Cultrex® BME Cell Invasion Assay

Catalog Number 3455-096-K

96-well assay for investigating chemotaxis, cell migration, and/or cell invasion for adhesive cell types.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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MANUFACTURED FOR AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems GmbH
Borsigstrasse 7
65205 Wiesbaden-Nordenstadt
Germany

TELEPHONE: +49 (0)6122 90980
FAX: +49 (0)6122 909819
E-MAIL: infogmbh@RnDSystems.co.uk

R&D Systems Europe
77 boulevard Vauban
59041 LILLE CEDEX
France

FREEPHONE: +0800 90 72 49
FAX: +0800 77 16 68
E-MAIL: info@RnDSystems.co.uk

INTRODUCTION

The Cultrex® Cell Invasion Assay is designed to accelerate the screening process for compounds that influence cell migration through extracellular matrices, which is a fundamental function of cellular processes such as angiogenesis, embryonic development, immune response, and metastasis of cancer cells. This assay offers a flexible, standardized, high-throughput format for quantitating the degree to which invasive cells penetrate a barrier consisting of basement membrane components *in vitro* in response to chemoattractants and/or inhibiting compounds.

Since different cell lines and different treatments can result in a wide range of invasive potentials, the permissiveness of the matrix may be optimized to fit each experiment by adjusting the coating concentration. A 1X coating solution is recommended for highly invasive cell lines, and the coating solution may be diluted up to 0.1X for less invasive cell lines. The process of transfection itself may also alter the invasive capacity of cells, and as a result, may require a more permissive barrier.

REAGENTS PROVIDED

Component	Part Number	Amount Provided	Storage
Cell Invasion Chamber	3455-096-01	1 Chamber	Room Temperature
5X BME Solution	3455-096-02	1 mL	≤ -20° C*
10X Coating Buffer	3455-096-03	1 mL	≤ -20° C
25X Cell Wash Buffer	3455-096-04	2 x 1.5 mL	≤ -20° C
10X Cell Dissociation Solution	3455-096-05	2 x 1.5 mL	≤ -20° C
Calcein AM	4892-010-01	50 µg	≤ -20° C

Components should be stored at ≤ -20° C in a manual defrost freezer.

*For long-term storage, store at ≤ -80° C until the expiration date of the kit.

MATERIALS REQUIRED BUT NOT PROVIDED

Equipment

- Pipettes and pipette tips (1 - 20 μL, 20 - 200 μL, and 200 - 1000 μL)
- Pipette helper
- Timer
- Ice bucket
- Vortex mixer
- 37° C CO_2 incubator
- Low speed centrifuge and tubes (for cell harvesting)
- Hemocytometer or other means to count cells
- 50 mL and 500 mL graduated cylinders
- Standard or inverted light microscope
- Fluorescent 96-well plate reader, top reader (485 nm excitation, 520 nm emission)
- Black 96-well microplate for standard curve (R&D Systems, Catalog # DY991)
- Graphing software

Reagents

- Cell Harvesting Buffer (EDTA, trypsin, or other cell detachment buffer)
- Tissue Culture Growth Medium (as recommended by cell supplier)
- Serum-Free Medium (tissue culture growth medium without serum)
- Chemoattractants or pharmacological agents for addition to culture medium
- Quenching Medium (Serum-Free Medium with 5% BSA)
- Sterile PBS or HBSS to wash cells
- Sterile deionized or distilled water
- Sterile DMSO (to reconstitute Calcein)
- Trypan blue or equivalent viability stain

Disposables

- 1.5 mL and 10 mL serological pipettes
- Cell culture flask (25 cm^2 or 75 cm^2)
- 10 mL syringe
- 50 mL tubes
- 0.2 μm filter

PRECAUTIONS

The physical, chemical, and toxicological properties of the chemicals and reagents in this kit may not yet have been fully investigated. The use of gloves, lab coats, and eye protection is recommended.

The Cultrex Cell Invasion Assay contains reagents that may be harmful if swallowed, or come in contact with skin or eyes. In case of contact with eyes, rinse immediately with water and seek medical advice. Material Safety Data Sheets are available on request.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.

PREPARATION OF REAGENTS

Completely thaw frozen reagents prior to performing dilutions.

1. **1X Coating Buffer**
   Dilute 1 mL of 10X Coating Buffer in 9.0 mL of sterile, deionized water to make the 1X Coating Buffer (0.2 μm filtration is recommended).

