

CULTREX[®] Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

CultreCoat[®] 24 Well High BME Cell Invasion Assay

**Reagent kit for evaluating cell invasion for
highly invasive cell models**

24 Inserts

Catalog #: 3483-024-K

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I. Quick Reference Procedure for CultreCoat® 24 Well High BME Cell Invasion Assay (cat# 3483-024-K): Read through the complete *Instructions for Use* prior to using this kit. This page is designed to be copied and used as a checklist.

Prior to Day 1

- 1. Culture cells per manufacturer's recommendation. Adherent cells should be passaged at least one time and cultured to 80% confluence. Plan accordingly for sufficient numbers of cells per well.
- 2. Assay cells for standard curve to determine optimal seeding densities if needed (please see section VII B).
- 3. 16-24 hours prior to assay, starve cells in a serum-free medium (0.5% FBS may be used if needed).

Day 1 (Preparation of cells and CultreCoat® rehydration)

- 4. Transfer the 24 well cell invasion inserts to room temperature, and let sit for one hour.
- 5. Rehydrate the inserts by adding 80 µl of serum-free medium (~37 °C, see section VII.D.5.) to each insert, and incubating at 37 °C in a CO₂ incubator for 1 hour.
- 6. After 16 hours serum starvation, harvest and count cells.
- 7. Dilute to working concentration (1 x 10⁶ cells/ml is recommended) in a serum-free medium (0.5% FBS may be used if needed). Inhibitors may also be added to cells at this time.
- 8. After rehydration, add 40 µl of cells per well to each insert (40,000 cells recommended).
- 9. Add 360 µl of medium per well to bottom well (with or without chemoattractants and/or inhibitors).
- 10. Assemble chambers and incubate at 37 °C in a CO₂ incubator for 4-48 hours (24 hours recommended).

Day 1-3

- 11. Add 12 µl of Calcein AM (see section VI.3.) solution to 12 mL of Cell Dissociation Solution (see section VI.2.).
- 12. Invert and lightly shake inserts into a dish to remove contents.
- 13. Wash each insert twice with 100 µl of 1X Wash Buffer (see section VI.1.); invert and lightly shake inserts into a dish to remove contents.
- 14. Transfer inserts to the new 24 well plate.
- 15. Add 360 µl of Cell Dissociation Solution/Calcein AM to bottom wells, and incubate at 37 °C in a CO₂ incubator for one hour.
- 16. Lightly tap the side of the plate ten times to remove cells from membranes.
- 17. Remove inserts, and read 24 well plate at 485 nm excitation, 520 nm emission. As an option, resuspend cells, and transfer 100 µl to a 96 well plate for evaluation.
- 18. Analyze data.

II. Background

Cell invasion is fundamental to angiogenesis¹, embryonic development², immune responses³, and tumor cell metastasis⁴. Trevigen's **CultreCoat® 24 well Cell Invasion Assays** were created in an effort to accelerate *in vitro* screening for modulators of these processes, and to further enable the investigation of pathways that influence cell invasion through extracellular matrices (Figure 1). These assays offer three distinct, standardized, high throughput formats for quantitating the degree to which invasive cells penetrate a barrier consisting of basement membrane components, in response to chemoattractants and/or inhibiting compounds. Basement membranes are continuous sheets of specialized extracellular matrix that form an interface between endothelial, epithelial, muscle, or neuronal cells and their adjacent stroma. Basement membranes are degraded and regenerated during development and wound repair. They not only support cells and cell layers, but they also play an essential role in tissue organization that affects cell adhesion, migration, proliferation, and differentiation. Basement membranes provide major barriers to invasion by metastatic tumor cells.

Neoplastic transformation is the result of multiple genetic and epigenetic alterations, affecting a variety of gene products and resulting in a range of metastatic potentials. As a result, different cancer cell lines may exhibit differing capacities for cell invasion. While Boyden chambers that are pre-coated with a reconstituted basement membrane have provided standardized solutions for evaluating cell invasion, the use of one coating density has become a limiting factor for evaluating cancer cell models because many exhibit different basal levels of invasive potential. As a result, inhibition of invasion may be masked in cell models that have a very high invasive capacity because the barrier is insufficient to limit invasion. Alternatively, few cells may be able to penetrate this barrier for models with lower invasive capacities; this results in a very small dynamic range and high signal to noise ratio, making it difficult to generate statistically significant data. To address these issues, Trevigen has developed an *in vitro* cell invasion assay system composed of three different coating densities that may be used for cell models with either high, medium, or low invasive potentials. If the optimal coating density is not known in advance, the requirements for each cell model should be determined by using the **CultreCoat® 24 well BME Optimization Cell Invasion Assay**.

