



Cultrex[®] 96 Well Collagen I Cell Invasion Assay

Catalog# 3457-096-K

96 Samples

96 Well Collagen I Cell Invasion Assay

Reagent kit for investigating chemotaxis,
cell migration and/or cell invasion.

96 samples

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I. Quick Reference Procedure for Cultrex® 96 Well Collagen I Cell Invasion Assay (Cat# 3457-096-K):

Read through the complete Instructions for Use prior to using this kit.

Prior to Day 1

1. Culture cells per manufacturer's recommendation; adherent cells should be cultured to no more than 80% confluence. Each well requires 50,000 cells, so plan accordingly.
2. Twenty-four hours prior to beginning assay, starve cells in a serum-free medium (0.5% FBS may be used if needed) to allow expression of free receptors.
3. Coat membrane of top invasion chamber (leave three chambers uncoated for a migration control) with 50 µl of 0.1X to 1X Collagen I Solution, and incubate for 4 hours or overnight at 37 °C in a CO₂ incubator.

Day 1

4. After 24 hours serum starvation (optional), harvest and count cells.
5. Centrifuge cells at 250 x g for 10 min, remove supernatant, wash with 1X wash buffer, count and resuspend at 1 x 10⁶ cells/ml in a serum free medium (0.5% FBS may be used if needed).
6. Aspirate top chamber of cell invasion device. DO NOT ALLOW TOP OR BOTTOM CHAMBERS TO DRY.
7. Add 50 µl of cells per well to top chamber (with or without inhibitors/stimulants).
8. Using access port, add 150 µl of medium per well to bottom chamber (with or without chemoattractants).
9. Incubate chamber at 37 °C in CO₂ incubator for 24 hours.
10. Assay remaining cells for standard curve (section VII. B.); each cell type will require a separate standard curve (optional).

Day 2

11. Carefully aspirate top chamber (do not puncture membrane), and wash each well with 100 µl of 1X Wash Buffer.
12. Aspirate bottom chamber, and wash each well twice with 200 µl 1X Wash Buffer. Transfer top chambers to assay chamber plate (black).
13. Add 12 µl of Calcein-AM solution to 10 mL of Cell Dissociation Solution.
14. Add 150 µl of Cell Dissociation Solution/Calcein-AM to bottom chamber, assemble cell invasion device, and incubate at 37 °C in CO₂ incubator for one hour.
15. Remove top chamber, and read plate at 485 nm excitation, 520 nm emission.
16. Using standard curve(s), convert RFU to Cell Number; determine percent invasion.

II. Background

Trevigen's Cultrex® 96 Well Cell Invasion Assays were created in an effort to accelerate the screening process for compounds that influence cellular digestion and migration across extracellular matrices, which is a fundamental component of cellular processes such as angiogenesis, embryonic development, immune responses, wound healing and metastasis¹⁻⁴. These assays offer a flexible, standardized, high-throughput 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.

Trevigen's Cultrex® Cell Invasion Assays are provided in multiple formats so that cell invasion may be evaluated against different extracellular matrices and matrix components:

- Laminin I
- Collagen I
- Collagen IV
- Basement Membrane Extract (BME), Regular and PathClear™

These assays employ a simplified Boyden chamber design with an 8 micron polyethylene terephthalate (PET) membrane. Ports within the migration chamber (top) allow the access to the assay chamber (bottom) without dismantling the device. This design is easier to use, prevents contamination, and is adaptable for robotic high throughput systems. The assay chamber may be directly analyzed in a 96 well plate reader, eliminating transfer steps that introduce additional variability to the assay.

Since different cell lines and different treatments can result in a wide range of invasive potentials, the permissiveness of each matrix may also be optimized to fit each experiment by adjusting the coating concentration. A 1X Coating solution is recommended for highly invasive cells, whereas 0.1X coatings may be sufficient for less invasive cells.

Detection of cell invasion is quantified using Calcein-AM. Calcein-AM is internalized by the cells, and intracellular esterases cleave the acetomethylester (AM) moiety to generate free Calcein. Free Calcein fluoresces brightly, and this fluorescence may be used to quantitate the number of cells that have migrated or invaded using a standard curve. Sufficient reagents are included to assess cell migration/invasion in 96 wells, as well as to calculate multiple standard curves.

