

CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

96 Well 3D Spheroid BME Cell Invasion Assay

**Reagent kit for investigating cell invasion
in 3D Culture**

96 samples

Catalog #: 3500-096-K

Cultrex® 96 Well 3D Spheroid BME Cell Invasion Assay

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I. Quick Reference Procedure for Trevigen's 96 Well 3D Spheroid BME Cell Invasion Assay:

Read through the complete Instructions for Use prior to using this kit. Determine the optimal seeding density for each cell line used. In general, 3,000 cells per well is a good starting point.

1. Culture cells per manufacturer's recommendation; adherent cells should be cultured to no more than 80% confluence.
2. Thaw 10X Spheroid Formation ECM on ice for two hours or overnight in a 4 °C refrigerator.
3. Harvest cells (section VII.A) and resuspend in 1X Spheroid Formation ECM (section VI.1).
4. Aliquot 50 µl of cell suspension per well of the 3D Culture Qualified 96 Well Spheroid Formation Plate.
5. Centrifuge at 200 x *g* for 3 minutes at room temperature in a swinging bucket rotor.
6. Incubate at 37 °C in a tissue culture incubator for 72 hours to promote spheroid formation.
7. Thaw Invasion Matrix on ice for four hours or overnight in a 4 °C refrigerator.
8. Place 3D Culture Qualified 96 Well Spheroid Formation Plate on ice in refrigerator for 5 minutes to cool wells.
9. Working on ice, add 50 µl of Invasion Matrix per well of the 3D Culture Qualified 96 Well Spheroid Formation Plate (section V.2). Centrifuge plates at 300 x *g* at 4 °C for 5 minutes in a swinging bucket rotor to eliminate bubbles and position spheroids within the Invasion Matrix towards the middle of the well. **Caution: centrifugation at high speeds and/or extended periods of time can adversely impact subsequent spheroid invasion.**
10. Transfer plate to a tissue culture incubator set at 37 °C for one hour to promote gel formation.
11. After one hour, add 100 µl of cell culture medium containing chemoattractant and invasion modulating compounds, if applicable. See section V.3.
12. Incubate at 37 °C in a tissue culture incubator for 3 to 6 days, and photograph the spheroid in each well every 24 hours using the 4X objective.
13. Analyze images to evaluate 3D culture cell invasion.

II. Background

The 96 Well 3D Spheroid BME Cell Invasion Assay offers a standardized, three-dimensional, high content format for quantitating the degree to which invasive cells penetrate a barrier, consisting of basement membrane components, *in vitro* in response to chemoattractants and/or inhibiting compounds, which is fundamental for angiogenesis¹, embryonic development², immune response³, and tumor cell metastasis⁴. Basement membranes are continuous sheets of specialized extracellular matrix that form an interface between endothelial, epithelial, muscle, or neuronal cells and their adjacent stroma. They not only support cells and cell layers, but they also play an essential role in tissue organization that influences cell adhesion, migration, proliferation, and differentiation. Basement membranes are degraded and regenerated during development and wound repair, and they are major barriers to invasion by metastatic tumor cells. Current methods for assessing cell invasion through basement membrane barrier most commonly utilize a Boyden chamber approach, such as the Cultrex® BME Cell Invasion Assays. This method does provide a quick, easy, and quantitative approach. However, it is very difficult to visualize invasion microscopically, and the assay starts with a single cell suspension which may negate early steps in the metastatic process, such as the initial epithelial to mesenchymal transition (EMT).

The need for more complete and physiologically predictive cancer invasion models has driven the development of the 96 Well 3D Spheroid BME Cell Invasion Assay. There is growing evidence that tumor cell aggregates or spheroids are more representative of tumors *in vivo*, and they exhibit several physiological traits including similar morphology, the formation of cell-cell bonds, decreased proliferation rates, increased cell survival, tumor dormancy, and a hypoxic core⁵⁻⁸. Applying this model to a 3D culture invasion assay provides a more physiological approach for assessing tumor invasion and offers a visual component that can be quantitated through image analysis.