The **CultreCoat® 24 Well High BME Cell Invasion Assay** contains 24 inserts that are coated with a standard density of BME for cell models that exhibit an accelerated invasive capacity. The high amount of BME used for coating provides the highest sensitivity for highly invasive cell models, giving the maximum signal to noise ratio and dynamic range.

The **CultreCoat® 24 Well High BME Cell Invasion Assay** utilizes a simplified Boyden chamber design with an 8 µm polycarbonate (PC) membrane coated with PathClear® growth factor reduced BME. Detection of cell invasion is quantified using Calcein AM. Calcein AM is internalized, 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 by using a standard curve.

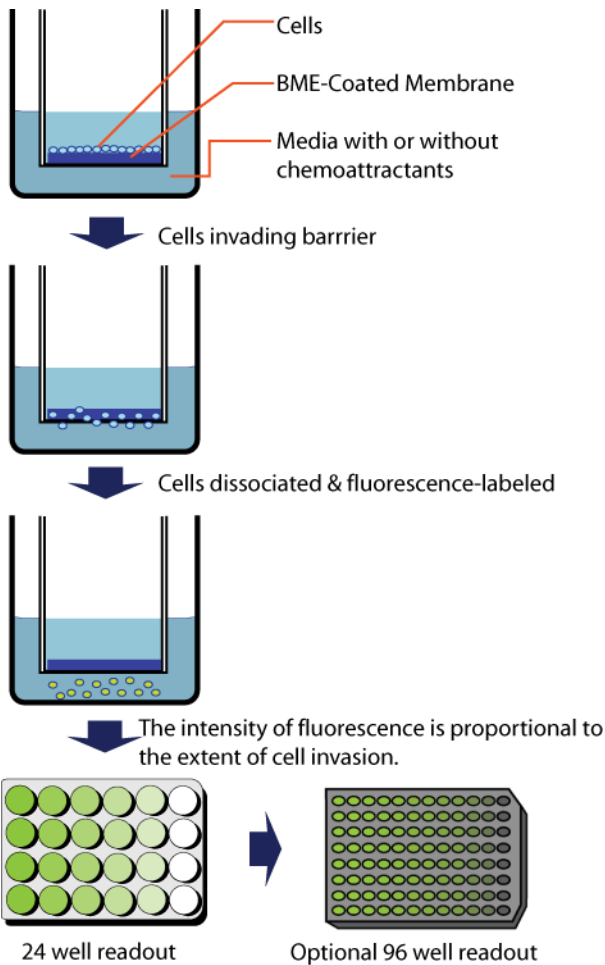


Figure 1. Illustration of protocol for Trevigen's CultreCoat® 24 well BME Cell Invasion Assays.

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. CultreCoat® 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>
24 Well High BME Cell Invasion Inserts	24 Inserts	≤-20 °C*	3483-024-01
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

*Store in a manual defrost freezer.

V. Materials/Equipment Required But Not Supplied

Equipment

- 1 - 1 - 20 µl, 20 - 200 µl, and 200 - 1000 µl pipettors
2. 37°C CO₂ incubator
3. Low speed centrifuge and tubes for cell harvesting
4. Hemocytometer or other means to count cells
5. -20°C and 4°C storage
6. Ice bucket
7. Standard light microscope (or inverted)
8. Pipette helper
9. Timer
10. Vortex mixer
11. Fluorescent 24 well (or 96 well) plate reader, which detects signals above the wells (485 nm excitation, 520 nm emission)
12. Computer and graphing software, such as Microsoft® Excel®.
13. Clear, Flat bottom 24 (or 96) Well Plates (if generating standard curves).

Reagents

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

Disposables

1. Cell culture flask, 25 cm² or 75 cm²
2. 15 ml tubes
3. 1 - 200 μ l and 200 - 1000 μ l pipette tips
4. 1, 5 and 10 ml serological pipettes
5. Gloves
6. 10 ml syringe
7. 0.2 μ m filter
8. 24 well TC plate

VI. Reagent Preparation

(Thaw reagents completely before diluting!)