2. **BME Solution**
   For highly invasive cells, dilute 1 mL of 5X BME Solution in 4 mL of 1X Coating Buffer on ice immediately before coating. Less invasive cell types may require a more permissive barrier, so the BME may be diluted as far as 0.1X. Aliquot and freeze any remaining 5X BME. Avoid repeated freeze-thaw cycles.

3. **1X Wash Buffer**
   Dilute 3 mL of the 25X Cell Wash Buffer in 72 mL of sterile, deionized water to make the 1X Wash Buffer.

4. **1X Cell Dissociation Solution**
   Dilute 3 mL of the 10X Cell Dissociation Solution in 27 mL of sterile, deionized water to make the 1X Cell Dissociation Solution.

5. **Calcein AM**
   Centrifuge the microtube momentarily to pellet the powder before opening the tube. Add 30 μL of sterile DMSO to make the stock solution. Pipette up and down to mix and store the solution at ≤ -20° C.

QUICK REFERENCE PROCEDURE

Prior to Day 1

1. Culture cells per the supplier's recommendation. Adherent cells should be cultured to 80% confluence. Each well requires 50,000 cells, so plan accordingly.

Day 1

2. Twenty-four hours prior to beginning the assay, starve the cells in Serum-Free Medium (0.5% FBS may be used if needed).
3. Coat the top chamber of the cell invasion device with 50 µL of 0.1X to 1X BME Solution, and incubate at 37° C in a CO_2 incubator for 4 hours or overnight.

Day 2

4. After 24 hours, harvest and count the cells.
5. Add Quenching Medium, centrifuge cells at 250 x g for 10 minutes, remove the medium, and resuspend at 1 x 10^6 cells/mL in Serum-Free Medium (0.5% FBS may be used if needed).
6. Aspirate the top chamber of the cell invasion device. **Do not allow the top or bottom chambers to dry.**
7. Add 50 µL of cells per well to the top chamber.
8. Add 150 µL of Medium per well to the bottom chamber (with or without chemoattractants).
9. Assemble the chamber, and incubate at 37° C in a CO_2 incubator for 24 - 48 hours.
10. Assay the remaining cells for the standard curve. Each cell type will require a separate standard curve.

Day 3

11. Aspirate the top chamber, and wash each well with 100 µL of 1X Wash Buffer.
12. Aspirate the bottom chamber, and wash each well twice with 200 µL of 1X Wash Buffer.
13. Add 12 µL of Calcein AM Stock Solution to 10 mL of 1X Cell Dissociation Solution.
14. Add 100 µL of diluted Calcein AM to the bottom chamber, assemble the cell invasion device, and incubate at 37° C in a CO_2 incubator for 1 hour.
15. Remove the top chamber, and read the bottom plate at 485 nm excitation and 520 nm emission.
16. Compare the data to the standard curve to determine the number of cells that have invaded, as well as the percentage of cell invasion.

ASSAY PROTOCOL

These procedures should be performed in a biological hood using 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 individual cell types.

1. Cells should be passaged 2 or 3 times prior to use in the assay and adherent cells need to be 80% confluent. Each well requires 50,000 - 100,000 cells. A 25 cm^2 or 75 cm^2 flask will yield approximately 3 x 10^6 or 9 x 10^6 cells, respectively. Calculate to include enough cells for a standard curve and the cell invasion assay.
2. Starve the cells by incubating for 18 - 24 hours in Serum-Free Medium prior to assay (0.5% fetal bovine serum may be used if needed).
3. Prior to harvest, visually inspect the cells and record cell health, relative number, and morphology.
4. Wash the 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 the cells. For a 25 cm^2 or 75 cm^2 flask, add 1 mL or 2 mL, respectively, of Cell Harvesting Buffer and incubate at 37° C for 5 - 15 minutes (until cells have dissociated from the bottom of the flask).
6. Transfer the cells to a 15 mL conical tube and add 5 mL of Quenching Medium.
7. Centrifuge the cells at 250 x g for 10 minutes to pellet, remove the Quenching Medium, and resuspend the cells in 2 mL of Serum-Free Medium (0.5% fetal bovine serum may be used if needed). Cells may need to be gently pipetted up and down with a serological pipette to break up clumps.
8. Count cells and dilute to 1 x 10^6 cells per mL in Serum-Free Medium (0.5% fetal bovine serum may be used if needed).

