CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

Cultrex® 24 Well BME Cell Invasion Assay

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

24 inserts

Catalog #: 3455-024-K

interchim

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I. Quick Reference Procedure for CULTREX® 24 well BME Cell Invasion Assay (Cat# 3455-024-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.

Day 1 or 2 (Preparation of cells and Coating of Insert Membranes)

- ☐ 2. Twenty-four hours prior to beginning assay, starve cells in serum-free media (0.5% FBS may be used if needed).
- ☐ 3. Working on ice, prepare 0.1X to 1.0X BME stock solution in a sterile 15 ml conical tube, invert to mix.
- ☐ 4. Aliquot 100 µl of BME coat (section VI item 5) per well. All wells should be evenly coated.
- ☐ 5. Incubate coated chambers at 37°C for 4 hours or overnight.

Day 2

- ☐ 6. After 24 hours under serum starvation, harvest and count cells.
- ☐ 7. Centrifuge cells at 250 x g for 10 min, remove supernatant, wash with 1X wash buffer, and suspend at 1 x 10^6 cells/ml in serum free media (0.5% FBS may be used if needed). Inhibitors may also be added to cells at this time (for final concentration of 5 x 10^5 cells/ml, or other customized number).
- ☐ 8. Carefully aspirate top chamber. DO NOT ALLOW TOP OR BOTTOM CHAMBERS TO DRY, and add 100 µl of cells per well to top chamber.
- ☐ 9. Add 500 µl of media per well to bottom chamber (with or without chemoattractants).
- ☐ 10. Assemble chamber and incubate at 37°C in a CO_2 incubator for 4-48 hours.
- ☐ 11. If desired, assay remaining cells for standard curve (please see control section).

Day 3

- ☐ 12. Carefully aspirate top chamber, and wash each well with 100 µl of 1X Wash Buffer. Do not puncture membrane.
- ☐ 13. Aspirate bottom chamber, and wash each well with 500 µl 1X Wash Buffer.
- ☐ 14. Add 12 µl of Calcein-AM solution to 12 mL of Cell Dissociation Solution.
- ☐ 15. Add 500 µl of Cell Dissociation Solution/Calcein-AM to bottom chamber, assemble cell migration device, and incubate at 37°C in CO_2 incubator for one hour.
- ☐ 16. Remove top chambers, and read dissolved Calcein-AM at 485 nm excitation, 520 nm emission (some users will transfer this solution to a 96 well plate for reading).

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]. To provide a 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 **Cultrex® 24 Well BME Cell Invasion Assay** kit. The modular format of the **Cultrex® 24 Well BME Cell Invasion Assay offers flexible utility, and sufficient insert size for informative results.**

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.

The **Cultrex® 24 Well BME Cell Invasion Assay** utilizes a simplified Boyden chamber design with an 8 µm polycarbonate (PC) membrane. Detection of cell migration 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 invaded or migrated using a standard curve.

Trevigen's Cultrex® Cell Invasion Assays allow cell invasion to be evaluated on:

- **Basement Membrane Extract (BME)**
- **Laminin I**
- **Collagen I**
- **Collagen IV**

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 for the cell types of interest by adjusting the coating concentration. A 1X Coating Solution is recommended for highly invasive cells, whereas the Coating Solution may be diluted up to 0.1X for less invasive cell types. The process of transfection itself may also alter the invasive capacity of the cells under investigation, and as a result, may require a more permissive barrier.

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

Component	Quantity	Storage	Catalog#
24 well cell invasion/migration plate	2	room temp	3455-024-01
5X BME Solution	1 ml	≤-20°C	3455-096-02
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

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. **10X BME Coating Buffer**
   Dilute 1 ml of 10X stock in 9 ml of sterile, deionized water to make 1X solution.
5. **BME coat**
   Dilute one volume 5X BME in 4 volumes of 1X BME Coating Buffer to make a 1X solution. Dilute one volume 5X BME in 49 volumes 1X BME Coating Buffer to make a 0.1X solution.

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^{0}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 (please see table 1 below). 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. Determine the saturation range for your cells (e.g. 50,000 to 100,000 cells), beyond which, additional invasion is difficult to detect.
2. For a standard curve, a serial dilution series is aliquotted as appropriate, whereas untreated controls are allowed to invade coated or uncoated membranes in triplicate wells.
3. Add 12 µl of Calcein-AM Solution (section VI, item 3) to 12 ml of 1X Cell Dissociation Solution (section VI, item 2), cap tube, and invert to mix.
4. Add 500 µL of 1X Cell Dissociation Solution/Calcein-AM to each well, and incubate for one hour; omit cells (and inserts) from at least three wells to calculate background.
5. Read supernatants at 485 nm excitation, 520 nm emission, and then subtract the average background value (see Table 1 for sample data) to obtain relative fluorescence units (RFU).
6. Calculate the average values for each condition (see Table 1).
7. If desired, plot standard curve RFU values vs. number of cells (see Fig. 1).
8. Insert a trend line (best fit) and use the line equation for each cell line in calculating number of cells that migrated (See Figure 1).