1. 25X Cell Wash Buffer

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

2. 10X Cell Dissociation Solution

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

3. Calcein AM

Centrifuge microtube momentarily to pellet powder before opening tube, and add 30 μ l of sterile DMSO to make working solution. Pipet up and down to mix, and store solution at -20 °C.

VII. Assay Protocol

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

A. Cell Culture and Harvesting

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

1. Cells should be passaged 2 or 3 times prior to use in the assay, and adherent cells need to be no more than 80% confluent. Each insert can accommodate 1×10^5 – 5×10^5 cells depending upon cell type. A 25 cm² or 75 cm² flask will yield approximately 2×10^6 or 6×10^6 cells, respectively. Plan to have enough cells for a standard curve, if used, controls and cell invasion assay.
2. Prior to harvest, visually inspect cells, and record cell health, relative number, and morphology.
3. 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.

4. 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).
5. Transfer cells to a 15 ml conical tube, and add 5 ml of Quenching Medium (see *Materials Required But Not Supplied*).
6. Centrifuge cells at 200 x *g* for 3 minutes to pellet, remove quenching medium, and resuspend cells in 2 ml of desired medium.
7. Cells may need to be gently pipetted up and down with serological pipet to break up clumps.
8. Count cells, and dilute to 1 x 10⁶ cells per ml in desired medium.

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 into number of cells, standard curves are recommended. It is not necessary to use inserts in order to do this. If used, a separate standard curve may be run for each cell type and assay condition. Control and experimental replicates should be performed in triplicate.

C. Standard Curve Determination

1. Your data should fall in the linear range of the curve. Determine the saturation range for your cells (e.g. 25,000 to 50,000 cells), beyond which, additional invasion would be difficult to detect because an increase in signal is no longer linear and approaches an asymptote.
2. Add 10 µl of Calcein AM Solution (VI.3.) to 10 ml of 1X Cell Dissociation Solution (VI.2.), cap tube, and invert to mix.
3. Pellet cells, count, and resuspend in Calcein AM/Cell Dissociation Solution. A 1 x 10⁶ cells/ml stock can be serially diluted to provide the dilution series needed.
4. Serially dilute cells in Calcein AM/Cell Dissociation Solution (e.g. 140,000, 70,000, 35,000, 17,500, 8,750, 4,350, etc. cells/ml), and dispense 360 µl in triplicate into an empty 24 well plate (not provided) (e.g. 50,000, 25,000, 12,500, 6,250, 3,125, 1,563, etc. cells/well). Omit cells from at least three wells to calculate background. As an option, a standard curve may be made using 100 µl samples in a 96 well plate.
5. Incubate for one hour at 37°C in a CO₂ incubator.
6. Read at 485 nm excitation, 520 nm emission (see Table 1 for sample data) to obtain RFU values.
7. Average your values for each condition; then subtract the background from each value (Table 1).
8. Plot standard curve RFU values vs. number of cells (see Figure 2).
9. Insert a trend line (best fit) and use the equation $y = mx + b$ for each cell line to calculate the number of cells that invaded (Figure 2).
10. Experimental data should be analyzed using same parameters as standard curve (time, gain, well format, etc.).

Table 1. Sample Data for Standard Curve (actual results may vary):

Cells/Well	Wells			Avg.	Background = 254	
	1	2	3		- Bg. =	
50,000	15710	15415	16135	15663	- Bg. =	15409
25,000	9118	8702	8644	8821	- Bg. =	8567
10,000	4454	4257	4091	4267	- Bg. =	4013
5,000	2609	2541	2599	2583	- Bg. =	2329
1,000	930	922	881	911	- Bg. =	657

