Cultrex™

Basement Membrane Extract Cell Invasion Assay

Catalog Number: 3455-024-K (24-well Kit)
3455-096-K (96-well Kit)

Reagent kits for investigating chemotaxis, cell migration, or cell invasion.

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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BACKGROUND

Cultrex™ Cell Invasion Assays were created in an effort to accelerate the screening process for compounds that influence cellular digestion and migration across extracellular matrices, which is a fundamental component of cellular processes such as angiogenesis, embryonic development, immune responses, wound healing and metastasis(1-7). These assays offer 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.

Cultrex Cell Invasion Assays are provided in multiple formats so that cell invasion may be evaluated against different extracellular matrices and matrix components:

- Basement Membrane Extract (BME)
- Laminin I (R&D Systems®, Catalog # 3456-024-K and 3456-096-K)
- Collagen I (R&D Systems, Catalog # 3457-024-K and 3457-096-K)
- Collagen IV (R&D Systems, Catalog # 3458-024-K and 3458-096-K)

These assays employ 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.

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 to fit each experiment by adjusting the coating concentration. A 1X Coating Buffer is recommended for highly invasive cells, whereas 0.1X coatings may be sufficient for less invasive cells.

Detection of cell invasion 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 or invaded using a standard curve. Sufficient reagents are included to assess cell migration/invasion in 96 wells, as well as to calculate multiple standard curves.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTION

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

Note: *Components for this kit require different storage/shipping temperatures and will arrive in separate packaging.*

CATALOG #	PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
3455-024-K	10X Coating Buffer	3455-096-03	1 mL	Store at 2-8 °C.
	25X Cell Wash Buffer	3455-096-04	2 vials (1.5 mL/vial)	
	10X Cell Dissociation Solution	3455-096-05	2 vials (1.5 mL/vial)	
	Cell Invasion/Migration Plates, 24-well	3455-024-01	24-well plate	
	Calcein AM	4892-010-01	50 µg	Store at ≤ -70 °C.
	Cultrex BME Solution, 5X	3455-096-02	1 mL	
3465-096-K	10X Coating Buffer	3455-096-03	1 mL	Store at 2-8 °C.
	25X Cell Wash Buffer	3455-096-04	2 vials (1.5 mL/vial)	
	10X Cell Dissociation Solution	3455-096-05	2 vials (1.5 mL/vial)	
	Cell Invasion/Migration Plates, 96-well	3455-096-01	96-well plate	
	Calcein AM	4892-010-01	50 µg	Store at ≤ -70 °C.
	Cultrex BME Solution, 5X	3455-096-02	1 mL	

OTHER SUPPLIES REQUIRED

Reagents

- Cell Harvesting Buffer (EDTA, trypsin, or other cell detachment buffer)
- Cell Culture Medium (as recommended by cell supplier)
- Serum-free Cell Culture Medium
- Chemoattractants or pharmacological agents for addition to culture medium
- Sterile PBS or HBSS
- Distilled, deionized water
- Trypan blue or equivalent viability stain

Equipment:

- Pipettes and pipette tips
- 37 °C CO_2 incubator
- Low speed centrifuge and tubes for cell harvesting
- Hemocytometer or other means to count cells
- 50 and 500 mL graduated cylinders
- ≤ -20 °C and 2-8 °C storage
- Ice bucket
- Standard light microscope
- Timer
- Vortex mixer
- Fluorescent 96-well plate reader, top reader \(485 nm excitation, 520 nm emission)
- Black 96 Well Plate (for standard curve)

Disposables:

- Treated Glass Microscope Slides (or alternative support)
- 50 mL tubes
- Microcentrifuge tubes
- 1, 5 and 10 mL serological pipettes
- Gloves
- Hydrophobic coverslips (optional)
- Glass coverslips

REAGENT PREPARATION

1X Coating Buffer - Dilute 500 µL of 10X Coating Buffer in 4.5 mL of sterile, distilled water to make 1X Coating Buffer; filtration at 0.2 µm recommended. Store at 2-8 °C.

Cultrex BME Solution, 5X - For highly invasive cells, dilute 1 mL of Cultrex 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. Avoid freeze-thaws.

1X Cell Wash Buffer - Dilute 3 mL of 25X Cell Wash Buffer in 72 mL of sterile, distilled water to make 1X solution.

1X Cell Dissociation Solution - Dilute 3 mL of 10X Cell Dissociation Solution in 27 mL of sterile, distilled water to make a 1X solution.