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. Cultrex® Cell Invasion Assays contain 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>
Cell Invasion Chamber	each	Room Temp	3455-096-01
5X Collagen I Solution	1 ml	4 °C*	3457-096-02
10X Coating Buffer	1 ml	4 °C	3455-096-03
25X Cell Wash Buffer	2 x 1.5 ml	4 °C	3455-096-04
10X Cell Dissociation Solution	2 x 1.5 ml	4 °C	3455-096-05
Calcein AM	50 µg	≤-20 °C	4892-010-01

*Do not freeze.

V. Materials/Equipment Required But Not Supplied

Equipment

- 1 - 20 µl, 20 - 200 µl, and 200 - 1000 µl pipettors
- 37 °C CO₂ incubator
- Low speed centrifuge and tubes for cell harvesting
- Hemocytometer or other means to count cells
- 50 and 500 ml graduated cylinders
- 20 °C and 4 °C storage
- Ice bucket
- Standard light microscope (or inverted)
- Pipette helper
- Timer
- Vortex mixer
- Fluorescent 96-well plate reader, top reader (485 nm excitation, 520 nm emission)
- Black 96 Well Plate (For standard curve)
- Computer and graphing software, such as Microsoft[®] Excel[®].

Reagents

- Cell Harvesting Buffer; EDTA, trypsin, or other cell detachment buffer.
- Tissue Culture Growth Media, as recommended by cell supplier.
- Serum-Free Media, Tissue Culture Growth Media without serum.
- Chemoattractants or pharmacological agents for addition to culture medium.
- Quenching medium: serum-free media with 5% BSA.
- Sterile PBS or HBSS to wash cells.
- Distilled, deionized water
- Trypan blue or equivalent viability stain

Disposables

- Cell culture flask, 25 cm² or 75 cm²
- 50 ml tubes
- 1 - 200 µl and 200 - 1000 µl pipette tips
- 1.5 and 10 ml serological pipettes
- Gloves
- 10 ml syringe
- 0.2 µm filter

VI. Reagent Preparation

(Thaw reagents completely before diluting!)

1. 10X Coating Buffer

Dilute 500 µl in 4.5 ml of sterile, deionized water to make 1X buffer; filtration at 0.2 µm recommended. Store at 4 °C.

2. 5X Collagen I Solution

For highly invasive cells, dilute 1 ml in 4 ml of 1X Coating Buffer on ice immediately before coating (800 µg/cm²). Less invasive cell types may require a more permissive barrier, so the Collagen I may be diluted as far as 0.1X (80 µg/cm²). Avoid freeze-thaws.

3. 25X Cell Wash Buffer

Dilute 3 ml in 72 ml of sterile, deionized water to make 1X solution.

4. 10X Cell Dissociation Solution

Dilute 3 ml of 10X stock in 27 ml of sterile, deionized water to make a 1X solution.

5. Calcein-AM Solution

Centrifuge microtube momentarily to pellet powder before opening tube, and add 30 µl of sterile DMSO to make working solution. Pipet up and down to mix, and store solution at -20 °C (do not foam).

VII. Assay Protocol

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

A. Cell Harvesting

Subject cells may be prepared for investigation as desired. The following procedure is suggested and may be optimized to suit the cell type(s) of interest.

- Cells should be passaged 2 or 3 times prior to use in the assay, and adherent cells need to be no greater than 80% confluent. Each chamber requires 50,000 – 100,000 cells, and a 25 cm² or 75 cm² flask will yield approximately 3 x 10⁶ or 9 x 10⁶ cells, respectively. Plan to have enough cells for a standard curve, if used, migration controls and cell invasion assay.

2. Starve cells by incubating 18-24 hours in Serum-Free medium (see *Materials/Equipment Required But Not Supplied*) prior to assay (0.5% FBS may be used if needed) to allow for the expression of free receptors.
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 or 75 cm² flask, add 1 ml or 2 ml, respectively, of Cell Harvesting Buffer (see *Materials/Equipment 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 Quenching Medium (see *Materials/Equipment Required But Not Supplied*).
7. Centrifuge cells at 250 x g for 10 minutes to pellet, remove quenching medium, and resuspend cells in 2 ml of Serum-Free Medium (0.5% FBS may be used if needed). Cells may need to be gently pipetted up and down using a serological pipette to break up clumps.
8. Count cells, and dilute to 1 x 10⁶ cells per ml in Serum-Free Medium (0.5% FBS may be used if needed).