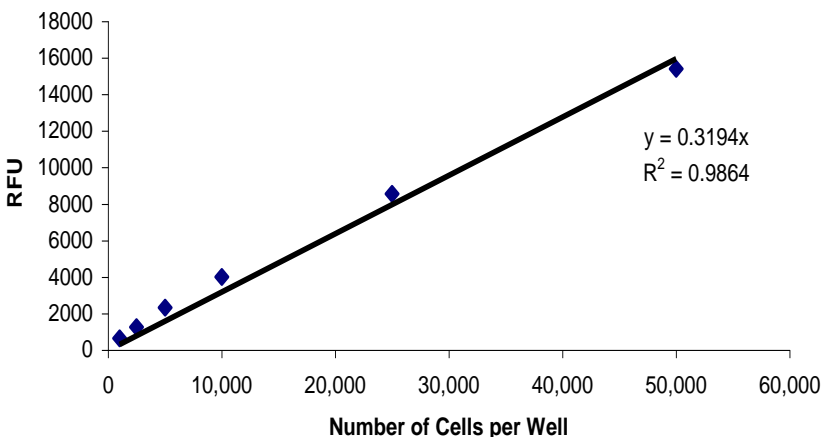


Figure 2. Standard Curve for a Cell Invasion Assay. HT-1080 cells were harvested (see section VII.A.), diluted, incubated for one hour with Calcein AM, and assayed for fluorescence (see section VII.C.). The trend line and line equation are included on the graph. A separate standard curve for each tested cell line is recommended.

D. Cell Invasion Assay

Prior to Day 1:

1. Culture cells to be assayed to 80% confluence. Adherent cells should be passaged at least one time prior to assay. Plan accordingly for sufficient cell numbers for each experiment (See section VII.A.).
2. Assay cells for standard curve to determine optimal seeding densities if needed (please see section VII.B).

3. Starve cells by incubating for 16-24 hours in Serum-Free medium (see Materials Required but Not Supplied) prior to assay (0.5% FBS may be used if needed).

Day 1 (Preparation of cells and CultreCoat® rehydration)

4. Transfer 24 well cell invasion inserts to room temperature and let sit for 1 hour.
5. Rehydrate the inserts by adding 80 µl of warm serum-free medium (37 °C), and incubating at 37 °C in a CO₂ incubator for one hour.
6. After serum starvation centrifuge cells at 200 x g for 3 min, remove supernatant, wash with 1X wash buffer, count, and resuspend.
7. Dilute to working concentration (1 x 10⁶ cells/ml is recommended) in a serum-free medium (0.5% FBS may be used if needed). Inhibitors may also be added to cells at this time.
8. After rehydration, add 40 µl of cells per well to each insert (40,000 cells per well recommended).
9. Add 360 µl of test media to bottom wells (with or without chemo-attractants and/or inhibitors). Assemble units.
10. Incubate at 37 °C in CO₂ incubator; incubation times may be varied (4-48 hours). The recommended incubation period is 24 hours.

Day 1-3:

11. Add 12 µl of Calcein AM solution (VI.3.) to 12 mL of 1X Cell Dissociation Solution (VI.2.).
12. After incubation, invert each insert and carefully shake to remove medium. Transfer the inserts to the new 24 well plate. **Be careful not to touch the bottom of the insert to any surface; this could remove cells that have invaded!**
13. Wash each insert with 100 µl of warm (37 °C) 1X Wash Buffer (VI.1.); invert the insert and carefully shake to remove wash buffer.
14. Place insert in the new 24 well plate.
15. Add 360 µl of Cell Dissociation Solution/Calcein AM to the bottom well of each well.
16. Incubate at 37 °C in a CO₂ incubator for 60 minutes to fluorescently label cells and dissociate them from the membrane. Periodic light tapping on the side of the assembled unit will aid in dissociation (every 15-30 minutes).
17. Disassemble units (remove inserts), and read the 24 well plate at 485 nm excitation, 520 nm emission using the same parameters (time and gain) as standard curve(s), or controls. Samples may be resuspended and transferred to a 96 well plate for analysis if needed.
18. Compare experimental data to controls to determine the number of cells that have invaded, or failed to invade according to experimental design.

VIII. Example Results

Typical results using MDA-MB-231 and HT-1080 cell lines are shown (Figure 3):