Calcein AM Solution - 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 **(do not foam)**.

Quenching Medium - Prepare Serum-free Cell Culture Medium containing 5% BSA.

ASSAY PROTOCOL

These procedures should be performed in a biological hood utilizing aseptic technique to prevent contamination.

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 no greater than 80% confluent. Each chamber requires 50,000-100,000 cells, and 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, migration controls and cell invasion assay.
2. Starve cells by incubating 18-24 hours in Serum-free Cell Culture Medium prior to assay (0.5% FBS may be used if needed) to allow for the expression of free receptors.
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, and incubate at 37 °C for 5-15 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.
7. Centrifuge cells at 250 x g for 10 minutes to pellet, remove Quenching Medium, and resuspend cells in 2 mL of Serum-Free Cell Culture Medium (0.5% FBS may be used if needed). Cells may need to be gently pipetted up and down using a serological pipette to break up clumps.
8. Count cells, and dilute to 1 x 10^6 cells/mL in Serum-Free Cell Culture Medium (0.5% FBS may be used if needed).

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 cells and experimental replicates should be performed in triplicate.

1. Determine the saturation range for your cells (*e.g.* 50,000 cells), beyond which, additional migration or invasion would be difficult to detect.
2. Determine the total number of cells needed per standard curve, and the required volume of medium (Table 1).
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 1X Cell Dissociation Solution at 1×10^6 cells/mL.
5. Dilute cells for 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 remaining stock with 1X Cell Dissociation Solution to deposit 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. Prepare Calcein AM/Dissociation Solution:
   a) 24-well Assay - Add 12 µL of Calcein AM to 12 mL of 1X Cell Dissociation Solution. Invert to mix.
   b) 96-well Assay - Add 12 µL of Calcein AM to 5 mL of 1X Cell Dissociation Solution. Invert to mix.
7. Add Calcein AM/Dissociation Solution to each well, and incubate for one hour; omit cells from at least three wells to calculate background.
   a) 24-well Assay - Add 500 µL of Calcein AM/Dissociation Solution to each well.
   b) 96-well Assay - Add 50 µL of Calcein AM/Dissociation Solution/well.
8. Read plate at 485 nm excitation, 520 nm emission (see Table 2) 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/invaded (see Figure 1).

Table 1. Sample Calculations for Standard Curve:

CELLS NEEDED

Cell/Well X	Conditions =	Cells Needed:
50,000	3	150,000
25,000	3	75,000
10,000	3	30,000
5000	3	15,000
2000	3	6000
1000	3	3000
Total cells needed		279,000

VOLUME OF HARVESTED CELLS NEEDED (ML)

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

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

Average Cells/Well	Wells		
	1	2	3
50,000	15,710	15,145	16,135
25,000	9118	8702	8644
10,000	4454	4257	4091
5000	2609	2541	2599
2000	1486	1476	1585
1000	930	922	881
0	243	254	264

Subtract Background and plot the data			
Cells/Well	Average	Background	Correct
50,000	15663	-254	15409
25,000	8821	-254	8567
10,000	4267	-254	4013
5000	2583	-254	2329
2000	1516	-254	1262
1000	911	-254	657
0	254	Background	

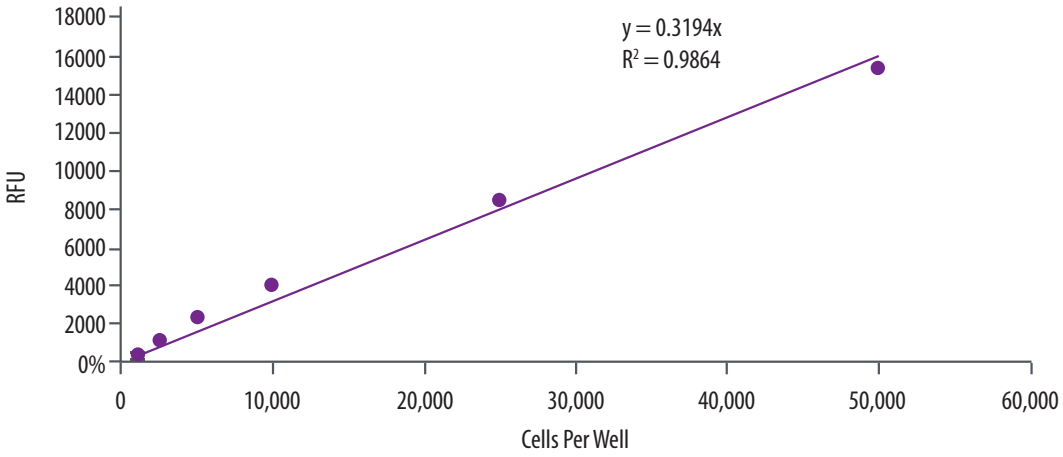

Figure 1. Standard Curve for Cell Invasion Assay. HT-1080 cells were harvested, diluted, incubated for one hour with Calcein AM, and assayed for fluorescence. The trend line and line equation are included on the graph.