The 96 Well 3D Spheroid BME Cell Invasion Assay utilizes a 3D Culture Qualified 96 Well Spheroid Formation Plate alongside a specialized Spheroid Formation ECM to drive aggregation and/or spheroid formation of cells. Upon completion of spheroid formation, the spheroid is embedded in an invasion matrix composed of basement membrane proteins. This matrix forms a hydrogel network on which invasive cells can travel. At this point, invasion modulating agents can be applied to the system to evaluate the impact on cell response. Cell invasion is visualized microscopically and can be quantitated through image analysis software.

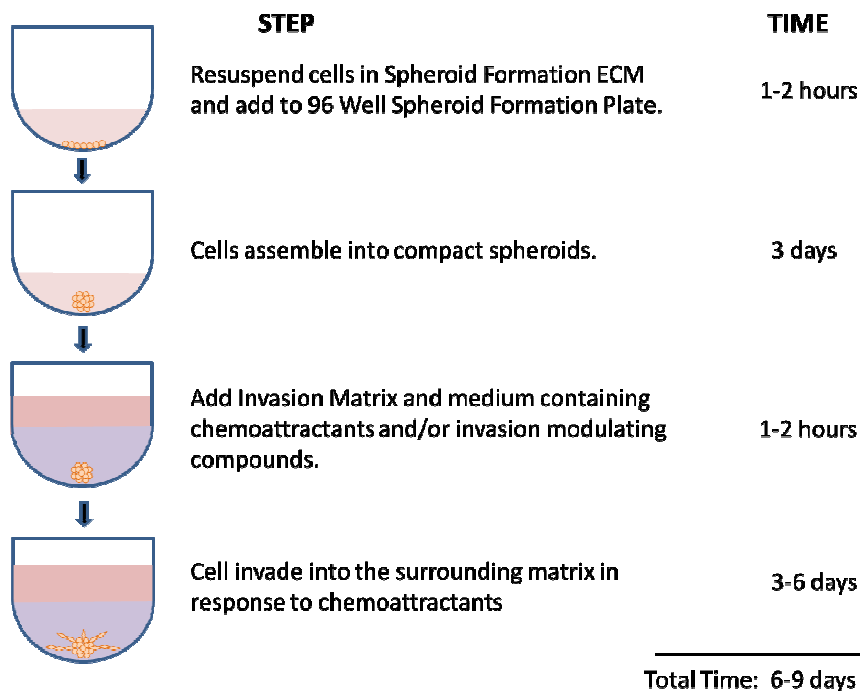


Figure 1. Steps comprising the 96 Well 3D Spheroid BME Cell Invasion Assay.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of these products may not yet have been fully investigated; therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. The **CULTREX® 3D Culture 96 Well BME Invasion Assay** contains reagents that may be harmful if swallowed, or come in contact with skin or eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Material safety data sheets are available on request.

IV. Materials Supplied

<u>Component</u>	<u>Quantity</u>	<u>Storage</u>	<u>Catalog #</u>
10X Spheroid Formation ECM	600 µl	-80°C	3500-096-01
3D Culture Qualified 96 Well Spheroid Formation Plate	Each	Room Temp	3500-096-02
Invasion Matrix	6 ml	-80°C	3500-096-03

V. Materials/Equipment Required But Not Supplied

Equipment

1. 1 - 20 µl pipettor, 20 - 200 µl pipettor, and 200 - 1000 µl pipettor
2. Laminar flow hood or clean room
3. 37°C CO₂ incubator
4. Low speed swinging bucket 4°C centrifuge and tubes for cell harvesting
5. Hemocytometer or other means to count cells
6. -80°C storage
7. ice bucket
8. standard light microscope (or inverted)
9. pipette aid
10. timer
11. Bright Field Microscope with 4X objective and digital camera
12. Computer
13. Graphing software, such as Microsoft® Excel®.
14. Image analysis software, such as ImageJ.