B. Conversion of Relative Fluorescence Units (RFU) into Cell Number

Many investigators express their results relative to untreated cells. In order to convert relative fluorescence units (RFU) into number of cells, standard curves are recommended. It is not necessary to use inserts to generate a standard curve. If used, a separate standard curve may be run for each cell type and assay condition. Control cells, and experimental replicates should be performed in triplicate.

1. Determine the saturation range for your cells (e.g. 50,000 cells), beyond which, additional migration or invasion would be difficult to detect.
2. Determine the total number of cells needed per standard curve, and the required volume of medium (Table 1, below).
3. Transfer volume of harvested cells needed to a 15 ml conical tube, and centrifuge at 250 x g for 10 minutes to pellet cells.
4. Remove supernatant, and resuspend cells in Cell Dissociation Solution at 1 x 10⁶ cells/ml.
5. Dilute cells for highest condition for a final volume of 50 µl (e.g. 50,000 cells/50 µl = 1.0 x 10⁶ cells/ml) in 1X Cell Dissociation Solution (section VI, item 4), add 50 µl/well, and serially dilute remaining stock with 1X Cell Dissociation Solution to deposit the number of cells needed in each well (in 50 µl of 1X Cell Dissociation Solution). Repeat dilutions until all conditions have been satisfied.
6. Add 12 µl of Calcein-AM Solution (section VI, item 5) to 5 ml of 1X Cell Dissociation Solution (section VI, item 4), cap tube, and invert to mix.

7. Add 50 µl of 1X Cell Dissociation Solution/Calcein-AM (VII.B6) to each well, and incubate for one hour; omit cells from at least three wells to calculate background.
8. Read plate at 485 nm excitation, 520 nm emission (see Table 2 for sample data) to obtain relative fluorescence units (RFU).
9. Average values for each condition; then subtract background from each value (see Table 2).
10. Plot standard curve of RFU vs. number of cells (see Figure 1).
11. Insert a trend line (best fit) and use the line equation for each cell line in calculating number of cells that migrated/invaded (see Figure 1).

Table 1. Sample Calculations for Standard Curve:

Cells Needed:		
Cells/Well X	Conditions =	Cells Needed
50,000	3	150,000
25,000	3	75,000
10,000	3	30,000
5,000	3	15,000
2,000	3	6,000
1,000	3	3,000
Total Cells Needed		279,000

Volume of Harvested Cells Needed (ml):

$$\frac{\text{Total Cells Needed}}{\text{Concentration Harvested Cells}} =$$

$$\frac{279,000}{1.00E+06} = 0.279$$

Table 2. Sample Data for Standard Curve (actual results may vary):

Average Cells/Well	Wells		
	1	2	3
50,000	15710	15145	16135
25,000	9118	8702	8644
10,000	4454	4257	4091
5,000	2609	2541	2599
2,500	1486	1476	1585
1,000	930	922	881
0	243	254	264

Table 2. Sample Data for Standard Curve (cont.):

Subtract Background and plot the data:

Cells/Well	Average	Background	Corrected
50,000	15663	-254	15409
25,000	8821	-254	8567
10,000	4267	-254	4013
5,000	2583	-254	2329
2,500	1516	-254	1262
1,000	911	-254	657
0	254	(Background)	

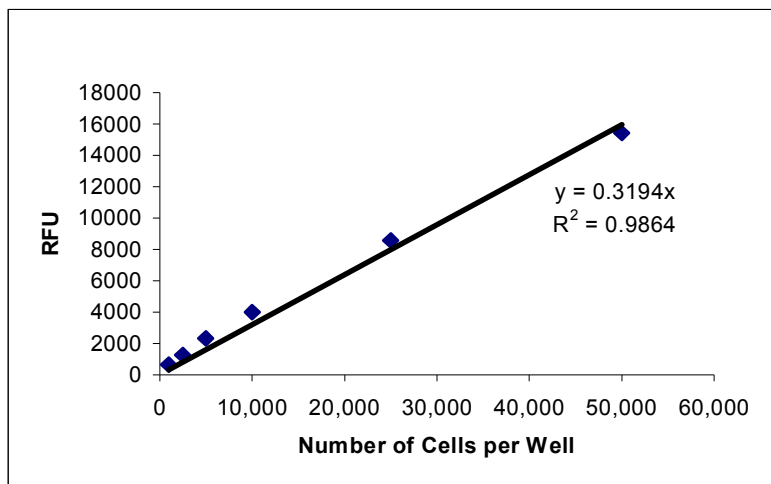


Figure 1. Standard Curve for Cell Migration Assay. HT-1080 cells were harvested (section VII. A), diluted, incubated for one hour with Calcein-AM, and assayed for fluorescence (section VII. B). The trend line and line equation are included on the graph.

