached cells. Do not aspirate using an in-house vacuum.

    Optional: If the plate was coated with an ECM (in Step 1), an overlay of ECM may be introduced in the wells to facilitate a 3-D invasion assay. Optimization of experimental conditions will be required to establish invasion conditions for a given cell line.

14. Add 100 µL of fresh culture media to each well.

15. Cells may be examined microscopically throughout the incubation period to monitor progression of migration. Migration time will vary depending upon cell type, experimental design, and ECM.

16. If performing an endpoint analysis of cell migration, stain cells with a fluorescent stain after sufficient migration 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.

VI. DATA ACQUISITION

The readout of the Oris™ Universal Cell Migration Assembly Kit can be conducted at any time, allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Universal Cell Migration Assembly Kit is designed to be used with any commercially available stain or labeling technique. 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: Microscopy observations are possible using phase contrast or bright field microscopy.
- No need to attach the Oris™ Detection Mask to the Oris™ plate.
- Sample Data using a colorimetric stain is shown in Figure 7. Wells populated with Oris™ Cell Seeding Stoppers were seeded with 50,000 HT-1080 cells (i.e., 100 µL of $5x10^5$ cells/mL) and incubated for 4 hours. The stoppers were removed from test wells, but remained in place in the pre-migration reference wells until the time of the assay readout. The seeded plate was incubated in a humidified chamber for 24 hours to permit cell migration. Stoppers were removed from the reference wells and all cells were fixed and treated with Wright-Giemsa stain. Images were captured using bright field microscopy (7A) and then imported to Image J software for analysis using thresholding. The images below, captured without a detection mask in place, illustrate representative data from pre-migration (t=0 hrs) and post-migration (t=24 hrs) wells. The graph (7B) depicts the average pixel number +/- S.D. in the detection zones for each condition (n = 8 wells/condition).

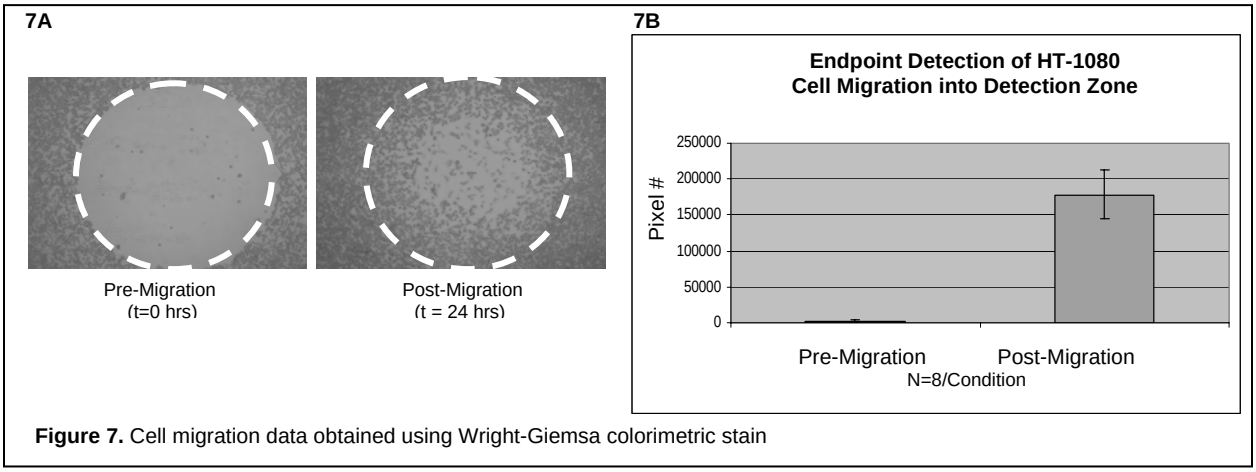


Figure 7. Cell migration data obtained using Wright-Giemsa colorimetric stain

Microplate Reader Analysis

- Attach the Oris™ Detection Mask to the bottom of the Oris™ plate (see Step 3 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.
- Sample data using a fluorescent stain and microplate reader analysis are shown in Figure 8. HT-1080 cells were fluorescently stained with CellTracker™ Green and wells populated with Oris™ Cell Seeding Stoppers were seeded with 50,000 cells (i.e., 100 µL of $5x10^5$ cells/mL). After a 4 hour incubation, stoppers were removed from test wells, but remained in place in the pre-migration reference wells until the time of the assay readout. The seeded plate was incubated in a humidified chamber for 28 hours and at various time points the fluorescence signals in the detection zones were measured using a microplate reader. The images below (Figure 8A), captured without a detection mask in place, illustrate representative data from pre-migration (t=0 hrs) and post-migration (t = 21 hrs) wells. The graph depicts a real-time analysis of cell migration that was prepared by transposing the fluorescent signal into cell numbers (Figure 8B).