B. Standard Curve

Note: *A separate standard curve should be run for each cell type. Conditions should be run in triplicate.*

1. Determine an appropriate standard curve based upon the number of cells per well (e.g. 50,000 cells, 25,000 cells, 10,000 cells, 5,000 cells, 2,000 cells, 1,000 cells, 500 cells, 0 cells).
2. Determine the total number of cells needed for the standard curve (see Table 1):

   cells/well x wells/condition = cells needed/condition

   Sum(cells needed/condition) = total number of cells needed
3. Calculate the volume of harvested cells needed (see Table 1):

   = total number of cells needed/1 x 10^6 cells/mL

Table 1: Sample calculations for the Standard Curve.

Cells/Well	x	Wells/Condition	=	Cells Needed/Condition
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 Number of Cells Needed				**279,000**
Volume of Harvested Cells Needed (mL) Total Number of Cells Needed (279,000) / Concentration of Harvested Cells (1 x 10^6)			=	0.279 mL

4. Transfer the volume of harvested cells needed to a 15 mL conical tube and centrifuge at 250 x g for 10 minutes to pellet cells.
5. Remove the supernate and resuspend the cells in 1X Cell Dissociation Solution at 1 x 10^6 cells/mL.

6. In a black 96-well microplate, prepare a standard curve in triplicate by diluting the cells for the highest condition for a final volume of 50 µL (e.g. 50,000 cells/50 µL = 1.0×10^6 cells/mL) in 1X Cell Dissociation Solution, add 50 µL/well, and serially dilute the 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 the dilutions until all conditions have been satisfied. Omit cells from at least three wells to calculate the background.
7. Add 12 µL of Calcein AM Stock Solution to 5 mL of 1X Cell Dissociation Solution, cap the tube, and invert to mix.
8. Add 50 µL of diluted Calcein AM to each well and incubate for 1 hour.
9. Read the plate at 485 nm excitation, 520 nm emission (see Table 2 for sample data).

C. Cell Invasion Assay

Note: *Assay the cells for the standard curve. Each cell type will require a separate standard curve.*

1. Working on ice, prepare 5 mL of 0.1X to 1X BME Solution in a sterile 15 mL conical tube and label it “BME Coat”. Cap the tube and gently invert it to mix.
2. Add 50 µL of BME Coat per well to the top chamber of the Cell Invasion device. Gently tap the side of the device a few times and visually inspect the wells for dispersion of coating. All wells should be coated.
3. Incubate the device at 37° C in a CO_2 incubator for 4 hours or overnight.
4. Aspirate the top chamber of the cell invasion device. **Do not allow the top or bottom chambers to dry.**
5. Harvest the cells and dilute to the working concentration (1.0×10^6 cells/mL is recommended) in Serum-Free Medium.
6. Add 50 µL of cells per well to the top chamber. To compensate for background, omit cells from at least three wells.
7. Add 150 µL of Medium per well to the bottom chamber (with or without chemoattractants). Assemble the device.
8. Incubate at 37° C in a CO_2 incubator. Incubation times may be varied (24 - 48 hours).
9. After incubation, aspirate the top chamber, and wash each well with 100 µL of 1X Wash Buffer. Aspirate the top chamber to remove the Wash Buffer.
10. Aspirate the bottom chamber, and wash each well twice with 200 µL of 1X Wash Buffer. The device may be disassembled and the bottom chamber inverted to empty the wells.
11. Add 12 µL of Calcein AM stock solution to 10 mL of 1X Cell Dissociation Solution.
12. Add 100 µL of diluted Calcein AM to each well of the bottom chamber. Reassemble the device, and incubate for 30 minutes at 37° C in a CO_2 incubator.
13. Gently tap the side of the device 10 times, and incubate for an additional 30 minutes at 37° C in a CO_2 incubator. This helps to ensure optimal dissociation.
14. Disassemble the cell invasion device, and read the assay chamber (bottom) at 485 nm excitation and 520 nm emission using the same parameters (time and gain) as the standard curve (see Table 3 for sample data).
15. Compare the data to the standard curve to determine the number of cells that have invaded, as well as the percentage of cell invasion. Refer to the Calculation of Results section for more details.

CALCULATION OF RESULTS

Average the triplicate readings for each standard and sample then subtract the average blank RFU.