Cell Invasion Assay

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

1. Culture cells to sufficient numbers to accommodate the pre-determined number of cells per chamber. Cells should be harvested at < 80% confluence.
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. Working on ice, prepare 0.1X to 1X BME Solution in a sterile 15 mL conical tube, and label "BME Coat". Cap tube, and gently invert to mix.
   a) 24-well Assay - Prepare 100 µL of 0.1X - 1X BME Solution/well used.
   b) 96-well Assay - Prepare 50 µL of 0.1X - 1X BME Solution/well used.
4. Add BME Coat into each well (100 µL/well for the 24-well Assay, 50 µL/well for the 96-well Assay). Gently tap side of device a few times, and visually inspect wells for dispersion of coating. All wells should be coated except at least three migration control wells (optional). Coat for 4 hours or overnight at 37 °C in a CO_2 incubator.
5. Assay cells for standard curve; each cell type will require a separate standard curve.
6. Harvest cells and dilute to working concentration (1 x 10^6 cells/mL recommended) in a Serum-free Cell Culture Medium. Inhibitors/stimulants may be added to cells at this time.
7. Aspirate off the coating solution from the top chamber. DO NOT ALLOW THE TOP OR BOTTOM CHAMBERS TO DRY.
8. Add cells with or without inhibitors/stimulants) to the top chamber (100 µL of cell suspension/chamber for the 24-well assay, 50 uL of cell suspension/chamber for the 96-well assay). To compensate for background, omit cells from at least three wells.

ASSAY PROTOCOL *CONTINUED*

9. Add Cell Culture Medium per well to bottom chamber (with or without chemoattractants).

    a) 24-well Assay: Add 500 µL/well

    b) 96-well Assay: Add 150 µL/well

10. Incubate at 37 °C in CO_2 incubator; incubation times may be varied (24-48 hours).

11. After incubation, aspirate top chamber without puncturing the membrane, and wash each well with 100 µL of 1X Cell Wash Buffer. Aspirate out the 1X Cell Wash Buffer.

12. Aspirate bottom chamber, and wash each well with 1X Cell Wash Buffer. The device may be disassembled and the bottom chamber inverted to empty wells. Re-assemble device (if disassembled) using the assay chamber plate.

    a) 24-well Assay: use 100 µL of 1X Cell Wash Buffer.

    b) 96-well Assay: use 200 µL of 1X Cell Wash Buffer.

13. Prepare Calcein AM/Dissociation Solution.

    a) 24-well Assay - Add 12 µL Calcein AM Solution to 12 mL of 1X Cell Dissociation Solution.

    b) 96-well Assay - Add 12 µL Calcein AM Solution to 10 mL of 1X Cell Dissociation Solution.

14. Add Cell Dissociation Solution/Calcein AM to each bottom chamber well, and incubate at 37 °C in CO_2 incubator for 60 minutes.
    Note: For the 96-well assay, incubate plate for 30 minutes then gently tap device 10 times on the side, and incubate at 37 °C in CO_2 incubator for an additional 30 minutes (one hour total). This helps to ensure optimal dissociation.

    a) 24-well Assay: 500 µL of Calcein AM/Dissociation Solution/well.

    b) 96-well Assay: 100 µL of Calcein AM/Dissociation Solution/well.

15. 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.

16. Compare data to standard curve to determine the number of cells that have migrated (no coating) or invaded (through the BME), as well as percent cell invasion.

