CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

CultreCoat® 96 well Low BME Cell Invasion Assay

Reagent kit for evaluating cell invasion for minimally invasive cell models

96 Samples

Catalog #: 3481-096-K

CultreCoat® 96 Well Low BME Cell Invasion Assay

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96 Samples

Table of Contents

I. Quick Reference Procedure for CultreCoat® 96 Well Low BME Cell Invasion Assay (cat# 3481-096-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 96 well cell invasion chamber to room temperature, and let sit for one hour.
- ☐ 5. Rehydrate the inserts by adding 25 µl of serum-free medium (~37 ºC, page 4) to each insert, and incubating at 37 ºC in a CO_2 incubator for 1 hour.
- ☐ 6. After 16 hours serum starvation, harvest and count cells.
- ☐ 7. Dilute to working concentration (1 x 10^6 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 25 µl of cells per well to each top chamber (25,000 cells recommended).
- ☐ 9. Add 150 µl of medium per well to bottom chamber (with or without chemoattractants and/or inhibitors).
- ☐ 10. Assemble chamber and incubate at 37 ºC in a CO_2 incubator for 4-48 hours (24 hours recommended).

Day 1-3

- ☐ 11. Add 12 µl of Calcein AM (page 4) solution to 12 mL of Cell Dissociation Solution (page 4).
- ☐ 12. Invert and lightly shake top chamber into a dish to remove contents.
- ☐ 13. Wash each well with 50 µl of 1X Wash Buffer (page 4); invert and lightly shake top chamber into a dish to remove contents.
- ☐ 14. Transfer top chamber to black receiver plate.
- ☐ 15. Add 100 µl of Cell Dissociation Solution/Calcein AM to bottom chamber (black receiver), and incubate at 37 ºC in a CO_2 incubator for one hour.
- ☐ 16. Lightly tap the side of the chamber ten times to remove cells from membranes.
- ☐ 17. Remove top chamber, and read plate at 485 nm excitation, 520 nm emission.
- ☐ 18. Analyze data.

II. Background

Cell invasion is fundamental to angiogenesis[1], embryonic development[2], immune responses[3], and tumor cell metastasis[4]. Trevigen's **CultreCoat® 96 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® 96 Well BME Optimization Cell Invasion Assay.**

The **CultreCoat® 96 Well Low BME Cell Invasion Assay** contains one 96 well plate that is coated with a low density of BME, for cell models that exhibit a nominal invasive capacity. The low amount of BME coating provides the highest sensitivity for minimally invasive cell models, giving the maximum signal to noise ratio and dynamic range.

The **CultreCoat® 96 Well Low 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.

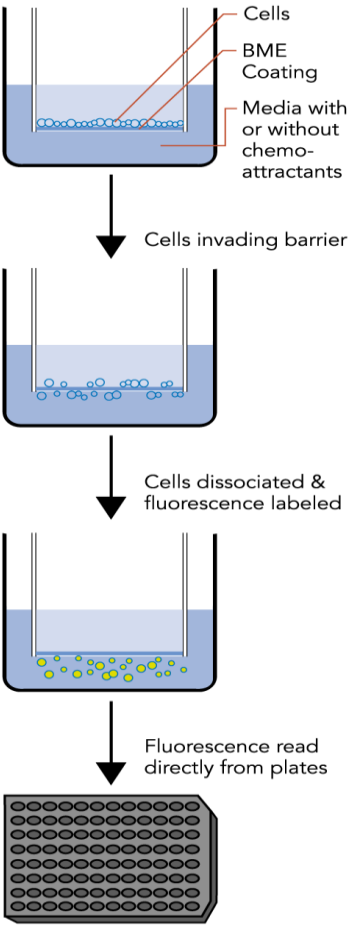

Figure 1. Illustration of protocol for Trevigen's CultreCoat® 96 Well BME Cell Invasion Assays.