Create a standard curve by reducing data using computer software capable of generating a linear curve-fit. As an alternative, construct a standard curve by plotting the corrected RFU on the y-axis against the cell number on the x-axis and insert the trendline (best fit), equation and R-squared value. Use the trendline equation to determine the number of cells present in each sample well. A standard curve should be generated for each cell type.

The number of cells may be compared for each condition to evaluate relative invasion or the number of invaded cells may be divided by the number of starting cells (e.g. 50,000) to determine the percent invasion.

Table 2: Typical sample data for the Standard Curve (actual results may vary).

Cells/Well	RFU Values	Average RFU	Corrected RFU
50,000	15,145 15,710 16,135	15,663	15,409
25,000	8644 8702 9118	8821	8567
10,000	4091 4257 4454	4267	4013
5000	2541 2599 2609	2583	2329
2000	1476 1486 1585	1516	1262
1000	881 922 930	911	657
0	264 254 243	254	—

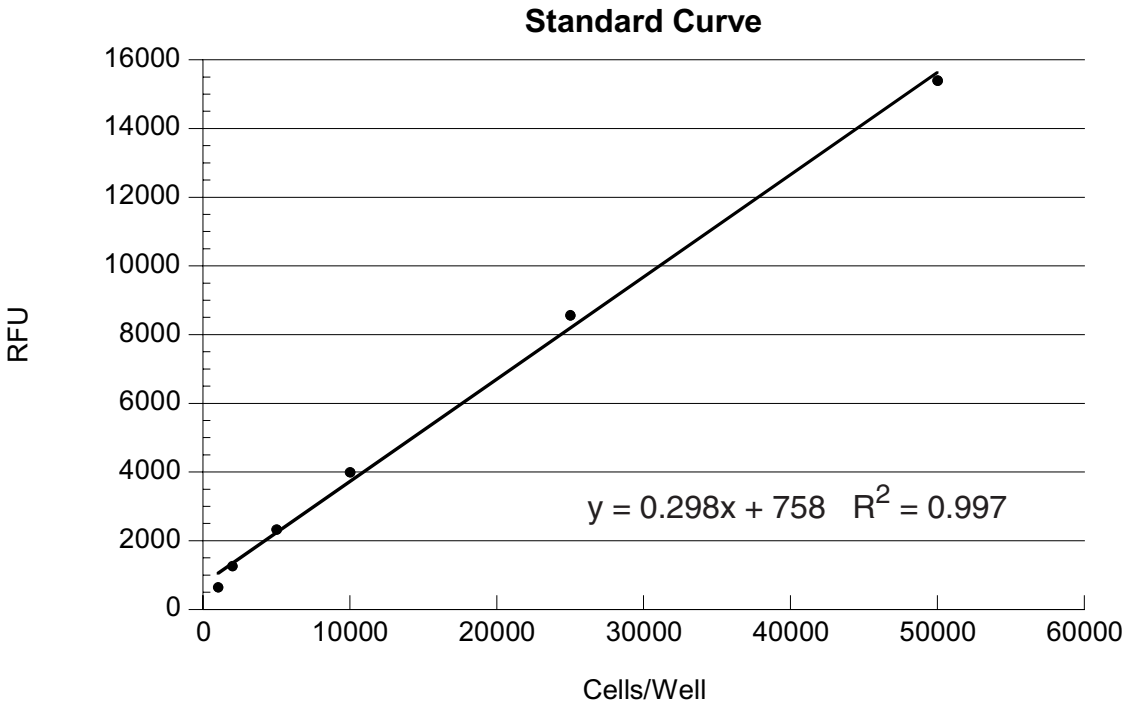


Figure 1: HT-1080 cells were harvested, diluted, incubated for 1 hour with Calcein AM, and assayed for fluorescence. The trendline and line equation are included on the graph.

TYPICAL DATA

Table 3: Typical data for each cell line.

