CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

CultreCoat® 24 Well BME-Coated Cell Invasion Assay

Reagent kit for investigating cell invasion

24 inserts

Catalog #: 3480-024-K

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24 inserts

Table of Contents

I. Quick Reference Procedure for CultreCoat® 24 Well BME-Coated Cell Invasion Assay (cat# 3480-024-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. Twenty-four 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)

- ☐ 3. Transfer 24 well cell invasion chambers to room temperature, and let sit for one hour.
- ☐ 4. Rehydrate the inserts by adding 100 µl of 1X Rehydration Solution (~37 ^{o}C, page 4) to each insert, and incubating at 37 ^{o}C in a CO_2 incubator for 1 hour.
- ☐ 5. After 24 hours under serum starvation, harvest and count cells.
- ☐ 6. 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).
- ☐ 7. After rehydration, carefully invert and tap inserts to remove excess buffer. Do not aspirate membrane. This may inadvertently puncture or disrupt the BME coating. Add 100 µl of cells per well to each top chamber. Inhibitors may also be added to cells at this time.
- ☐ 8. Add 500 µl of media per well to bottom chamber (with or without chemoattractants).
- ☐ 9. Assemble chamber and incubate at 37 **º**C in a CO_2 incubator for 4-48 hours.
- ☐ 10. If desired, assay remaining cells for standard curve (please see section VII B).

Day 1-3

- ☐ 11. Carefully aspirate top chamber, and wash each well with 100 µl of 1X Wash Buffer (page 4). Do not puncture membrane.
- ☐ 12. Aspirate bottom chamber, and wash each well with 500 µl 1X Wash Buffer.
- ☐ 14. Add 12 µl of Calcein-AM (page 4) solution to 12 mL of Cell Dissociation Solution (page 4).
- ☐ 15. Add 500 µl of Cell Dissociation Solution/Calcein-AM to bottom chamber, assemble cell migration device, and incubate at 37 **º**C in a CO_2 incubator for one hour.
- ☐ 16. Remove top chamber, and read plate at 485 nm excitation, 520 nm emission.
- ☐ 17. Using standard curve(s), convert RFU to Cell Number; determine percent invasion.

II. Background

Trevigen's Cultrex® Cell Invasion Assays were originally created in an effort to accelerate the screening process for compounds that influence cellular invasion through extracellular matrices, which is fundamental to angiogenesis[1], embryonic development[2], immune responses[3], and tumor cell metastasis[4]. 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.

Cultrex® Basement Membrane Extract is a soluble form of basement membrane purified from Engelbreth-Holm-Swarm (EHS) tumor. The extract gels at 37 °C to form a reconstituted basement membrane. The major components of the Basement Membrane Extract include laminin I, collagen IV, entactin, and heparin sulfate proteoglycan. BME can be used for promotion and maintenance of a differentiated phenotype in a variety of cell cultures including primary epithelial cells, endothelial cells, and smooth muscle cells. It has been employed in angiogenesis assays, tumor cell invasion assays, and as a vehicle to augment the tumorigenicity of injected tumor cells in nude mice.

To provide a ready-to-use platform for the analysis of responses to chemokines, toxins, drugs and other analytes of interest, for larger numbers of cells per well, Trevigen offers its **CultreCoat® 24 Well BME-Coated Cell Invasion Assay Kit**. Each insert is coated with PathClear® growth factor reduced BME, to allow investigators to control for matrix-induced effects. The modular format of the **CultreCoat® 24 Well BME-Coated Cell Invasion Assay** offers flexible utility, convenience, and sufficient insert size for informative results.

The **CultreCoat® 24 Well BME-Coated Cell Invasion Assay** utilizes a simplified Boyden chamber design with an 8 μm polycarbonate (PC) membrane. 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 using a standard curve.