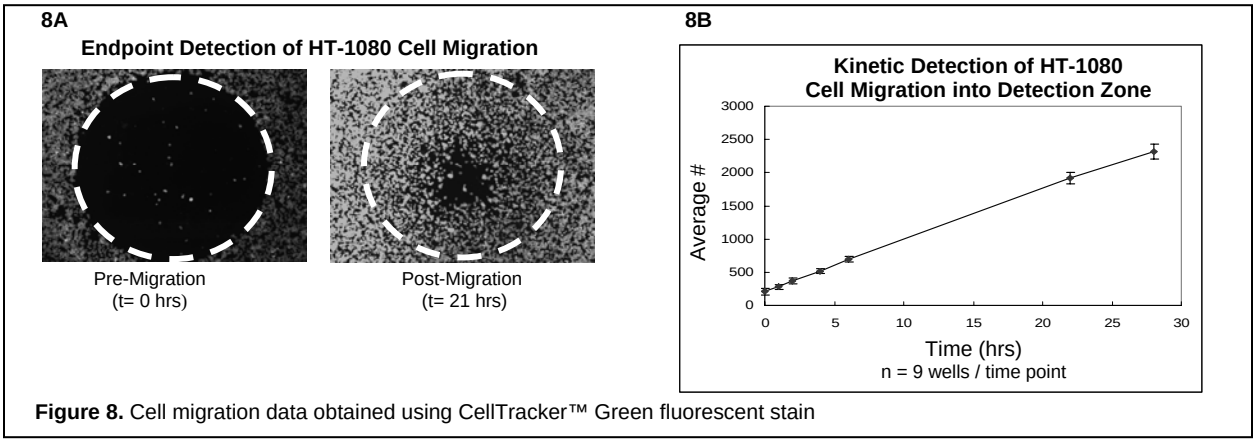


Figure 8. Cell migration data obtained using CellTracker™ Green fluorescent stain

VII. 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 Cell Invasion Assays	Collagen I Coated	1-pack (PROCIACC1) 2-pack (PROCIACC2)	Biocompatible Gel
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™ 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)

VIII. TERMS & CONDITIONS

Certain uses of these products may be covered by U.S. Pat. No. 7,018,838, No. 7,842,499, Appl. No. 11/342,413, Appl. No. 10/579,118, and Appl. No. 12/195,007 issued to or applied for by PLATYPUS. Certain applications of PLATYPUS products may require licenses from other parties. Determining the existence and scope of such third party intellectual property is the responsibility of the PURCHASER. Purchase of the product provides the PURCHASER with a limited non-transferable license under any PLATYPUS patents or patent applications to use the product for internal research unless there is a written limitation to this license in the product literature. PURCHASER is responsible for carefully reviewing the product literature and respecting any limitations to this license, e.g. limitations for commercial use or research by for-profit institutions. These products may not be resold, modified for resale, used to manufacture commercial products, or used to develop commercial products without the express written approval of PLATYPUS.

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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™ Universal Cell Migration Assembly Kit. 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 cm^2. Based on the typical seeding density of your particular cell line, you can infer a different cell number for your first serial dilution and adjust the numbers below accordingly.
2. Prepare a log-phase culture of the cell line to be tested. Collect cells and determine the total number of cells present.
3. Pellet cells by centrifugation. Prepare three serial dilutions at final concentrations of 1.0 x 10^6, 0.5 x 10^6 and 0.25 x 10^6 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% CO_2) 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™ Universal Cell Migration Assembly Kit via colorimetric or microscopic analysis, you have successfully determined the optimal cell seeding concentration to be used in Step 5 of the Universal Cell Migration Assembly 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™ Universal Cell Migration Assembly Kit.

1. Using the optimal cell seeding concentration determined in Appendix I, perform a cell migration assay per Section V, Universal Cell Migration Assembly Protocol using culture conditions expected to result in robust cell migration. Be sure to include equal numbers of pre-migration reference wells (stoppers left in place until staining) and post-migration 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™ Universal Cell Migration Assembly Kit has been designed to work with all types of fluorescent stains and staining techniques. The precise method for staining cells with fluorescent stains varies according to the nature of the individual stain. It is important to stain cells using a fluorescent reagent that uniformly stains cells. Probes affected by experimental conditions will increase variability of results and reduce correlation between fluorescence signal and cell migration. Please consult the manufacturer of your fluorescent stain for specific considerations.

   The following is an example Fluorescent Staining Protocol 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 (containing both Ca^{++} and Mg^{++}). 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 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 (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-migration and post-migration 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 5 of the Universal Cell Migration Assembly Protocol) and microplate reader settings for analysis of cell migration using a fluorescence microplate reader.

UK & Rest of the World
AMS Biotechnology (Europe) Ltd
184 Park Drive, Milton Park
Abingdon, UK
T: +44 (0)1235 828 200
F: +44 (0) 1235 820 482

North America
amsbio LLC
1035 Cambridge Street,
Cambridge, MA 02141
T: +1 617 945 5033 or
T: +1 800 987 0985
F: +1 617 945 8218

Germany
AMS Biotechnology (Europe) Ltd
Bockenheimer Landstr. 17/19
60325 Frankfurt/Main
T: +49 (0) 69 779099
F: +49 (0) 69 13376880

Switzerland
AMS Biotechnology (Europe) Ltd
Centro Nord-Sud 2E
CH-6934 Bioggio-Lugano
T: +41(0) 91 604 55 22
F: +41(0) 91 605 17 85