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

Component	Quantity	Storage	Catalog#
96 Well Low BME Cell Invasion Chamber	each	≤-20 °C*	3481-096-01
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 CO_2 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 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 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

Disposables

1. Cell culture flask, 25 cm^2 or 75 cm^2
2. 50 ml tubes
3. 1 - 200 µl and 200 - 1000 µl pipette tips
4. 1.5 and 10 ml serological pipettes
5. Gloves
6. 10 ml syringe
7. 0.2 µm filter
8. 96 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 µl of sterile DMSO to make working solution. Pipet up and down to mix, and store solution at –20 ^oC.

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 chamber can accommodate 1 x 10^5 – 5 x 10^5 cells depending upon cell type. A 25 cm^2 or 75 cm^2 flask will yield approximately 2 x 10^6 or 6 x 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 cm^2 flask and 10 ml per wash for a 75 cm^2 flask.

4. Harvest cells. For 25 cm^2 flask or 75 cm^2 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 10^6 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 chambers 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 5 µl of Calcein AM Solution (VI.3.) to 5 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 10^6 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. 500,000, 250,000, 125,000, 62,500, 31,250, 15,625, etc. cells/ml), and dispense 100 µl in triplicate into an empty 96 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.
5. Incubate for one hour at 37°C in a CO_2 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 Fig. 1).
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 (Fig. 2).

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

	Wells					
Cells/Well	**1**	**2**	**3**	**Avg.**	**Background = 254**	
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

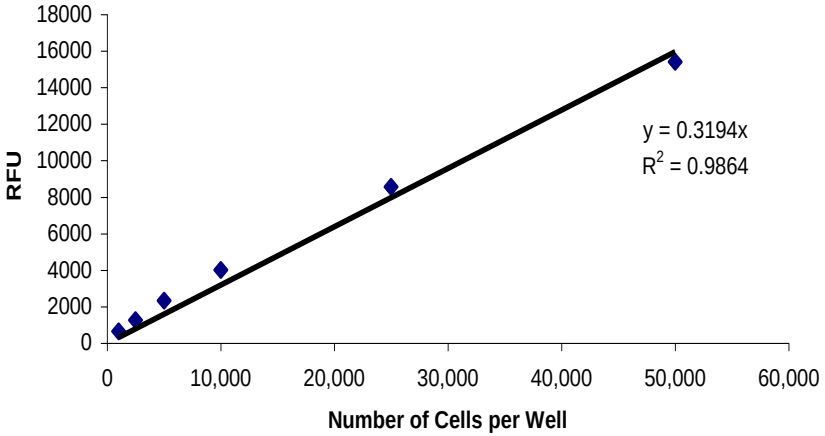

Figure 2. Standard Curve for a Cell Invasion Assay. HT-1080 cells were harvested (page 5), diluted, incubated for one hour with Calcein AM, and assayed for fluorescence (page 6). The trend line and line equation are included on the graph. A separate standard curve for each tested cell line is recommended.

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 96 well cell invasion chambers to room temperature and let sit for 1 hour.
5. Rehydrate the chambers by adding 25 µl of warm serum-free medium (37 ^{o}C), and incubating at 37 ^{o}C in a CO_2 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 10^6 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 25 µl of cells per well to each top chamber (25,000 cells per well recommended).
9. Add 150 µl of test media to bottom chambers (with or without chemo-attractants and/or inhibitors). Assemble chambers.
10. Incubate at 37 ^{o}C in CO_2 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 the top chamber and carefully shake to remove medium. Transfer the top chamber to the black receiver plate. **Be careful not to touch the bottom of the top chamber to any surface; this could remove cells that have invaded!**
13. Wash each well with 100 µl of warm (37 ^{o}C) 1X Wash Buffer (VI.1.); invert the top chamber and carefully shake to remove wash buffer.
14. Place top chamber back on black receiver plate.
15. Add 100 µl of Cell Dissociation Solution/Calcein AM to the bottom chamber of each well.
16. Incubate at 37 ^{o}C in a CO_2 incubator for 60 minutes to fluorescently label cells and dissociate them from the membrane. Periodic light tapping on the side of the assembled chamber will aid in dissociation (every 15-30 minutes).
17. Disassemble chambers (remove top chamber), and read plate (assay chamber solutions/bottom) at 485 nm excitation, 520 nm emission using the same parameters (time and gain) as standard curve(s), or controls.
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 in figure 3:

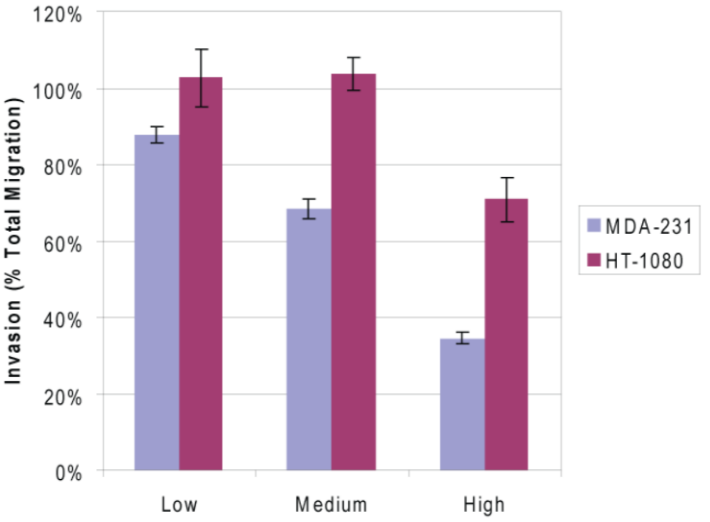

Figure 3. Different coating densities for the 96 Well BME Cell Invasion Chamber result in different invasion rates for invasive cell lines. HT-1080, human fibrosarcoma, and MDA-MB-231, breast cancer, cell lines were serum starved for 16 hours and seeded at 25,000 cells per well in pre-coated 8 µm chambers. 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.

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XI. Related products available from Trevigen.

Catalog#	Description	Size
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
3455-096-K	Cultrex® 96 Well 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
3480-024-K	CultreCoat® 24 Well BME-coated Cell Adhesion Assay	24 inserts
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	CellSperse™	15 ml

XII. Appendices

Appendix A. Reagent and Buffer Composition

1. **CultreCoat® 96 Well Low BME Cell Invasion Chamber (cat# 3481-096-01)**
   96 well BME-coated Boyden chamber, 8.0 um polycarbonate membrane. Clear culture chamber allows monitoring during invasion, and black receiver plate is compatible with 96 well fluorescent plate reader (figure 4). For the normal invasion protocol, the culture chamber plate should be used during rehydration, and observation of cell invasion, whereas the black assay chamber plate is used during detection/ quantitation. If a plate reader that reads from the bottom is used, the entire assay/detection may be conducted using the culture chamber plate (with clear wells). The contents of each culture chamber well may also be transferred to a black assay plate with a clear bottom for detection if signals from neighboring wells need to be minimized.

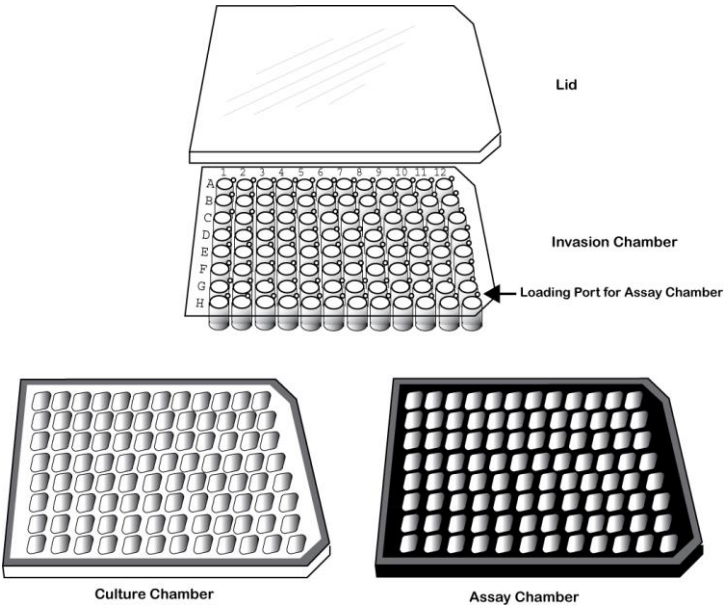

Figure 4. Diagram of Trevigen's 96 well version of the Low BME Cell Invasion Chamber and assay plates.

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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