CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

96 Well Cell Migration Assay

Reagent kit for investigating chemotaxis

96 samples

Catalog #: 3465-096-K

Cultrex® 96 Well Cell Migration Assay

Catalog# 3465-096-K

96 Samples

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I. Quick Reference Procedure for Cultrex® 96 Well Cell Migration Assay (Cat# 3465-096-K):

Read through the complete Instructions for Use prior to using this kit.

Prior to Day 1

1. Culture cells per manufacturers recommendation; adherent cells should be cultured to 80% confluence. Each well requires 50,000 cells, so plan accordingly.
2. Twenty-four hours prior to beginning 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 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).
5. Add 50 µl of cells per well to top chamber (with or without inhibitors/stimulants).
6. Using access port, add 150 µl of medium per well to the clear bottom chamber (with or without chemoattractants).
7. Incubate chamber at 37 ^oC in CO_2 incubator for 4-48 hours.
8. Assay remaining cells for standard curve (section VII. B.); each cell type will require a separate standard curve.

Day 2

9. Carefully aspirate top chamber (do not puncture membrane), and wash each well with 100 µl of 1X Wash Buffer.
10. Aspirate bottom chamber, and wash each well with 200 µl 1X Wash Buffer.
11. Add 12 µl of Calcein-AM solution to 10 mL of Cell Dissociation Solution.
12. Add 100 µl of Cell Dissociation Solution/Calcein-AM to bottom chamber, of the black assay plate, assemble cell migration device, and incubate at 37 ^oC in 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 migration.

II. Background

Trevigen's **Cultrex® 96 Well Cell Migration Assay** was created in an effort to accelerate the screening process for compounds that influence chemotaxis, which is a fundamental component of cellular processes such as angiogenesis[1], embryonic development[2], immune responses[3], and wound healing[4]. This assay offers a flexible, standardized, high-throughput format for quantitating the degree to which cells migrate *in vitro* in response to chemoattractants and/or inhibiting/stimulant compounds.

The **Cultrex® 96 Well Cell Migration Assay** utilizes a simplified boyden chamber design with an 8 micron polyethylene terephthalate (PET) membrane. Ports within the migration chamber (top) allow the access to the assay chamber (bottom) without dismantling the device. This design is easier to use, prevents contamination, and is adaptable for robotic high throughput systems. The assay chamber may be directly analyzed in a 96 well plate reader, eliminating transfer steps that introduce additional variability to the assay.

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 migrated using a standard curve. Sufficient reagents are included to assess cell migration in 96 wells, as well as to calculate multiple standard curves.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the **Cultrex® 96 Well Cell Migration Assay** 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#
Cell Migration Chamber	each	Room Temp	3455-096-01
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

V. Materials/Equipment Required But Not Supplied

Equipment

1. 1 - 20 µl, 20 - 200 µl, and 200 - 1000 µl pipettors
2. 37 ^{o}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 ^{o}C and 4 ^{o}C storage
7. Ice bucket
8. Standard light microscope (or inverted)
9. Pipette helper
10. Timer
11. Vortex mixer
12. Fluorescent 96-well plate reader, top reader (485 nm excitation, 520 nm emission)
13. Computer and graphing software, such as Microsoft® Excel®.

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 pipets
5. Gloves
6. 10 ml syringe
7. 0.2 µm filter
8. Black 96 Well Plate (e.g. Corning cat# 3924 or #3925, for standard curve)

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. **Calcien 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 ^{o}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 may 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 requires 50,000 – 100,000 cells, and a 25 cm^2 flask 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, and cell migration assay.
2. Starve cells by incubating 18-24 hours in Serum-Free medium (see *Materials/Equipment 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 or 75 cm^2 flask, add 1 ml or 2 ml, respectively, of Cell Harvesting Buffer (see *Materials/Equipment Required But Not Supplied*), and incubate at 37 ^{o}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/Equipment 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 using a 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 to generate a standard curve. If used, a separate standard curve may be run for each cell type and assay condition. Control cells, and experimental replicates should be performed in triplicate.