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

	Wells				
Cells/Well	**1**	**2**	**3**	**Avg.**	**Background = 566**
50,000	5656	4010	4472	4713	
25,000	3398	2988	2777	3054	
10,000	1396	969	827	1064	
5,000	774	610	434	606	
1,000	110	165	82	119	

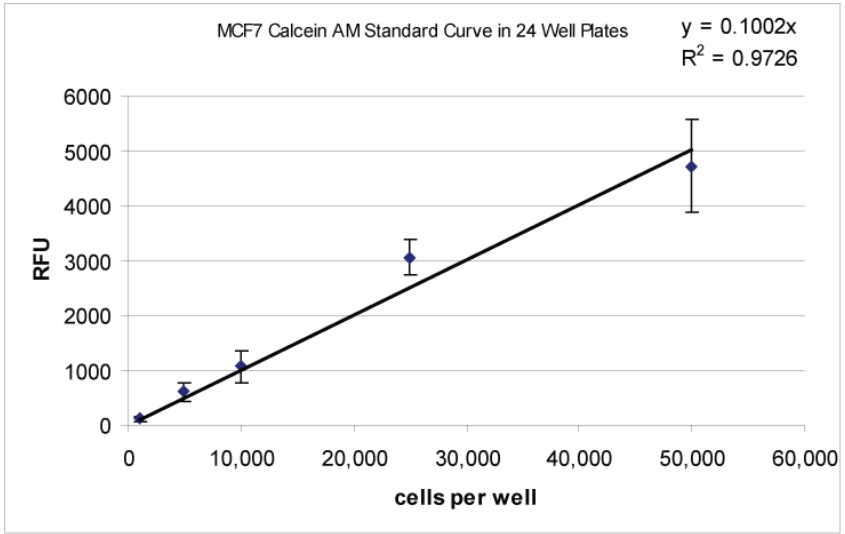

Figure 1. Standard Curve for a Cell Invasion Assay. MCF-7 cells were harvested (page 4), diluted, incubated for one hour with calcein AM, and assayed for fluorescence (page 4). The trend line and line equation are included on the graph.

D. Cell Invasion Assay

Prior to Day 1:

1. Culture cells to be assayed to 80% confluence. Plan accordingly for sufficient numbers of cells per insert.

Day 1:

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.

3. In the hood, working on ice, prepare 100 µl of 0.1X to 1X BME stock solution for each insert being used in a sterile 15 ml conical tube, invert to mix. Less invasive cell types will need less BME in order to traverse the coated membrane. Some optimization with varying amounts of BME may be required.
4. Aliquot 100 µl of BME coat per insert. Place lid on chamber, and visually assess each insert. The coating buffer should be evenly distributed across each insert. If necessary, the plate may be tapped lightly to evenly disperse the coating buffer.
5. Incubate coated chambers at 37°C, for 4 hours or overnight.

Day 2:

6. After serum starvation, if used, load invading cells into top chamber, and to compensate for background, omit cells from at least three wells (no inserts are needed). Alternatively, set aside uncoated inserts if migration is to be detected and compared with invasion.
7. Centrifuge cells at 250 x g for 10 min, remove supernatant, wash with 1X wash buffer, and suspend at 1 x 10^6 cells/ml in serum free media (0.5% FBS may be used if needed) or assay concentration of choice. Inhibitors may also be added to cells at this time.
8. Carefully aspirate top chamber. DO NOT ALLOW TOP OR BOTTOM CHAMBERS TO DRY, and add 100 µl of cells per well to top chamber. **Note**: It is important to avoid cell clumping and overcrowding.
9. Add 500 µl of test media to bottom chambers (with or without drugs, chemokines, etc.). Assemble chambers.
10. Incubate at 37°C in CO_2 incubator; incubation times may be varied (4 hours-48 hours).
11. After incubation, carefully aspirate top chamber, **without puncturing the membrane**.

Day 3:

12. Carefully aspirate top chamber and wash each well with 100 µl 1X Wash Buffer. Do not puncture membrane.
13. Aspirate bottom chamber, and wash each well with 500 µl 1X Wash Buffer (section VI, item 1).
14. Add 12 µl of Calcein AM solution (section VI, item 3) to 12 mL of 1X Cell Dissociation Solution (section VI, item 2).
15. Add 500 µl of Cell Dissociation Solution/Calcein-AM to the bottom chamber of each well, reassemble the chambers, and incubate at 37°C in a CO_2 incubator for 60 minutes.
16. Disassemble chambers, and read assay chamber solutions (bottom) at 485 nm excitation, 520 nm emission using same parameters (time and gain) as standard curve, or controls.
17. Compare data to standard curve or controls to determine the number of cells that have migrated, or invaded, or failed to migrate or invade according to experimental design.

