CULTREX® Instructions

*For Research Use Only. Not For Use In Diagnostic Procedures*

# 24 Well Cell Migration Assay

## Reagent kit for investigating chemotaxis

**24 inserts**

**Catalog #: 3465-024-K**


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## Table of Contents

**I. Quick Reference Procedure for Trevigen's 24 Well Cell Migration Assay (Cat# 3465-024-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**

- ☐ 3. After 24 hours serum starvation (optional), harvest and count cells.
- ☐ 4. Centrifuge cells at 250 x g for 10 minutes, 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).
- ☐ 5. Add 100 µl of cells per well to each top chamber. Inhibitors/stimulants may also be added to cells at this time.
- ☐ 6. Add 500 µl of media per well to bottom chamber (with or without chemoattractants).
- ☐ 7. Assemble chamber and incubate at 37 °C in a $CO_2$ incubator for 4-48 hours.
- ☐ 8. If desired, assay remaining cells for standard curve (please see section VII B).

**Day 1-3**

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



## II. Background

Trevigen's Cultrex® Cell Migration Assays were originally created in an effort to accelerate the screening process for compounds that influence chemotaxis, which is a fundamental component of angiogenesis[1], embryonic development[2], immune responses[3], and wound healing.[4] This assay offers a flexible, standardized, multi-well format for quantitating the degree to which cells migrate *in vitro* in response to chemoattractants in the presence of inhibitors or stimulants.

Trevigen's **Cultrex® 24 well Cell Migration Assay** utilizes a simplified Boyden chamber design with an 8 µm polyethylene terephthalate (PET) membrane. Ports within the migration chamber (top) allow access to the assay chamber (bottom). The inserts are sufficiently large to allow for subsequent analysis of migrating subpopulations, in response to chemokines, cytokines, drugs or other compounds of interest.

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. Cultrex® Cell Migration 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 Migration Chamber | each | Room Temp. | 3465-024-01 |
| 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 |

## 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.

## 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% (but not 100%) 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 migration 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 (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 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. 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. A 1 x $10^6$ cells/ml stock can be serially diluted to provide the dilution series shown in the parenthesis.
3. 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.
4. Add 500 µL of 1X Cell Dissociation Solution/Calcein-AM to each set of wells containing decreasing numbers of cells, 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 |