Reagents

1. Cell line(s) of interest.
2. Cell Harvesting Buffer; EDTA, trypsin, or other cell detachment buffer.
3. Tissue Culture Growth Media, as recommended by cell supplier.
4. Pharmacological agents for addition to culture medium, if necessary.
5. Sterile PBS or HBSS to wash cells.
6. Trypan blue or equivalent viability stain

Disposables

1. Cell culture flask, 25 cm² or 75 cm²
2. 10 ml and 50 ml conical tubes
3. 1 - 200 µl and 200 - 1000 µl pipette tips
4. 1, 5, and 10 ml serological pipettes
5. gloves

VI. Reagent Preparation

1. 10X Spheroid Formation ECM

10X Spheroid Formation ECM should be thawed on ice at 4 °C and diluted with Tissue Culture Growth Medium chilled to 4 °C; pipet up and down with a serological pipet to mix. Cells are resuspended in 1X Spheroid Formation ECM, and 50 µl of cell suspension is added to each well of the 3D Culture Qualified 96 Well Spheroid Formation Plate. See Table 1 for recommended dilution schedules.

2. Invasion Matrix

Invasion Matrix should be thawed on ice at 4 °C, and gently inverted to make a homogenous solution. If there are visible bubbles in the Invasion Matrix, centrifuge 300 x *g* for 5 minutes at 4 °C in a swinging bucket rotor. Add 50 µl of undiluted Invasion Matrix to each invasion well of a chilled 3D Culture Qualified 96 Well Spheroid Formation Plate.

3. Tissue Culture Medium with Invasion Modulating Compounds

Add 2X concentration of Invasion Modulating compounds within the tissue culture medium to compensate for changes in total volume due to the spheroid embedded within the Invasion Matrix. Tissue culture medium must be 37 °C when added to gelled invasion matrix to preserve gel properties.

VII. Assay Protocol

These procedures should be performed in a biological hood utilizing aseptic technique to prevent contamination.

A. Cell Harvesting

Culture cells per manufacturer's recommendation. The following procedure is suggested and may need to be optimized to suit the cell type(s) being studied.

1. Cells need to be healthy and proliferating prior to use in the assay. Cells should be passaged 2 or 3 times and evaluated for cell viability by trypan blue or equivalent assay. Do not start the assay until cell viability is greater than 90%.
2. Each well requires approximately 2,000 - 5,000 cells, and 25 and 75 cm² flasks yield at least 1 x 10⁶ and 3 x 10⁶ cells, respectively. Plan accordingly.
3. Prior to harvest, visually inspect cells, and record cell health, relative number, and morphology.
4. Wash cells two times with sterile PBS or HBSS. Use 5 ml per wash for a 25 cm² flask and 10 ml per wash for a 75 cm² flask.
5. Harvest cells. For 25 cm² flask or 75 cm² flask, add 1 ml or 2 ml, respectively, of Cell Harvesting Buffer (see Materials Required But Not Supplied), and incubate at 37°C for 5 to 15 minutes until cells have dissociated from bottom of flask.
6. Transfer cells to a 15 ml conical tube, and add 5 ml of cell culture medium.
7. Centrifuge cells at 200 x *g* for 3 minutes to pellet cells, remove medium, and resuspend cells in 2 ml of cell culture medium. Cells may need to be gently pipetted up and down with serological pipette to resuspend cells and break up cell aggregates. Visually inspect cells to verify formation of a single cell suspension, no aggregates prior to counting.
8. Count cells and evaluate cell viability by trypan blue exclusion or equivalent test. Do not start the assay if cell viability is less than 90%.
9. Dilute to 1 x 10⁶ cells per ml in cell culture medium.

B. 96 Well 3D Spheroid BME Cell Invasion Assay

1. Assay Preparation – Prior to Day 0

- a. Establish assay parameters and appropriate controls. Appropriate controls include samples with and without invasion modulating agents, as well as samples with and without Invasion Matrix.
- b. Determine optimal seeding density for each cell line used. In general, 3,000 cells per well is a good starting point. Optimal seeding density can be evaluated by analyzing the size of spheroid formation using the Spheroid Formation protocol (VII.B.2.).
- c. Culture cells per manufacturer's recommendation; adherent cells should be cultured to no greater than 80% confluence.
- d. Thaw 10X Spheroid Formation ECM on ice overnight in a 4°C refrigerator.