C. Cell Invasion Assay

Note: Two plate bottoms are provided for your convenience: optimization of assay conditions (clear), and background signal minimization (black).

1. Culture cells to be assayed to not greater than 80% confluence. Plan accordingly for sufficient numbers of cells per chamber.
2. 24 hours prior to assay, cells may need to be serum starved in order to express unbound receptors on their plasma membranes. This step may be omitted depending upon the cell types and ligands under investigation.
3. Working on ice, prepare 5 ml of 0.1X to 1X Collagen I Solution in a sterile 15 ml conical tube (section VI, item 2), and label "Collagen I Coat". Cap tube, and gently invert to mix.

4. Aliquot 50 μ l of Collagen I Coat (Section VII, C3) per well. Gently tap side of device a few times, and visually inspect wells for dispersion of coating. All wells should be coated except at least three migration control wells (optional). Coat for 4 hours or overnight at 37 $^{\circ}$ C in a CO₂ incubator.
5. Assay cells for standard curve; each cell type will require a separate standard curve (section VII. B).
6. Harvest cells, and dilute to working concentration (1 x 10⁶ cells/ml recommended) in a serum-free medium.
7. Aspirate off coating solution from top chamber of cell invasion device. DO NOT ALLOW TOP OR BOTTOM CHAMBERS TO DRY.
8. Add 50 μ l of cells per well to top chamber (with or without inhibitors/stimulants), and to compensate for background, omit cells from at least three wells.
9. Add 150 μ l of medium per well to bottom chamber (with or without chemoattractants).
10. Incubate at 37 $^{\circ}$ C in CO₂ incubator; incubation times may be varied (24 hours-48 hours).
11. After incubation, aspirate top chamber without puncturing the membrane, and wash each well with 100 μ l of 1X Wash Buffer (section VI, item 3). Aspirate out the wash buffer.
12. Aspirate bottom chamber, and wash each well with 200 μ l 1X Wash Buffer (section VI, item 3). The device may be disassembled and the bottom chamber inverted to empty wells. Re-assemble device (if disassembled) using the assay chamber plate.
13. Add 12 μ l of Calcein AM solution (section V, item 5) to 10 ml of 1X Cell Dissociation Solution (section VI, item 4).
14. Add 100 μ l of Cell Dissociation Solution/Calcein-AM (VII.C.12) to each bottom chamber well, and incubate at 37 $^{\circ}$ C in CO₂ incubator for 30 minutes.
15. Gently tap device 10 times on the side, and incubate at 37 $^{\circ}$ C in CO₂ incubator for an additional 30 minutes (one hour total). This helps to ensure optimal dissociation.
16. Disassemble cell migration device, and read assay chamber (bottom) at 485 nm excitation, 520 nm emission using same parameters (time and gain) as standard curve(s), or controls (see Table 3 for sample data).
17. Compare data to standard curve to determine the number of cells that have migrated (no coating) or invaded (through the Collagen I), as well as percent cell invasion (see VIII. Example Results).

VIII. Example Results

1. After plotting standard curve (section VII. B), insert trendline, best fit, with intercept equals zero, equation, and R-square value (coefficient of determination).

- For assay samples, first average all wells for each condition (Table 4).
- Next, subtract background from averages (Table 5).
- Use the trendline equation to determine the number of cells present in each well; for the equation, $y = mx + b$, replace Y value with RFU, and solve for X (Table 6). See an example of a trendline and equation in Figure 1.
- The number of cells may be compared for each condition to evaluate relative migration, or the number of migrated cells may be divided by the number of starting cells to determine percent migration (Table 7 and Figure 2).