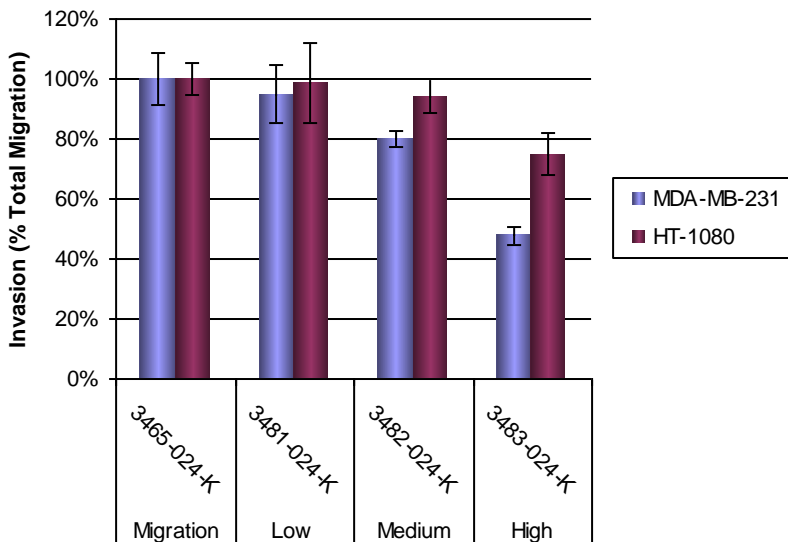


Figure 3. Different coating densities for the 24 Well BME Cell Invasion Inserts result in different invasion rates for invasive cell lines. HT-1080, human fibrosarcoma, and MDA-MB-231, breast cancer, cell lines were serum starved for 16 hours and seeded at 40,000 cells per well in pre-coated 8 μm inserts. Cells invaded in response to 10% FBS over a 24 hour period and were quantitated using Calcein AM. Samples were run in quadruplicate.

IX. Troubleshooting

Problem	Cause	Solution
No signal	Cells did not traverse the barrier	Cell type may be non-invasive or chemoattractant may be insufficient.
		There is inherent variability in FBS from lot to lot; this can affect the assay if used.
	Cells may have died as a result of treatment.	Test cells for viability in treatment regimen.

Problem	Cause	Solution
High background	Insufficient Washing - agents in media, FBS, and/ or chemoattractant may react with Calcein AM.	Re-assay, and make sure to wash well.
	Contamination – proteases released by bacteria or mold may activate 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

1. Tamilarasan KP, Kolluru GK, Rajaram M, Indhumathy M, Saranya R, Chatterjee S. 2006. Thalidomide attenuates nitric oxide mediated angiogenesis by blocking migration of endothelial cells. *BMC Cell Biol.* 7:17.
2. Borghesani PR, Peyrin JM, Klein R, Rubin J, Carter AR, Schwartz PM, Luster A, Corfas G, Segal RA. 2002. BDNF stimulates migration of cerebellar granule cells. *Development* 129:1435-1442.
3. 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.* 168:6420-6428.
4. Li G, Chen YF, Greene GL, Oparil S, Thompson JA. 1999 Estrogen inhibits vascular smooth muscle cell-dependent adventitial fibroblast migration *in vitro*. *Circulation* 100:1639-1645.

XI. Related products available from Trevigen.

Catalog#	Description	Size
3455-096-K	Cultrex® 96 Well BME Cell Invasion Assay	96 samples
3481-096-K	CultreCoat® 96 Well Low BME Cell Invasion Assay	96 samples
3482-096-K	CultreCoat® 96 Well Medium BME Cell Invasion Assay	96 samples
3483-096-K	CultreCoat® 96 Well High BME Cell Invasion Assay	96 samples
3484-096-K	CultreCoat® 96 Well BME-Coated Cell Invasion Optimization Assay	96 samples
3465-096-K	Cultrex® 96 Well Cell Migration Assay	96 samples
3455-024-K	Cultrex® 24 Well BME Cell Invasion Assay	24 inserts
3480-024-K	CultreCoat® 24 Well BME-coated Cell Invasion Assay	24 inserts
3481-024-K	CultreCoat® 24 Well Low BME Cell Invasion Assay	24 inserts
3482-024-K	CultreCoat® 24 Well Medium BME Cell Invasion Assay	24 inserts