2. Spheroid Formation – Day 0

- a. Harvest and count cells, as directed in section VII. A.
- b. Prepare a single cell suspension in 1X Spheroid Formation ECM. See section VI.1. for reagent preparation.

Table 1 Dilution Schedules (10% overage for multiwell dispensing):

Reagent	1 Well	24 Wells	96 Wells
10X Spheroid Formation ECM (4 °C)	5 µl	132 µl	528 µl
Tissue Culture Growth Medium (4 °C)	45-X µl	1188-X µl	4752-X µl
Cells	X µl	X µl	X µl
Total	50 µl	1320 µl	5280 µl

- c. Dispense 50 µl of the single cell suspension in 1X Spheroid Formation ECM per well of the 3D Culture Qualified 96 Well Spheroid Formation Plate.
 - d. Centrifuge at 200 x *g* for 3 minutes at room temperature in a swinging bucket rotor.
 - e. Incubate at 37 °C in a tissue culture incubator for 72 hours to promote spheroid formation.
- ### 3. 3D Culture Cell Invasion – Day 3
- a. Place the 3D Culture Qualified 96 Well Spheroid Formation Plate on ice for 5 minutes to cool wells.
 - b. Working on ice, add 50 µl of Invasion Matrix per well of the 3D Culture Qualified 96 Well Spheroid Formation Plate; see section V.2. for reagent preparation.
 - c. Centrifuge plates at 300 x *g* at 4°C for 5 minutes in a swinging bucket rotor to eliminate bubbles and position spheroids within the

Invasion Matrix towards the middle of the well. **Caution: centrifugation at high speeds and/or extended periods of time can adversely impact subsequent spheroid invasion.**

- d. Transfer plate to a tissue culture incubator set at 37 °C for one hour to promote gel formation of the Invasion Matrix.
- e. After one hour, add 100 µl of warm (37 °C) cell culture medium containing chemoattractant and invasion modulating compounds, if applicable. See section V.3 for reagent preparation.
- f. Incubate the plate at 37 °C in a tissue culture incubator for 3 to 6 days, and photograph the spheroid in each well every 24 hours using the 4X objective. Adjust the lighting and focus to provide the most contrast between the 3D structure and background. The use of fluorescence microscopy for cells that express fluorescent protein or have a fluorescent label may also improve contrast and subsequent analysis. Debris may transfer to the bottom of the 96 well plate during handling; wiping the bottom of the wells with lens paper prior to photographing may improve clarity. The assay may be conducted longer than 6 days if desired; the endpoint is usually when the structure size begins to exceed the field of analysis or the cells begin to expire.
- g. Analyze images using image analysis software to measure changes in the area of the invasive structures to determine the extent of 3D culture BME cell invasion for each sample.

4. Image Analysis

Note: Images may be analyzed using free software such as ImageJ (<http://rsb.info.nih.gov/ij/>); see below. Other image analysis software may be configured to make the same measurements; please consult your software supplier regarding capabilities and instructions.

- a. Photograph image of known size (eg. 1 mm) using 4X objective, and measure pixels in imageJ.
 - i. Open image. Go to File/Open, and select image.
 - ii. Select line tool.
 - iii. Draw a line the length of the object.
 - iv. Go to Analyze/Measure, and record pixel number.
 - v. Calculate the number of pixels in each mm (eg. 600 pixels/mm).

Record result _____ pixels/mm

- b. Set scale; this will need to be done each time ImageJ is opened.
 - i. Go to Analyze/Set Scale.
 - ii. For "Distance in pixels" input value from VII.B.4.v. (above).
 - iii. For "Know distance" input "1,000".
 - iv. For "Unit of Length" input "um".
 - v. Check "Global", and select "OK".

c. Analyze spheroid image.