VIII. Example Results

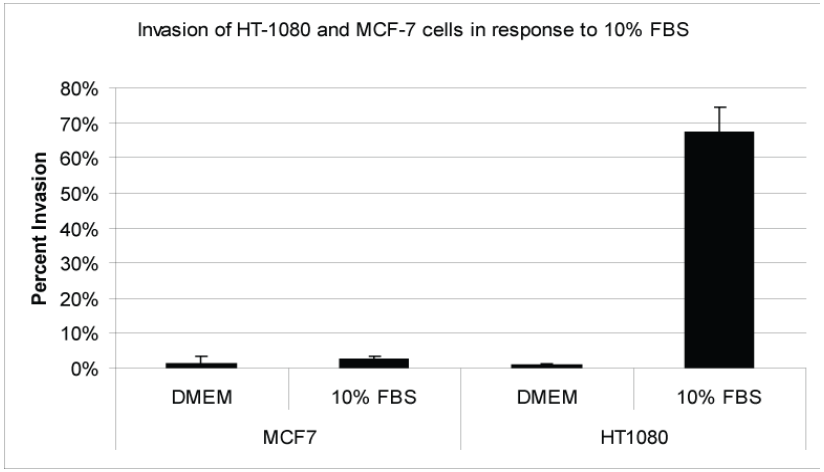

Figure 2. Example results for MCF7, and HT1080 cells processed as described under VII. Assay Protocol, Section D. Triplicate wells containing 50,000 cells/well were used, and invasion towards 10% FBS was measured as described above.

IX. Troubleshooting

Problem	Cause	Solution
No signal	Cells did not invade or migrate	Cell type may be non-invasive or chemoattractant may be insufficient.
		There is inherent variability in FBS from lot to lot; this can affect assay if used.
	Toxicity/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 chemo-attractant 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. Thalidomide attenuates nitric oxide mediated angiogenesis by blocking migration of endothelial cells. BMC Cell Biol. 2006 Apr 4;7:17.
2. Borghesani PR, Peyrin JM, Klein R, Rubin J, Carter AR, Schwartz PM, Luster A, Corfas G, Segal RA. BDNF stimulates migration of cerebellar granule cells. *Development*. 2002 Mar;129(6):1435-42.
3. Mohan K, Ding Z, Hanly J, Issekutz TB. 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.* 2002 Jun 15;168(12):6420-8.
4. Li G, Chen YF, Greene GL, Oparil S, Thompson JA. Estrogen inhibits vascular smooth muscle cell-dependent adventitial fibroblast migration in vitro. *Circulation.* 1999 Oct 12;100(15):1639-45.

XI. Related products available from Trevigen.

Catalog#	Description	Size
3460-024-K	Cultrex® CultreCoat® 24 Well BME-Coated Cell Invasion Assay	24 inserts
3465-024-K	Cultrex® 24 Well BME Cell Migration Assay	24 inserts
3465-096-K	Cultrex® 96 Well BME Cell Migration Assay	96 samples
3455-096-K	Cultrex® 96 Well BME Cell Invasion Assay	96 samples
3456-096-K	Cultrex® 96 Well Laminin I Cell Invasion Assay	96 samples
3457-096-K	Cultrex® 96 Well Collagen I Cell Invasion Assay	96 samples
3458-096-K	Cultrex® 96 Well Collagen IV Cell Invasion Assay	96 samples
3456-024-K	Cultrex® 24 Well Laminin I Cell Invasion Assay	24 samples
3457-024-K	Cultrex® 24 Well Collagen I Cell Invasion Assay	24 samples
3458-024-K	Cultrex® 24 Well Collagen IV Cell Invasion Assay	24 samples
3450-048-SK	Cultrex® Directed *in vivo* Angiogenesis Assay (DIVAA™) Starter Kit	48 samples
3450-048-K	Cultrex® Directed *in vivo* Angiogenesis Assay Activation Kit	48 samples
3450-048-IK	Cultrex® Directed *in vivo* Angiogenesis Assay Inhibition Kit	48 samples

Accessories:

Catalog#	Description	Size
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, No Phenol Red, PathClear®	5 ml
3433-005-02	Cultrex® BME, No Phenol Red, 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
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
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
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. Appendices

Appendix A. Reagent and Buffer Composition

1. **Cell Migration Inserts (Cat# 3455-024-01)**
   24 Well Boyden Chamber, 8.0 µm PC membrane, clear receiver plate compatible with 24 well fluorescent plate reader.

2. **25X Cell Wash Buffer (Cat# 3455-096-04)**
   PBS buffer for washing cells (250 mM Potassium Phosphate (pH 7.4), 3.625 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.

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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