1. Your data should fall in the linear range of the curve. Determine the saturation range for your cells (e.g. 50,000 cells), beyond which, additional migration would be difficult to detect, because an increase in signal is no longer linear, and approaches an asymptote.
2. Determine the total number of cells needed per standard curve (table 1), and the required volume of medium.
3. Transfer volume of harvested cells needed to a 15 ml conical tube, and centrifuge at 250 *x g* for 10 minutes to pellet cells.
4. Remove supernatant, and resuspend cells in Cell Dissociation Solution at 1 x 10^6 cells/ml. (Phenol red in culture media will interfere with your signal.)
5. Dilute cells for highest condition for a final volume of 50 µl (eg. 50,000 cells/ 50 µl = 1.0 x 10^6 cells/ml) in 1X Cell Dissociation Solution (section VI, item 2), add 50 µl/well, and serially dilute remaining stock with 1X Cell Dissociation Solution to generate the number of cells needed in each well (in 50 µl of 1X Cell Dissociation Solution). Repeat dilutions until all conditions have been satisfied.
6. Add 12 µl of Calcein-AM Solution (section VI, item 3) to 5 ml of 1X Cell Dissociation Solution (section VI, item 2), cap tube, and invert to mix.
7. Add 50 µL of 1X Cell Dissociation Solution/Calcien-AM (VII.B5) to each well, and incubate for one hour; omit cells from at least three wells to calculate background.
8. Read plate at 485 nm excitation, 520 nm emission (see Table 2 for sample data) to obtain relative fluorescence units (RFU).
9. Average values for each condition; then subtract background from each value (see Table 2).
10. Plot standard curve of RFU vs. number of cells (see Figure 1).
11. 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 Calculations for Standard Curve:

Cells Needed:

Cells/Well X	Conditions =	Cells Needed
50,000	3	150,000
25,000	3	75,000
10,000	3	30,000
5,000	3	15,000
2,000	3	6,000
1,000	3	3,000
Total Cells Needed		279,000

Volume of Harvested Cells Needed (ml):

$$\frac{\text{Total Cells Needed}}{\text{Concentration Harvested Cells}} = \frac{279{,}000}{1.00\text{E+}06} = 0.279$$

Table 2. Sample Data for Standard Curve (actual results may vary):

Average Cells/Well	Wells 1	2	3
50,000	15710	15145	16135
25,000	9118	8702	8644
10,000	4454	4257	4091
5,000	2609	2541	2599
2,500	1486	1476	1585
1,000	930	922	881
0	243	254	264

Subtract Background

Cells/Well	Average	Background	Corrected
50,000	15663	-254	15409
25,000	8821	-254	8567
10,000	4267	-254	4013
5,000	2583	-254	2329
2,500	1516	-254	1262
1,000	911	-254	657
0	254	(Background)	

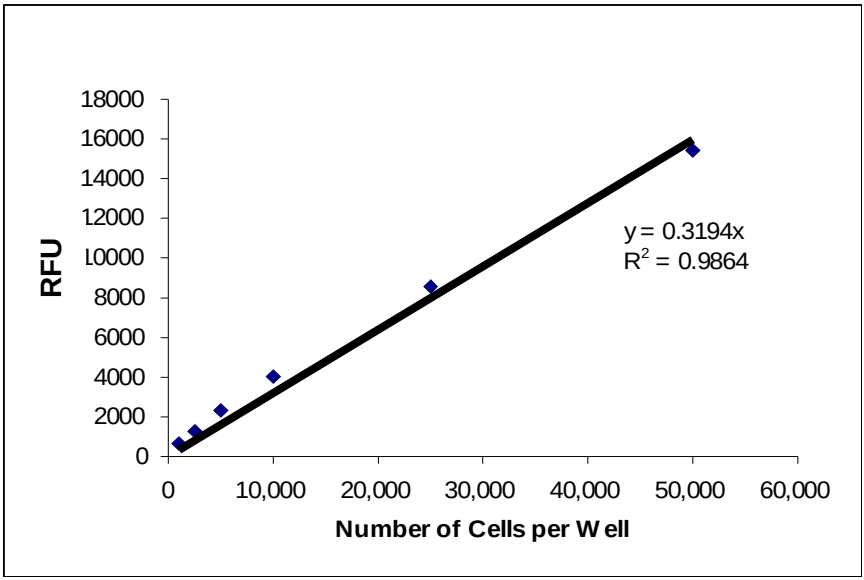


Figure 1. Standard Curve for Cell Migration Assay. HT-1080 cells were harvested (section VII. A), diluted, incubated for one hour with calcein AM, and assayed for fluorescence (section VII. B). The trendline and line equation are included on the graph.

C. Cell Migration Assay

Note: Two plate bottoms are provided for your convenience: optimization of assay conditions (clear), and background signal minimization (black).