Cell Line	Treatment	Corrected RFU	Cell Number	% Invasion
MCF-7 (non-invasive)	DMEM	2	14	0%
	FBS	29	201	0%
NIH-3T3 (non-invasive)	DMEM	32	157	0%
	FBS	339	1685	3%
HT-1080 (invasive)	DMEM	-12	-38	0%
	FBS	5855	18,295	37%
MDA-MB-231 (invasive)	DMEM	-17	-91	0%
	FBS	1797	9712	19%

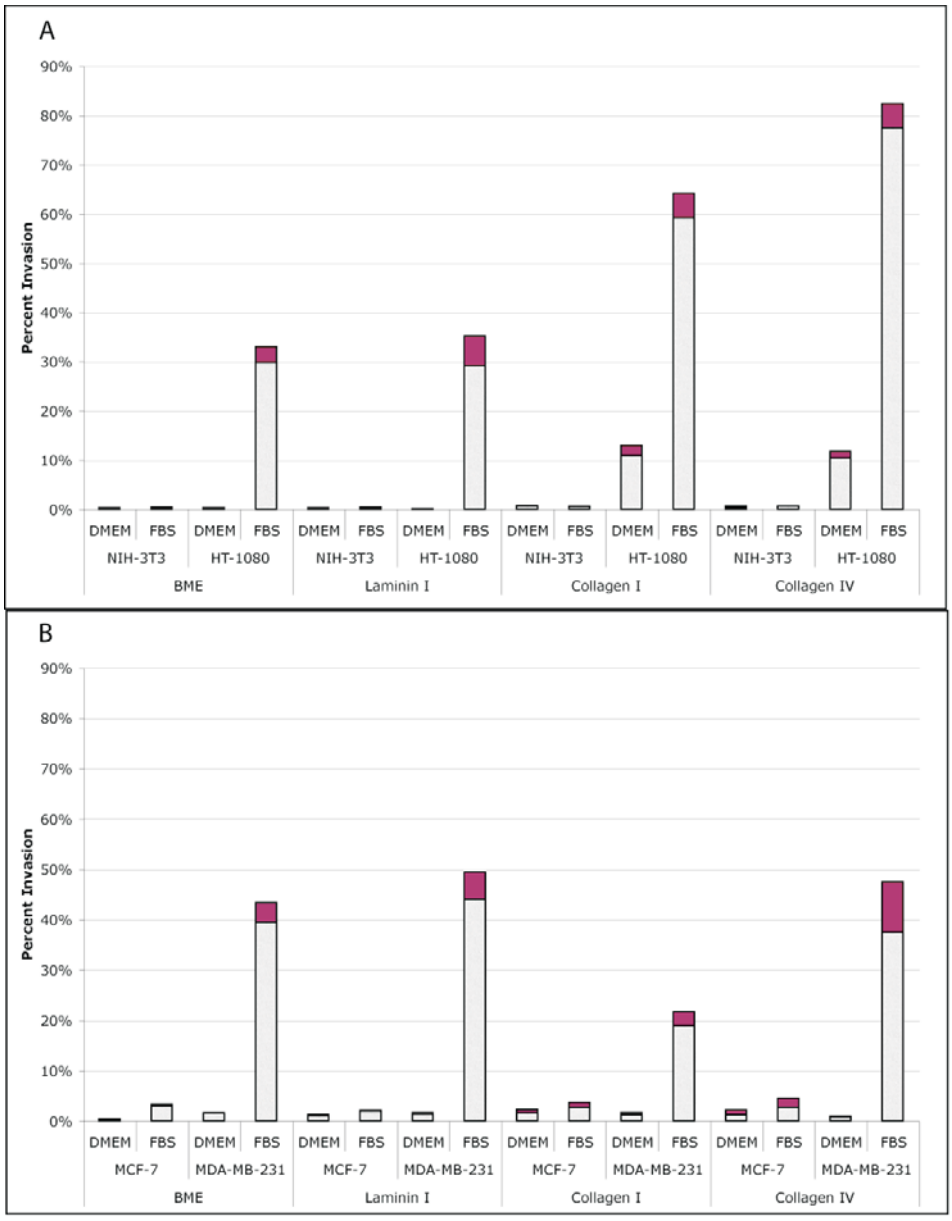


Figure 2: Quantitation of the ability of fibroblastic cell lines (A) and breast cancer cell lines (B) to cross a barrier consisting of an 8 μm polyester filter occluded with different extracellular components over a 24 hour period in response to 10% FBS. Samples were run in quadruplicate for non-invasive cell types, MCF-7 and NIH-3T3, and invasive cell types, HT-1080 and MDA-MB-231. Light area represents average and dark area represents the standard deviation.