- i. Open image. Go to File/Open, and select image.
- ii. Convert to an 8 bit image: Go to Image/Type/8 bit (check).
- iii. Adjust image threshold. Go to Image/Adjust/Threshold (check). The program will distinguish between dark and light pixels, and the threshold may be adjusted on the histogram using the sliding scale. Similar settings will work for photographs taken under the same conditions. Areas of dark pixels will be overlaid with red. Adjust the threshold so that only the spheroid image is overlaid.
- iv. Select spheroid. Select the circle tool and surround the image. This will eliminate any dark pixels outside of the spheroid from the measure.
- v. Go to Analyze/Measure. This will measure the area of the spheroid structure (µm²) in a table. Multiple measurements may be made in one table. Save table.
- vi. Convert the table to an Excel worksheet. Open with Excel and "Save As" an .xls or .xlsx file.

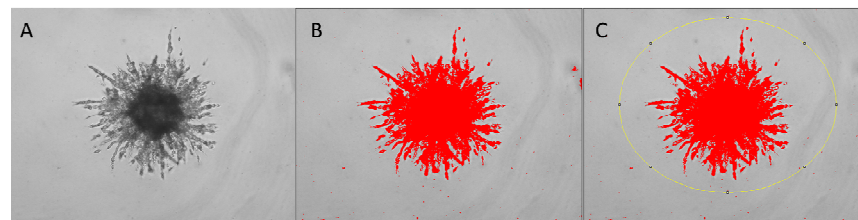


Figure 2. Process for analysis of 3D invasion. A) Capture image and convert to 8 bit; B) set threshold to capture the total structure; and C) select structure to calculate total area.

VIII. Data Interpretation

1. The Cultrex® 3D Culture 96 Well BME Invasion Assay provides morphological and quantitative analysis of cell invasion. Invading cells project out of the spheroid into the surrounding matrix as spindle-like projections; whereas, there are no projections in the absence of an invasion matrix, see figure 3. This results in large changes in surface area that occur in a relatively small amount of time for invasive cell lines which do not occur for non-invasive cell lines, see figure 4. These changes in surface area can be measured and used to compare relative invasiveness of different cell lines or cell lines subjected to genetic manipulation.

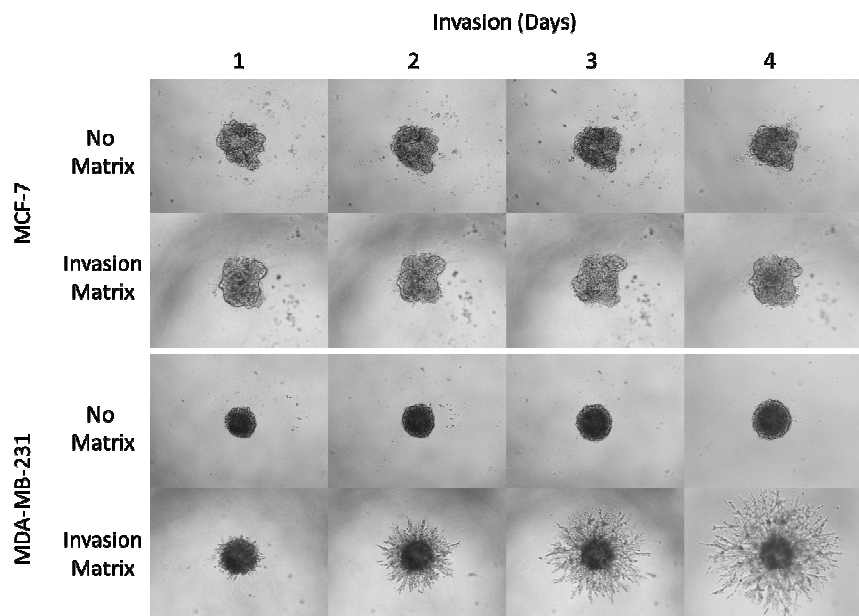


Figure 3. Morphology of 3D cell invasion over a four day period. Non-invasive cells (MCF-7) remain as cell aggregates and do not invade into the surrounding invasion matrix; whereas, invasive cells (MDA-MB-231) invade into the surrounding invasion matrix as spindle-like protrusions.