Table 3. Sample Data for Cell Invasion Assay (Actual Results May Vary):

Cell Line	Std Curve Slope (m value)
MCF-7	0.1428
NIH-3T3	0.2012
HT-1080	0.3194
MDA-MB-231	0.1850

	Non-Invasive		Invasive		Blank
	MCF-7	NIH-3T3	HT-1080	MDA-MB-231	
DMEM	273	327	234	224	227
	239	249	226	187	264
	238	255	233	243	233
	242	279	243	263	255
DMEM, 10% FBS	273	587	6591	2547	251
	205	572	5798	1674	241
	320	575	4913	1948	254
	301	606	7100	2002	243
			average	246	

Table 4. Average Data for Each Condition:

	MCF-7		NIH-3T3		HT-1080		MDA-MB-231	
	DMEM	FBS	DMEM	FBS	DMEM	FBS	DMEM	FBS
Average	248	275	278	585	234	6101	229	2043
Std Dev	17	50	35	15	7	956	32	366

Table 5. Subtract Background from Each Condition:

	MCF-7		NIH-3T3		HT-1080		MDA-MB-231	
	DMEM	FBS	DMEM	FBS	DMEM	FBS	DMEM	FBS
Average	2	29	32	339	-12	5855	-17	1797
Std Dev	17	50	35	15	7	956	32	366

Table 6. Divide Each Value by Line Equation to Determine Cell Number:

	MCF-7		NIH-3T3		HT-1080		MDA-MB-231	
	DMEM	FBS	DMEM	FBS	DMEM	FBS	DMEM	FBS
Average	14	201	157	1685	-38	18295	-91	9712
Std Dev	117	353	176	77	22	2987	175	1976

Table 7. Divide Cell Number for Each Condition by 50,000 to Determine Percent Invasion:

	MCF-7		NIH-3T3		HT-1080		MDA-MB-231	
	DMEM	FBS	DMEM	FBS	DMEM	FBS	DMEM	FBS
Average	0%	0%	0%	3%	0%	37%	0%	19%
Std Dev	0%	1%	0%	0%	0%	6%	0%	4%