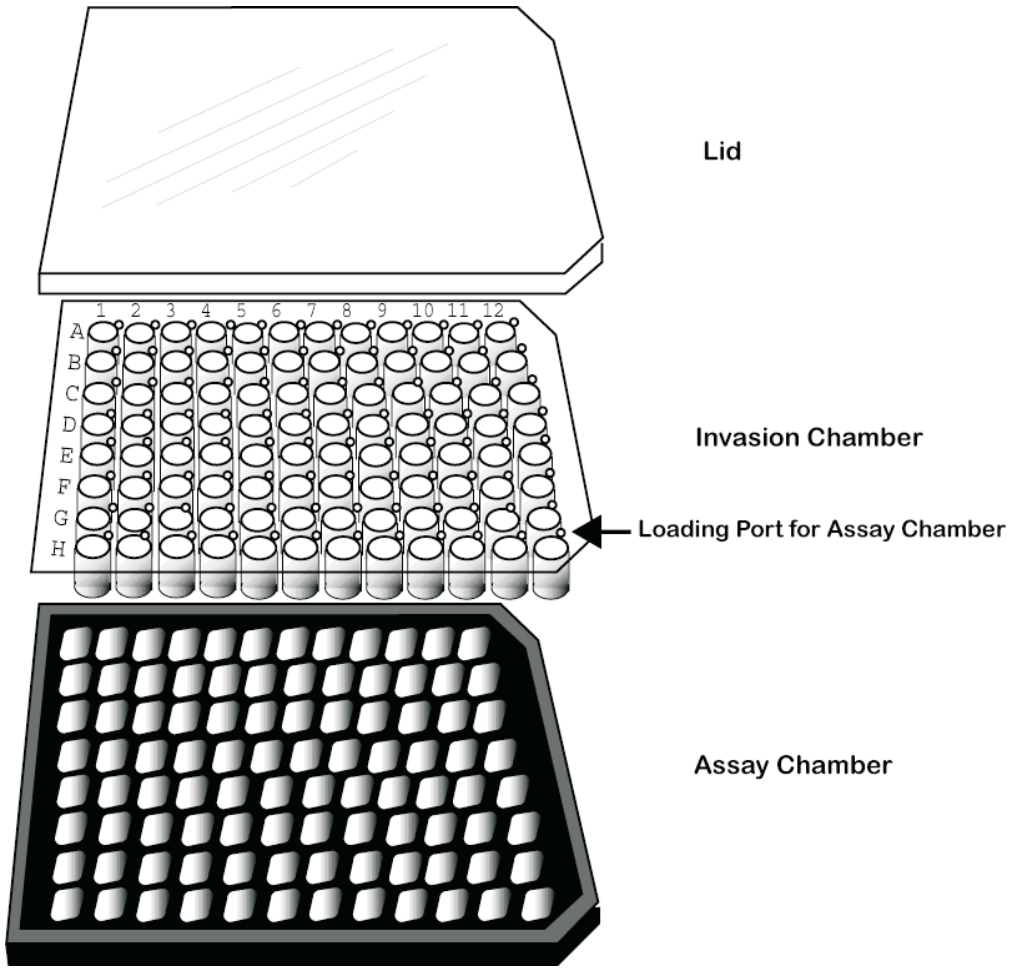
TROUBLESHOOTING GUIDE

Problem	Cause	Solution
No signal	Cells did not penetrate barrier.	Cell type may be non-invasive or chemoattractant may be insufficient.
		Barrier may not be permissive enough for cell type; use a more dilute coating solution.
		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 medium, FBS, and/or chemoattractants may react with Calcein AM.	Re-assay and make sure to wash thoroughly.
	Contamination - Proteases released by bacteria or mold may degrade the BME components, allowing cells to pass through barrier.	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 pipettes and monitor pipette tips for air bubbles.
	Membrane punctured with pipette tips.	Disregard data from wells that are punctured. Re-assay if necessary.

APPENDIX

Cell Invasion Device

96-well Boyden Chamber, 8.0 μm polyester membrane, and a black receiver plate compatible with a 96-well fluorescent plate reader.



Reagent and Buffer Composition

5X BME Solution

Basement Membrane Extract provided at 10 mg/mL, derived from EHS tumor. Avoid repeated freeze-thaw cycles.

10X Coating Buffer

Proprietary buffer optimized for coating BME to a polyester membrane.

25X Cell Wash Buffer

PBS buffer for washing cells (10 mM potassium phosphate (pH 7.4), 145 mM NaCl).

10X Cell Dissociation Solution

Proprietary formulation containing sodium citrate, EDTA, and glycerol.

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.

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