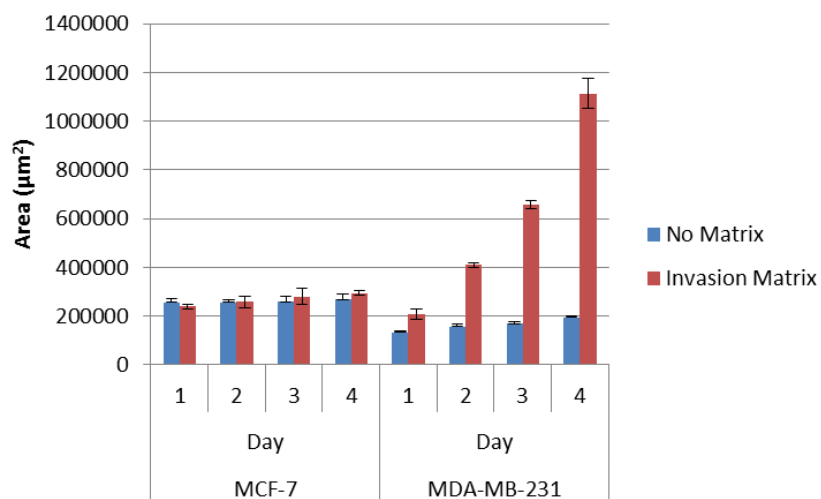
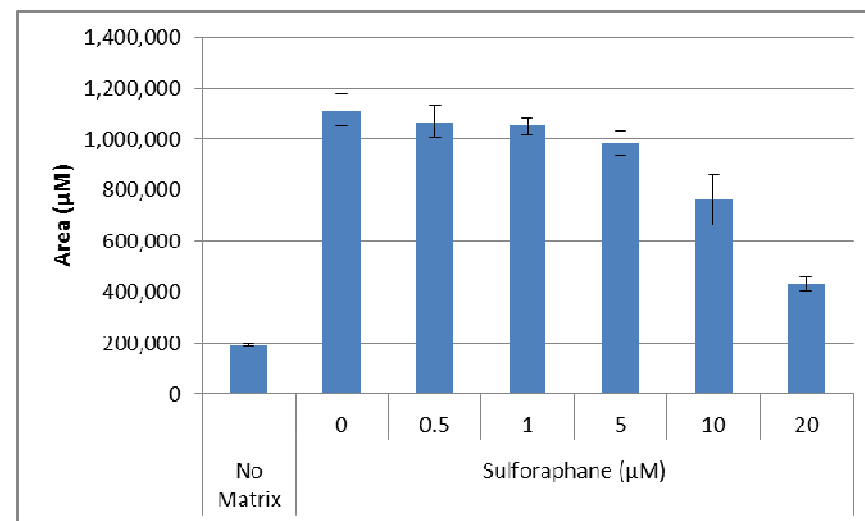
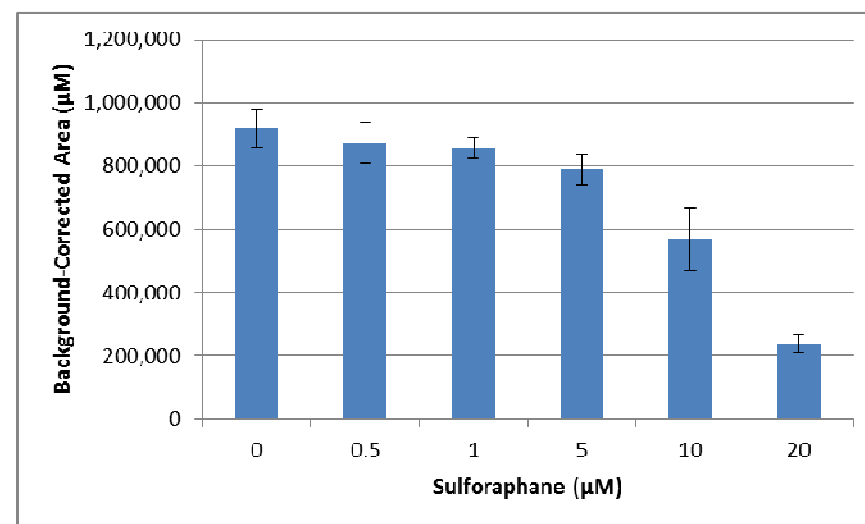


Figure 4. Quantitative analysis of surface area for non-invasive (MCF-7) and invasive (MDA-MB-231) cell lines over a four day period.