1. Culture cells to be assayed to not greater than 80% confluence. Plan accordingly for sufficient numbers of cells per chamber.
2. 24 hours prior to assay, cells may need to be serum starved in order to express unbound receptors on their plasma membranes. This step may be omitted depending upon the cell types and ligands under investigation.
3. Assay cells for standard curve; each cell type will require a separate standard curve (section VII. B).
4. Harvest cells, and dilute to working concentration (1 x 10^6 cells/ml recommended) in a serum-free medium.
5. Add 50 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 150 µl of medium per well to bottom chamber (with or without chemoattractants).
7. Incubate at 37 ^{o}C in CO_2 incubator; incubation times may be varied (4 hours-48 hours).
8. After incubation, carefully aspirate top chamber (do not puncture membrane), and wash each well with 100 µl of 1X Wash Buffer.
9. Aspirate bottom chamber, and wash each well with 200 µl 1X Wash Buffer (section VI, item 1). The device may be disassembled and the bottom chamber inverted to empty wells. Re-assemble device (if disassembled).
10. Add 12 µl of Calcein AM solution (section VI, item 3) to 10 mL of 1X Cell Dissociation Solution (section VI, item 2).
11. Add 100 µl of Cell Dissociation Solution/Calcein-AM (VII.C.8) to each well of bottom chamber, and incubate at 37 ^{o}C in CO_2 incubator for 30 minutes.
12. Gently tap device 10 times on the side, and incubate at 37 ^{o}C in CO_2 incubator for an additional 30 minutes (one hour total). This helps to ensure optimal dissociation.
13. Disassemble cell migration device, and read assay chamber (bottom) at 485 nm excitation, 520 nm emission using same parameters (time and gain) as standard curve(s), or controls.
14. Compare data to standard curve to determine the number of cells that have migrated, as well as percent cell migration (see VIII. Example Results).

VIII. Example Results

1. After plotting standard curve (section VII. B), insert trendline, best fit, with intercept equals zero, equation, and R-square value (coefficient of determination).
2. For assay samples, first average all wells for each condition (Table 3).
3. Next, subtract background from averages (Table 4).
4. Use the trendline equation to determine the number of cells present in each well; for the equation, y = mx + b, replace Y value with RFU, and solve for X (Table 5). See an example of a trendline and equation in Figure 1.
5. The number of cells may be compared for each condition to evaluate relative migration, or the number of migrated cells may be divided by the number of starting cells to determine percent migration (Table 6 and Figure 2).

Table 3. Average Data for Each Condition:

	MCF7		HT1080	
	DMEM	FBS	DMEM	FBS
Average	357	332	771	15507
Std Dev	38	59	299	3525

Table 4. Subtract Background from Each Condition:

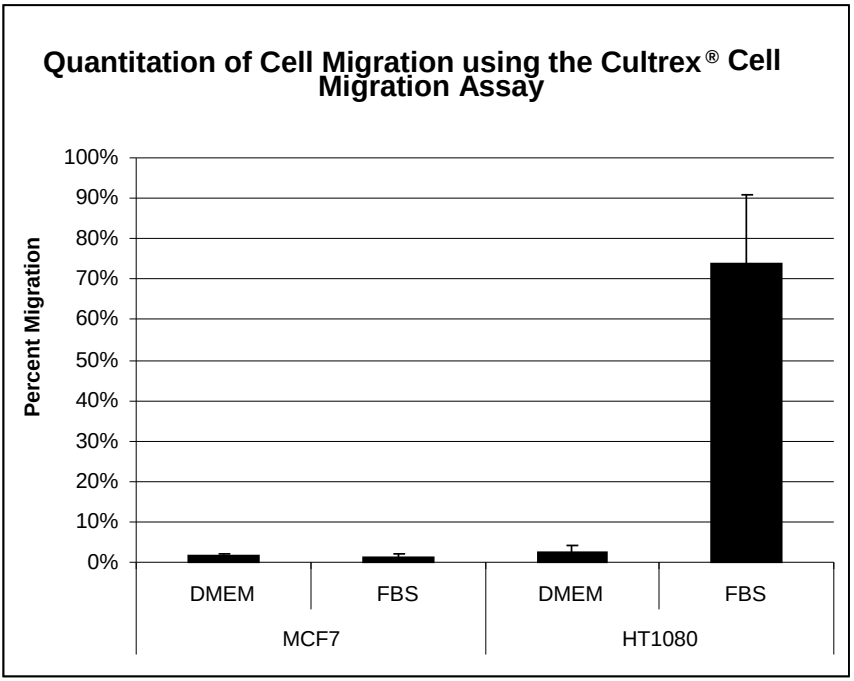
	MCF7		HT1080	
	DMEM	FBS	DMEM	FBS
Average	112	87	526	15262
Std Dev	38	59	299	3525