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#
24 Well BME-coated Cell Invasion Chamber	each	≤-20 °C*	3480-024-01
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
20X Rehydration Solution A	1 ml	Room Temp.	3480-024-02
20X Rehydration Solution B	1 ml	Room Temp.	3480-024-03

*Store in a manual defrost freezer; for long term storage, store at -80 °C

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. 50 and 500 ml graduated cylinders
6. -20°C and 4°C storage
7. Ice bucket
8. Standard light microscope (or inverted)
9. Pipette helper
10. Timer
11. Vortex mixer
12. Fluorescent 24 or 96 well plate reader, top reader (485 nm excitation, 520 nm emission)
13. Computer and graphing software, such as Microsoft® Excel®.
14. Clear, Flat bottom 24 Well Plates (if generating standard curve)

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 Media, Tissue Culture Growth Media without serum.
4. Chemoattractants or pharmacological agents for addition to culture medium.
5. Quenching medium: serum-free media 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

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 °C.
4. **1X Rehydration Solution**
   Add 1 ml of 20X Rehydration Solution A and 1 ml of 20X Rehydration Solution B to 18 ml of sterile water, and warm to 37 °C prior to use.

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 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 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 3 x 10^6 or 9 x 10^6 cells, respectively. Plan to have enough cells for a standard curve, if used, controls and cell invasion assay.
2. Starve cells by incubating 18-24 hours in Serum-Free medium (see Materials Required But Not Supplied) prior to assay (0.5% FBS may be used if needed).

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 cm^2 flask and 10 ml per wash for a 75 cm^2 flask.
5. 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).
6. Transfer cells to a 15 ml conical tube, and add 5 ml of Quenching Medium (see Materials 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 with serological pipet to break up clumps.
8. Count cells, and dilute to 1 x 10^6 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 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. 50,000 to 500,000 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 12 µl of Calcein-AM Solution (item 3, page 4) to 12 ml of 1X Cell Dissociation Solution (item 2, page 4), cap tube, and invert to mix.
3. For a standard curve, dispense, in triplicate, a serial dilution series (e.g. 100,000, 50,000, 25,000, 12,500, 6,250, 3,125, etc. cells/well) into an empty 24 well plate. Phenol red in culture media will interfere with your signal, so aliquot your cells in 1X Cell Dissociation Solution containing Calcein-AM. A 1 x 10^6 cells/ml stock can be serially diluted to provide the dilution series shown in the parenthesis.
4. Add 500 µL of each dilution series in 1X Cell Dissociation Solution/Calcein-AM to each set of wells , and incubate for one hour; omit cells from at least three wells to calculate background.
5. Read at 485 nm excitation, 520 nm emission (see Table 1 for sample data) to obtain RFU values.
6. Average your values for each condition; then subtract the background from each value (Table 1).
7. Plot standard curve RFU values vs. number of cells (see Fig. 1).
8. 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. 1).

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

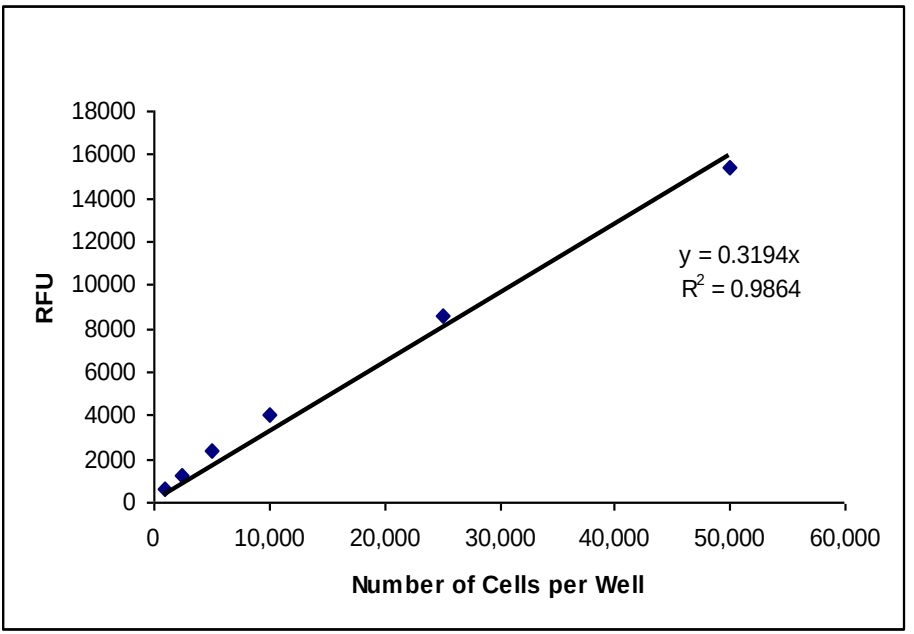

Figure 1. Standard Curve for a Cell Invasion Assay. HT-1080 cells were harvested (page 4), diluted, incubated for one hour with calcein AM, and assayed for fluorescence (page 5). The trend line and line equation are included on the graph. A separate standard curve for each tested cell line is recommended.