Catalog#	Description	Size
3484-024-K	CultreCoat® 24 Well BME Cell Invasion Optimization Assay	24 inserts
3465-024-K	Cultrex® 24 Well Cell Migration Assay	24 inserts
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
3490-096-K	CultreCoat® BME 96 Well Cell Adhesion Assay	96 samples
3491-096-K	CultreCoat® Laminin 1 96 Well Cell Adhesion Assay	96 samples
3492-096-K	CultreCoat® Collagen I 96 Well Cell Adhesion Assay	96 samples
3493-096-K	CultreCoat® Collagen IV 96 Well Cell Adhesion Assay	96 samples
3494-096-K	CultreCoat® Fibronectin 96 Well Cell Adhesion Assay	96 samples
3495-096-K	CultreCoat® Vitronectin 96 Well Cell Adhesion Assay	96 samples
3496-096-K	CultreCoat® Adhesion Protein Array Kit	96 samples
3450-048-SK	Cultrex® Directed in vivo Angiogenesis Assay (DIVAA™) Starter Kit	48 samples
3450-048-K	Cultrex® DIVAA™ Activation Kit	48 samples
3450-048-IK	Cultrex® DIVAA™ Inhibition Kit	48 samples

Accessories:

Catalog#	Description	Size
3400-010-01	Cultrex® Mouse Laminin I	1 mg
3446-005-01	Cultrex® 3-D Culture Matrix™ Laminin I	5 ml
3440-100-01	Cultrex® Rat Collagen I	100 mg
3442-050-01	Cultrex® Bovine Collagen I	50 mg
3447-020-01	Cultrex® 3-D Culture Matrix™ Collagen I	100 mg
3410-010-01	Cultrex® Mouse Collagen IV	1 mg
3415-001-02	Cultrex® Human BME, PathClear®	1 mg/ 1 ml
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
3445-048-01	Cultrex® 3-D Culture Matrix™ BME	15 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
3420-001-01	Cultrex® Human Fibronectin	1 mg
3416-001-01	Cultrex® Bovine Fibronectin	1 mg
3421-001-01	Cultrex® Human Vitronectin	50 µg
3417-001-01	Cultrex® Bovine Vitronectin	50 µg
3438-100-01	Cultrex® Poly-L-Lysine	100 ml
3439-100-01	Cultrex® Poly-D-Lysine	100 ml
3437-100-K	Cultrex® Cell Staining Kit	100 ml
3450-048-05	CellSpere™	15 ml

XII. Appendices

Appendix A. Reagent and Buffer Composition

1. **CultreCoat® 24 Well High BME Cell Invasion Inserts (cat# 3483-024-01)**

24 well BME-coated Boyden chamber inserts, 8.0 μm polycarbonate membrane. Clear culture wells allow monitoring during invasion (Figure 4). Fluorescence may be quantitated directly in the 24 well plate. To evaluate in a 96 well plate format, resuspend cells and transfer 100 μl to a black 96 well plate for detection.

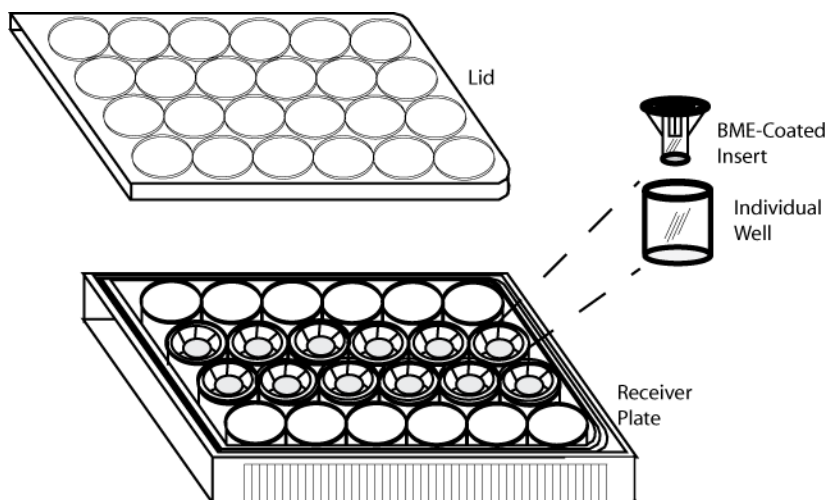


Figure 4. Diagram of Trevigen's 24 well version of the High BME Cell Invasion Inserts and receiver plate.

2. **25X Cell Wash Buffer (cat# 3455-096-04)**

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

3. **10X Cell Dissociation Solution (cat# 3455-096-05)**

Proprietary formulation containing sodium citrate, EDTA, and glycerol.

4. **Calcein AM (cat# 4892-010-01)**

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 of viable cells.

**The product accompanying this document is intended
for research use only and is not intended for
diagnostic purposes or for use in humans.**

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