Table 5. Divide Each Value by Line Equation to Determine Cell Number

	MCF7		HT1080	
	DMEM	FBS	DMEM	FBS
Average	756	589	1273	36954
Std Dev	256	401	724	8536

Table 6. Divide Cell Number for Each Condition by 50,000 to Determine Percent Migration

	MCF7		HT1080	
	DMEM	FBS	DMEM	FBS
Average	2%	1%	3%	74%
Std Dev	1%	1%	1%	17%



IX. Troubleshooting

Problem	Cause	Solution
No signal	Cells did not migrate: Cell type may lack the needed receptor or chemoattractant may be insufficient or sub-optimal.	Use early passage cells; check for cell surface receptor expression; run dilution series for chemoattractant of interest to determine optimal concentration.
	There is inherent FBS variability from lot to lot; this can affect assay if used.	Screening of different FBS lots may be necessary.
	Cells may have died as a result of treatment.	Test cells for viability in treatment regimen.
	Insufficient Washing - agents in media, FBS, and/or chemo-attracts may react with Calcein-AM.	Re-assay, and make sure to wash well.
High background	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.
High background Well to well variability	Inconsistent pipetting	Calibrate pipettors, and monitor pipet tips for air bubbles.
Well to well variability	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.

Related Products:

Catalog#	Description	Size
3465-024-K	Cultrex® 24 well BME Cell Migration Assay	24 inserts
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
3456-024-K	Cultrex® 24 Well Laminin I Cell Invasion Assay	24 inserts
3457-024-K	Cultrex® 24 Well Collagen I Cell Invasion Assay	24 inserts
3458-024-K	Cultrex® 24 Well Collagen IV 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
3496-096-K	CultreCoat® 96 Well Adhesion Protein Array	96 samples

Accessories:

Catalog#	Description	Size
3415-001-02	Cultrex® Human BME, PathClear®	1 ml
3432-005-02	Cultrex® BME, PathClear®	5 ml
3432-005-01	Cultrex® BME without 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
3430-005-02	Cultrex® BME with Phenol Red, PathClear®	5 ml
3431-005-02	Cultrex® BME with Phenol Red, Reduced Growth Factor PathClear®	5 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® Ploy-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
3437-100-K	Cultrex® Cell Staining Kit	100 ml
3450-048-05	CellSperse™	15 ml

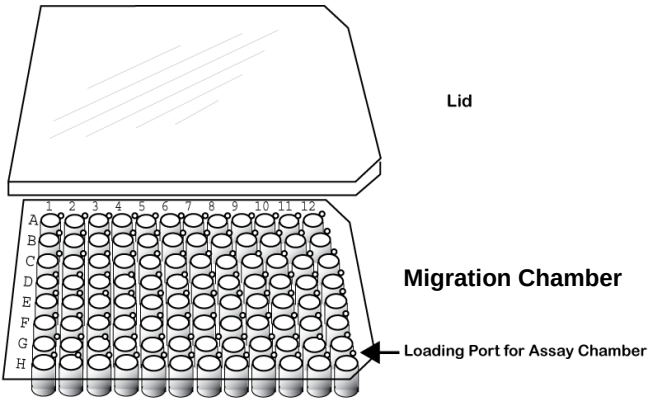
*New Zealand Herd Derived

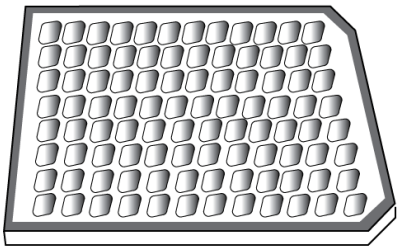
XII. Appendix

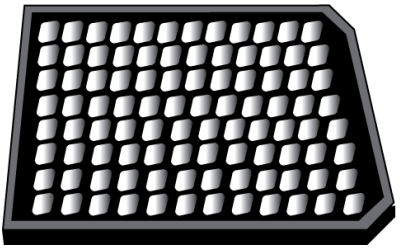
Appendix A. Reagent and Buffer Composition

1. Cell Migration Chamber

96 Well Boyden Chamber, 8.0 µm PET membrane, black receiver plate compatible with 96 well fluorescent plate reader.




Culture Chamber


Assay Chamber

2. 25X Cell Wash Buffer

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

3. 10X Cell Dissociation Solution

Proprietary formulation containing sodium citrate, EDTA, and glycerol.

4. Calcein-AM

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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