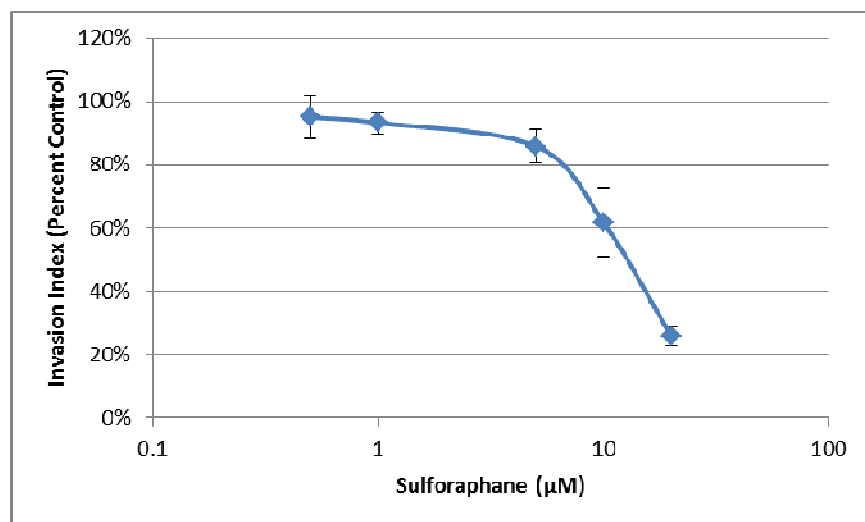
2. The changes in area can also be used to evaluate the effect of pharmacological compounds on 3D cell invasion. Once optimal assay conditions have been established, this can be evaluated as an endpoint assay. The following assay was conducted using MDA-MB-231 cells treated with varying concentrations of the inhibitor Sulforaphane.
 - a. First the areas were calculated for each condition, and these values were used to generate average and standard deviations:



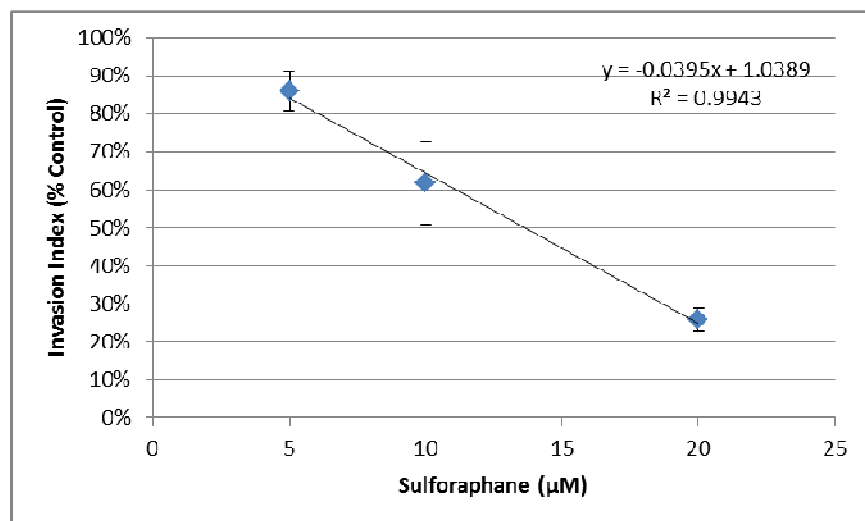
- b. Next the area for the spheroid with no Invasion Matrix was used as a background value and subtracted from the average of each condition:



- c. Finally, the resulting areas were divided by the area obtained for the no inhibitor control, providing the invasion index as a percentage of control:



- d. Using the linear portion of the inhibition curve, the IC₅₀ may be calculated:



IC₅₀ is calculated by determining x where $y = 0.5$:

-Based on the line equation, $x = (y - 1.0389) / -0.0395$.