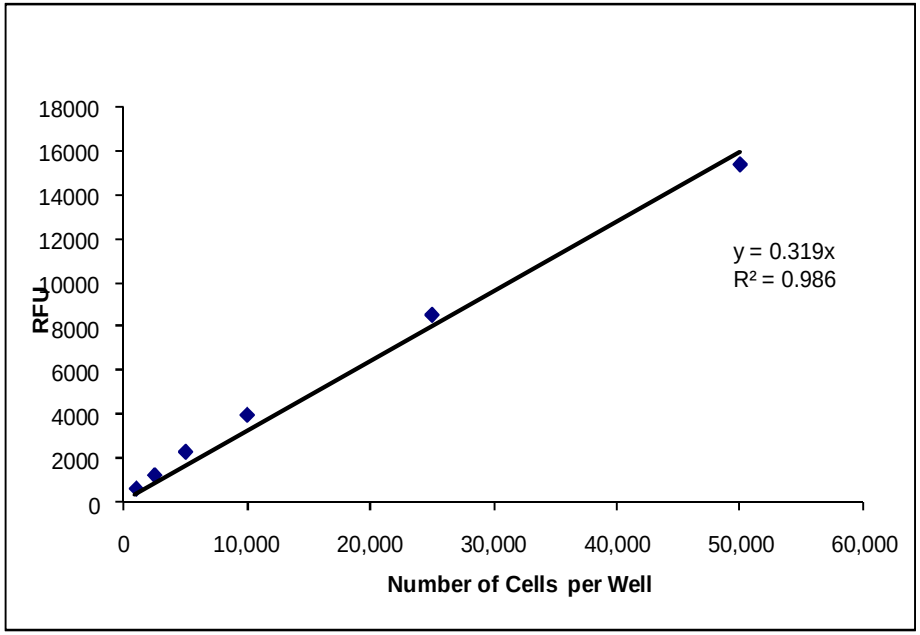


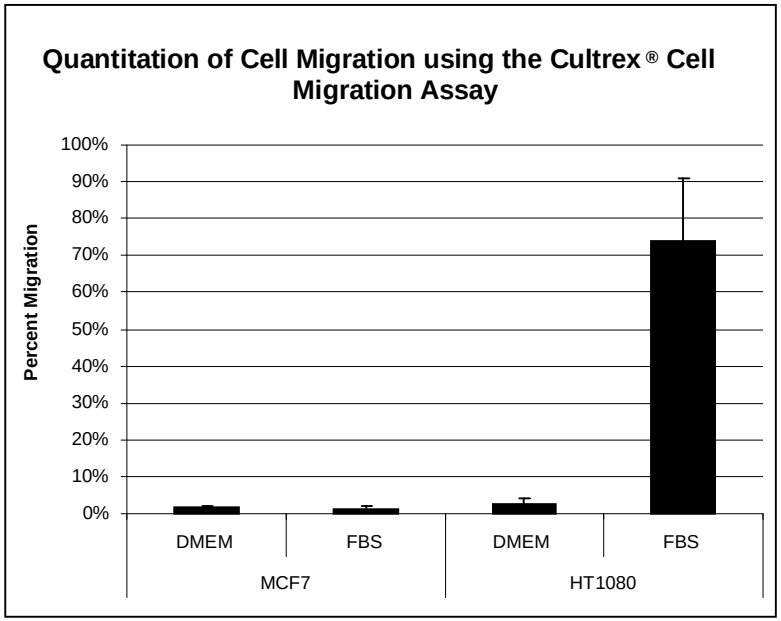

**Figure 1. Standard Curve for a Cell Migration Assay.** HT-1080 cells were harvested (page 4), diluted, incubated for one hour with calcein-AM, and assayed for fluorescence (page 5). The trend line and line equation are included on the graph. A separate standard curve for each tested cell line is recommended.

### D. Cell Migration Assay

1. Culture cells to be assayed to 80% confluence (try to avoid exceeding 80%). 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 and ligands under investigation.
3. 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).
4. If desired, assay cells for standard curve; each cell line will require a separate standard curve (Section VII. B).
5. Add 100 µl of cells per well to top chamber (with or without inhibitors/stimulants), and to compensate for background, omit cells from at least three wells.
6. Add 500 µl of test media to bottom chambers (with or without drugs, chemokines, cytokines, etc.). Assemble chambers.

7. Incubate at 37 $^{o}$C in $CO_2$ incubator; incubation times may be varied (4-48 hours).
8. 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).
9. Aspirate bottom chamber, and wash each well twice with 500 µl warm (37 $^{o}$C) 1X Wash Buffer.
10. Add 12 µl of Calcein AM solution (item 3, page 4) to 12 mL of 1X Cell Dissociation Solution (item 2, page 4).
11. 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 30 minutes.
12. Gently tap device 10 times on the side, and incubate at 37 $^{o}$C in a $CO_2$ incubator for 30 minutes (one hour in total) for optimal dissociation.
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 4) to determine the number of cells that have migrated, or failed to migrate according to experimental design.

## VIII. Example Results (figure 2):






Typical results using MCF-7 and HT-1080 cell lines are shown in figure 2: The human fibroblastic cell line HT-1080 readily traverses through a perforated PET membrane with 8 µm pores in response to 10% FBS in the lower well whereas static MCF-7 cells do not migrate.

## IX. Troubleshooting

| Problem | Cause | Solution |
|---|---|---|
| No signal | Cells did not migrate/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. |
| 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 – pro-teases 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 |
|---|---|---|
| 3455-024-K | Cultrex® 24 Well BME Cell Invasion Assay | 24 inserts |
| 3460-024-K | Cultrex® CultreCoat® 24 Well BME-Coated Cell Invasion Assay | 24 inserts |
| 3455-096-K | Cultrex® 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 |
| 3471-096-K | Cultrex® 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 |
| 3442-050-01 | Cultrex® Bovine Collagen I | 50 mg |
| 3410-010-01 | Cultrex® Mouse Collagen IV | 1 mg |
| 3447-020-01 | Cultrex® 3-D Culture Matrix Collagen I | 100 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, No Phenol Red, PathClear® | 5 ml |
| 3433-005-02 | Cultrex® BME, No Phenol Red, 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. Appendices

### Appendix A. Reagent and Buffer Composition

**1. 24-well Cell Migration Chamber (Cat# 3465-024-01)**
Perforated PET membrane with 8.0 µm pores, within two 24-Well Plates compatible with 24 well fluorescent plate reader (figure 3).

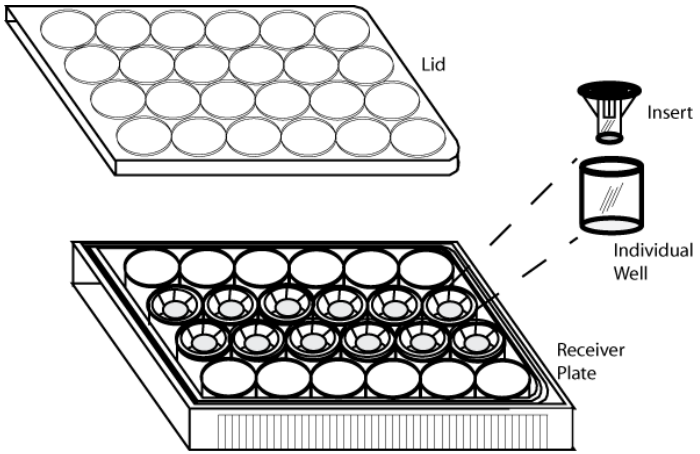



Figure 3. Diagram of 24-Well Cell Invasion Chamber.

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