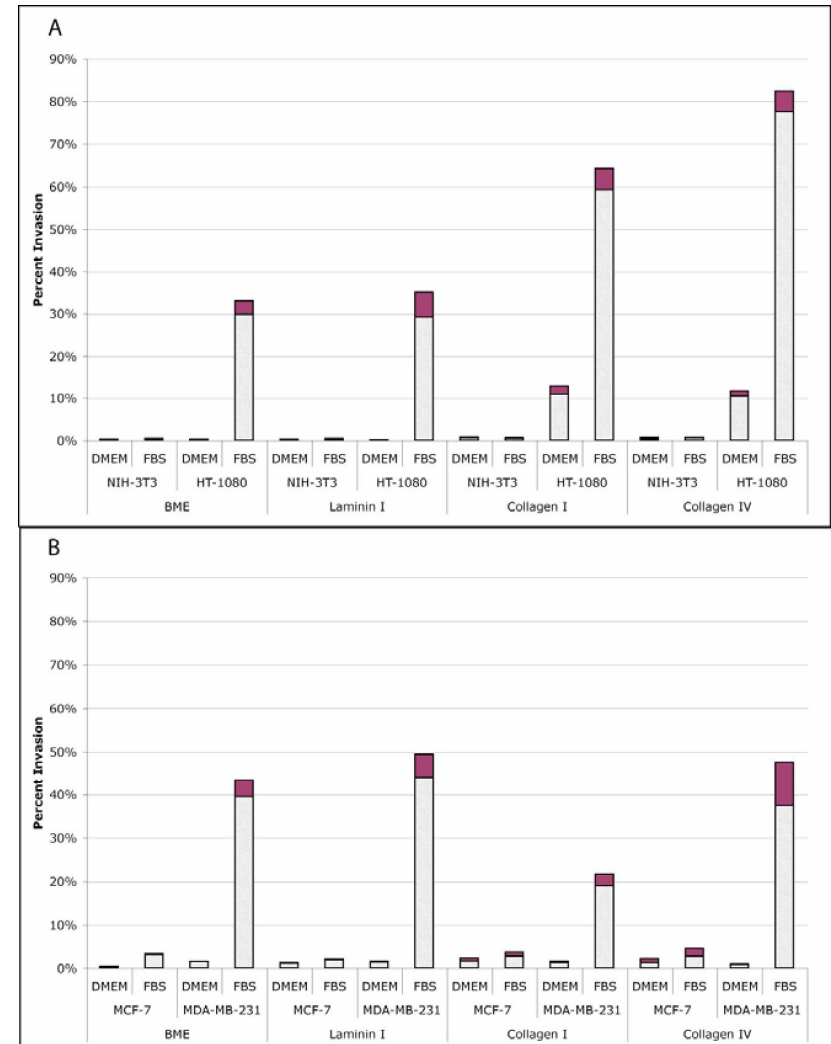


Figure 2. Quantitation of the ability of fibroblastic cell lines (A) and breast cancer cell lines (B) to cross a barrier consisting of an 8 micron polyester filter occluded with different extracellular components over a 24 hour period in response to 10% FBS. Samples were run in quadruplicate for non-invasive cell types, MCF-7 and NIH-3T3, and invasive cell types, HT-1080 and MDA-MB-231. Light area represent average invasion, and dark area represents standard deviation.

IX. Troubleshooting

Problem	Cause	Solution
No signal	Cells did not penetrate barrier	Cell type may be non-invasive or chemoattractant may be insufficient.
		Barrier may not be permissive enough for cell type; use more dilute coating solution.
		There is inherent variability in FBS from lot to lot; this can affect assay if used.
	Cells may have died as a result of treatment.	Test cells for viability in treatment regimen.
High background	Insufficient Washing - agents in media, FBS, and/or chemo-attracts may react with Calcein-AM.	Re-assay, and make sure to wash well.
	Contamination – enzymes released by bacteria or mold may degrade the Collagen I and cleave Calcein-AM.	Start a new culture from seed stocks, and re-assay. If seed stock is contaminated, then it may be prudent to get new cells.
Well to well variability	Inconsistent pipetting	Calibrate pipettors, and monitor pipet tips for air bubbles.
	Puncture membrane with pipet tips	Disregard data from wells that are punctured; re-assay if necessary.

X. References

- Kleinman HK, Martin GR. 2005. Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol.* 15:378-86.
- Venneri MA, De Palma M, Ponzoni M, Pucci F, Scielzo C, Zonari E, Mazzieri R, Doglioni C, Naldini L. 2007. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 109: 5276-85.
- Mohan K, Ding Z, Hanly J, Issekutz TB. 2002. IFN-gamma-inducible T cell alpha chemoattractant is a potent stimulator of normal human blood T lymphocyte transendothelial migration: differential regulation by IFN-gamma and TNF-alpha. *J Immunol.* 168:6420-8.
- Koblinski JE, Kaplan-Singer BR, VanOsdol SJ, Wu M, Engbring JA, Wang S, Goldsmith CM, Piper JT, Vostal JG, Harms JF, Welch DR, Kleinman HK. 2005. Endogenous osteonectin/SPARC/BM-40 expression inhibits MDA-MB-231 breast cancer cell metastasis. *Cancer Res.* 65:7370-7.

XI. Related products available from Trevigen.

Catalog#	Description	Size
3455-024-K	24 Well BME Cell Invasion Assay	24 inserts
3460-024-K	CultreCoat™ 24 Well BME-Coated Cell Invasion Assay	24 inserts
3465-096-K	Cultrex® 96 Well Cell Migration Assay	96 samples
3465-024-K	Cultrex® 24 Well Cell Migration Assay	12 samples
3455-096-K	Cultrex® 96 well BME Cell Invasion Assay	96 samples
3456-096-K	Cultrex® Laminin I Cell Invasion Assay	96 samples
3458-096-K	Cultrex® Collagen IV Cell Invasion Assay	96 samples
3471-096-K	In vitro Angiogenesis Assay Endothelial Cell Invasion	96 samples

Accessories:

Catalog#	Description	Size
3400-010-01	Cultrex® Mouse Laminin I	1 mg
3440-100-01	Cultrex® Rat Collagen I	100 mg
3410-010-01	Cultrex® Mouse Collagen IV	1 mg
3430-005-02	Cultrex® BME with Phenol Red PathClear™	5 ml
3431-005-02	Cultrex® BME with Phenol Red Growth Factor Reduced PathClear™	5 ml
3432-005-02	Cultrex® BME PathClear™	5 ml
3433-005-02	Cultrex® BME Growth Factor Reduced PathClear™	5 ml
3430-005-01	Cultrex® BME with Phenol Red	5 ml
3432-005-01	Cultrex® BME; no Phenol Red	5 ml
3431-005-01	Cultrex® BME with Phenol Red; Reduced Growth Factors	5 ml
3433-005-01	Cultrex® BME; no Phenol Red; Reduced Growth Factors	5 ml
3416-001-01	Cultrex® Bovine Fibronectin	1 mg
3417-001-01	Cultrex® Bovine Vitronectin	50 µg
3438-100-01	Cultrex® Poly-L-Lysine	100 ml

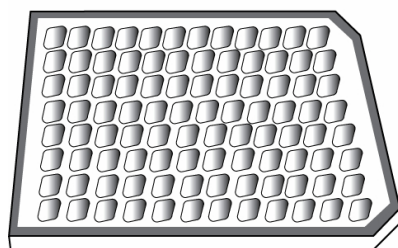
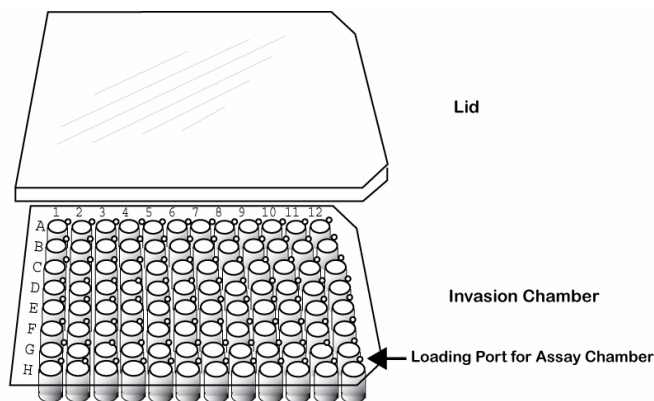
Catalog#	Description	Size
3443-050-03	Cultrex [®] Murine VEGF	1 µg
3443-050-02	Cultrex [®] Human FGF-2	5 µg
3443-050-01	Cultrex [®] Human EGF	50 µg
3443-050-04	Cultrex [®] Human β-NGF	2 µg
3437-100-K	Cultrex [®] Cell Staining Kit	100 ml
3439-100-01	Cultrex [®] Cell Recovery Solution	100 ml
3450-048-05	CellSperse [™]	15 ml

XII. Appendix

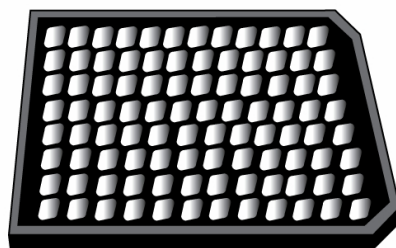
Appendix A. Reagent and Buffer Composition

1. Cell Invasion Chamber

96 Well Boyden Chamber, 8.0 µm PET membrane, black receiver plate compatible with 96 well fluorescent plate reader.



Culture Chamber



Assay Chamber

2. 5X Collagen I Solution

Collagen I is provided at 10 mg/ml, derived from Rat tails. Avoid repeated freeze-thaw cycles.

3. 10X Coating Buffer

Proprietary buffer optimized for coating Collagen I to the PET membrane.

4. 25X Cell Wash Buffer

PBS buffer for washing cells (10 mM Potassium Phosphate (pH 7.4), 145 mM NaCl).

5. 10X Cell Dissociation Solution

Proprietary formulation containing sodium citrate, EDTA, and glycerol.

6. Calcein-AM

A non-fluorescent, hydrophobic compound that easily permeates intact, live cells. Once in the cell, Calcein-AM is hydrolyzed by intracellular esterases to produce Calcein, a hydrophilic, strongly fluorescent compound that is retained in the cell cytoplasm.



211 bis Avenue Kennedy - BP 1140
03103 Montluçon - France
33 (0) 4 70 03 88 55
Fax 33 (0) 4 70 03 82 60
e-mail interchim@interchim.com

Agence Paris - Normandie
33 (0) 1 41 32 34 40
Fax 33 (0) 1 47 91 23 90
e-mail interchim.paris@interchim.com

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