-Thus IC₅₀ = $(0.5 - 1.0389) / -0.0395 = 13.6 \mu\text{M}$ Sulforaphane

IX. Troubleshooting

Troubleshooting Guide		
Problem	Cause	Solution
Cells do not form spheroids.	Cells are not healthy.	Assess cell doubling rates and check cell viability prior to spheroid formation.
	The cell line does not form spheroids.	While many cancer cell lines of epithelial origin have been shown to form spheroids, there may be exceptions. The kit may not be compatible with this cell line.
Cells do not invade into the Invasion Matrix	Cells are not healthy.	Assess cell doubling rates and check cell viability prior to spheroid formation.
	The model lacks factors critical for inducing cell invasion.	Consult the scientific literature and cell provider for information regarding your cell line.
	The cell line is not invasive.	
Spheroids are not fully embedded or located near the sides of the wells	The spheroids were not in the correct location during Invasion Matrix polymerization.	The well of the Spheroid Formation Plate and the Invasion Matrix must be chilled prior to adding Invasion Matrix.
		The plates must be centrifuged $300 \times g$ in a swinging bucket rotor at 4°C for 5 minutes.

X. References

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XI. Appendix A: Reagent and Buffer Composition

1. 10X Spheroid Formation ECM

Proprietary mixture of extracellular matrix proteins derived from murine EHS sarcoma cells optimized for spheroid formation. It is qualified for 3D Culture Invasion and provided in DMEM with 10 µg/ml gentamycin. Avoid freeze-thaws.

2. 3D Culture Qualified 96 Well Spheroid Formation Plate

96 Well, U bottom plate with low adhesion surface to promote spheroid formation.

3. Invasion Matrix

Proprietary extracellular matrix blend comprised of basement membrane extract, derived from murine EHS sarcoma cells, and collagen. It is qualified for 3D Culture Cell Invasion and provided in DMEM. Avoid freeze-thaws.

XII. Related products available from Trevigen.

Catalog #	Description	Size
3445-048-01	Cultrex® 3-D Culture Matrix™ BME	15 ml
3446-005-01	Cultrex® 3-D Culture Matrix™ Laminin I	5 ml
3447-020-01	Cultrex® 3-D Culture Matrix™ Rat Collagen I	20 ml
3445-096-K	3D Culture BME Cell Proliferation Assay Kit	96 samples
3446-096-K	3D Culture Laminin-I Cell Proliferation Assay Kit	96 samples
3447-096-K	3D Culture Collagen I Cell Proliferation Assay Kit	96 samples
3448-096-K	3D Culture Cell Harvesting Kit	20 samples
3455-096-K	Cultrex® BME Cell Invasion Assay	96 samples
3456-096-K	Cultrex® Laminin I Cell Invasion Assay	96 samples
3457-096-K	Cultrex® Collagen I Cell Invasion Assay	96 samples
3458-096-K	Cultrex® Collagen IV Cell Invasion Assay	96 samples
4892-010-K	Cultrex® Calcein-AM Cell Viability Kit	1000 samples
4890-025-K	TACS® MTT Cell Proliferation Assay	2500 samples
4891-025-K	TACS® XTT Cell Proliferation Assay	2500 samples
4822-96-K	HT TiterTACS™ Assay Kit	96 samples
4817-60-K	FlowTACS™ Apoptosis Detection Kit	60 samples
4830-01-K	TACS® Annexin V FITC Kit	100 samples
4835-01-K	TACS® Annexin V Biotin Kit	100 samples
6300-100-K	DePsipher™ Mitochondrial Potential Assay Kit	100 samples
6305-100-K	MitoShift™ Mitochondrial Potential Assay Kit	100 samples

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