D. Cell Invasion Assay

Prior to Day 1:

1. Culture cells to be assayed to 80% confluence. Adherent cells may need to be passaged at least one time prior to assay. Plan accordingly for sufficient numbers of cells per insert.
2. 24 hours prior to assay, cells may need to be serum starved in order to allow ligands to bind to free receptors. This step may be omitted, depending on the cell types under investigation.

Day 1 (Preparation of cells and CultreCoat® rehydration)

3. Transfer 24 well cell invasion chambers to room temperature and let sit for 1 hour.
4. Rehydrate the inserts by adding 100 µl of warm 1X Rehydration Solution (37 ^{o}C), and incubating at 37 ^{o}C in a CO_2 incubator for one hour.
5. After 24 hours of serum starvation, if used, centrifuge cells at 250 x g for 10 min, remove supernatant, wash with 1X wash buffer, count and resuspend at 1 x 10^6 cells/ml in a serum free medium (0.5% FBS may be used if needed).
6. After rehydration, carefully invert and tap inserts to remove excess buffer. Do not aspirate membrane. This may inadvertently puncture or disrupt the BME coating. Add 100 µl of cells per well to each top chamber. Inhibitors may also be added to cells at this time.
7. Add 500 µl of test media to bottom chambers (with or without drugs, chemokines, etc.). Assemble chambers.
8. Incubate at 37 ^{o}C in CO_2 incubator; incubation times may be varied (4-48 hours). If desired, assay remaining cells for standard curve (please see section VII B).

Day 1-3:

9. After incubation, carefully aspirate top chamber, **without puncturing the membrane,** and wash each well with 100 µl of warm (37 ^{o}C) 1X Wash Buffer (item 1, page 4).
10. Aspirate bottom chamber, and wash each well with 500 µl warm (37 ^{o}C) 1X Wash Buffer.
11. Add 12 µl of Calcein AM solution (item 3, page 4) to 12 mL of 1X Cell Dissociation Solution (item 2, page 4).
12. Add 500 µl of Cell Dissociation Solution/Calcein AM to the bottom chamber of each well, reassemble the chambers, and incubate at 37 ^{o}C in a CO_2 incubator for 60 minutes.
13. Disassemble chambers (remove inserts), and read plate (assay chamber solutions/bottom) at 485 nm excitation, 520 nm emission using same parameters (time and gain) as standard curve(s), or controls.
14. Compare experimental data to controls, and convert RFU into cell number (page 5) to determine the number of cells that have migrated, or invaded, or failed to migrate or invade according to experimental design.

VIII. Example Results

Typical results using MCF-7 and HT-1080 cell lines are shown in figure 2:

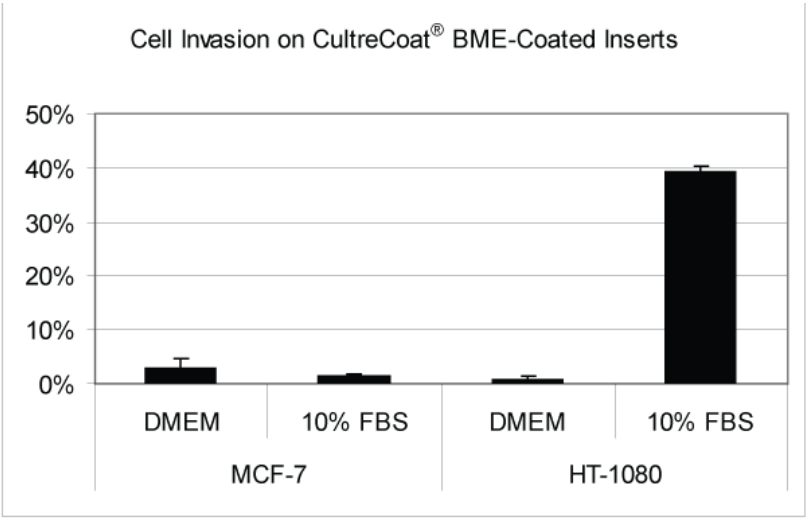

Figure 2. Quantitation of the ability of the human fibroblastic cell line, HT-1080, to cross a barrier consisting of an 8 micron polycarbonate filter occluded with Cultrex® Basement Membrane Extract over a 24 hour period in response to 10% FBS. Samples were run in triplicate for both HT-1080 and the non-invasive MCF-7, mammary epithelial cell line.

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.

Problem	Cause	Solution
No Signal (cont.)	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 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
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
3483-024-K	CultreCoat® 24 Well High BME Cell Invasion Assay	24 inserts
3484-024-K	CultreCoat® 24 Well BME-Coated Cell Invasion Optimization Assay	24 inserts
3455-024-K	Cultrex® 24 Well BME Cell Invasion 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

Accessories:

Catalog#	Description	Size
3434-005-02	Cultrex® Stem Cell Qualified BME, Growth Factor Reduced, PathClear®	5 ml
3415-001-02	Cultrex® Human BME, PathClear®	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 no phenol red; reduced growth factor PathClear®	5 ml
3400-010-02	Cultrex® Mouse Laminin I, PathClear®	1 ml
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
3420-001-01	Cultrex® Human Fibronectin, PathClear®	1 mg
3416-001-01	Cultrex® Bovine Fibronectin, NZHD*	1 mg
3421-001-01	Cultrex® Human Vitronectin, PathClear®	50 µg
3417-001-01	Cultrex® Bovine Vitronectin, NZHD*	50 µg
3438-100-01	Cultrex® Poly-L-Lysine	100 ml
3439-100-01	Cultrex® Poly-D-Lysine	100 ml
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™ Collagen I	100 mg
3430-005-01	Cultrex® BME with Phenol Red	5 ml
3432-005-01	Cultrex® BME without Phenol Red	5 ml
3431-005-01	Cultrex® BME with Phenol Red; Reduced Growth Factor	5 ml
3433-005-01	Cultrex® BME; no Phenol Red; Reduced Growth Factor	5 ml

*New Zealand Herd Derived

XII. Appendices

Appendix A. Reagent and Buffer Composition

1. CultreCoat® BME-Coated Cell Invasion Chamber (cat# 3480-024-01)
24 BME-Coated Boyden Chamber inserts, 8.0 um polycarbonate membrane, contained within two 24-Well Plates compatible with 24 well fluorescent plate reader (figure 3).

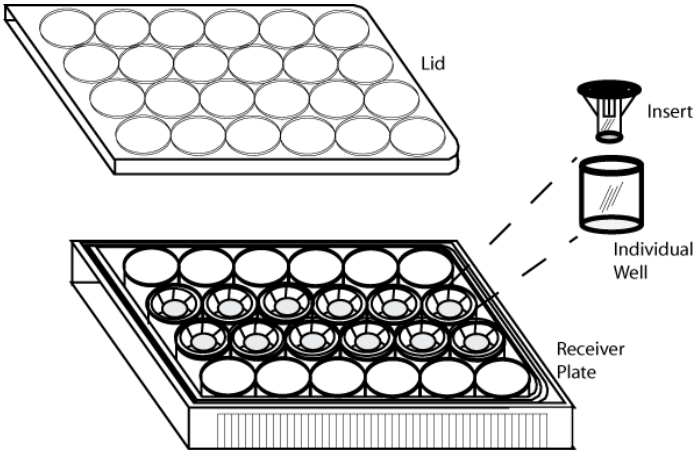

Figure 3. Diagram of 24-Well Cell Invasion Chamber.

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.

5. **20X Rehydration Solutions A and B (cat# 3480-024-02 and 3480-024-03)**
Proprietary formulation for rehydration of BME-Coated inserts containing buffered saline and divalent cations.

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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