EXAMPLE RESULTS

1. After plotting standard curve, 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.
3. Next, subtract background from averages.
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. 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 3. Sample Data for Cell Invasion Assay:

Cell Line	Standard Curve (m value)
MCF-7	0.1428
NIH-3T3	0.2012
HT-1080	0.3194
MDA-MB-231	0.1850

	NON-INVASIVE		INVASIVE		
	MCF-7	NIH-3T3	HT-1080	MDA-MB-231	
DMEM	273	327	234	224	227
	239	249	226	187	264
	238	255	233	243	233
	242	279	243	263	255
DMEM, 10% FBS	273	587	6591	2547	251
	205	572	5798	1674	241
	320	575	4913	1948	254
	301	606	7100	2002	243
				Average	246

EXAMPLE RESULTS *CONTINUED*

Table 4. Average Data for Each Condition:

	MCF-7		NIH-3T3		HT-1080		MDA-MB-231	
	DMEM	FBS	DMEM	FBS	DMEM	FBS	DMEM	FBS
Average	248	275	278	585	234	6101	229	2043
Standard deviation	17	50	35	15	7	956	32	366

Table 5. Subtract Background from Each Condition:

	MCF-7		NIH-3T3		HT-1080		MDA-MB-231	
	DMEM	FBS	DMEM	FBS	DMEM	FBS	DMEM	FBS
Average	2	29	32	339	-12	5855	-17	1797
Standard deviation	17	50	35	15	7	956	32	366

Table 6. Divide Each Value by Line Equation to Determine Cell Number:

	MCF-7		NIH-3T3		HT-1080		MDA-MB-231	
	DMEM	FBS	DMEM	FBS	DMEM	FBS	DMEM	FBS
Average	14	201	157	1685	-38	18295	-91	9712
Standard deviation	117	353	176	77	22	2987	175	1976

Table 7. Divide Cell Number for Each Condition by 50,000 to Determine Percent Invasion:

	MCF-7		NIH-3T3		HT-1080		MDA-MB-231	
	DMEM	FBS	DMEM	FBS	DMEM	FBS	DMEM	FBS
Average (%)	0%	0%	0%	3%	0%	37%	0%	19%
Standard deviation (%)	0%	1%	0%	0%	0%	6%	0%	4%

EXAMPLE RESULTS *CONTINUED*

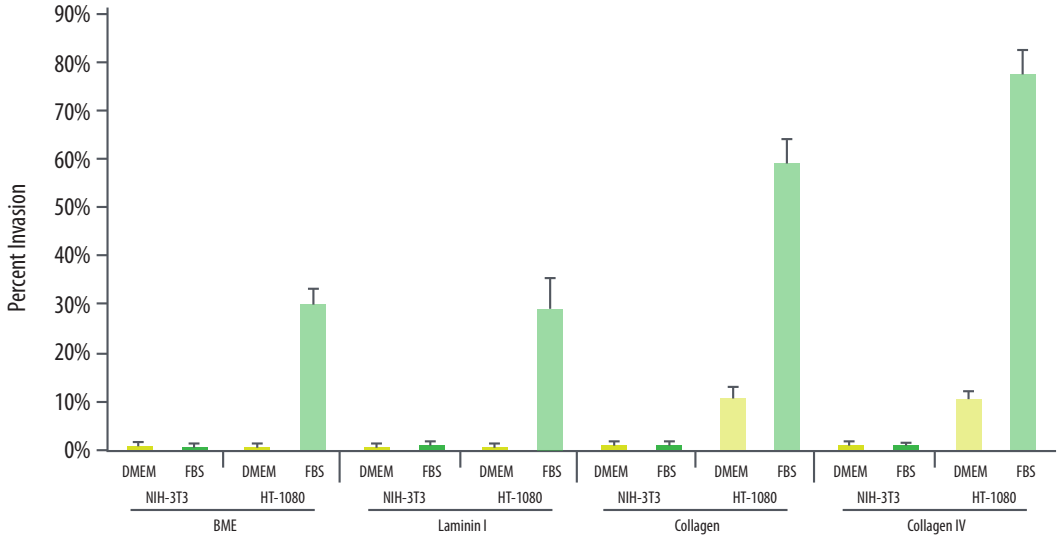

Figure 1. FBS Stimulates Migration of HT-1080 Cells. The NIH-3T3 mouse embryonic fibroblast cell line and the HT1080 human fibrosarcoma cell line were treated with 10% fetal bovine serum (FBS). The migration of untreated (yellow bars) and treated (green bars) NIH-3T3 and HT-1080 cells against different extracellular matrix components, including Cultrex BME, Laminin I, Collagen I, Collagen IV, were quantified using the Cultrex Cell Invasion Assay Kits (R&D Systems, Catalog # 3455-096-K, 3456-096-K, 3457-096-K, 3458-096-K, respectively). Data from four experiments was quantified for both non-invasive (NIH-3T3) and invasive (HT1080) cell types.

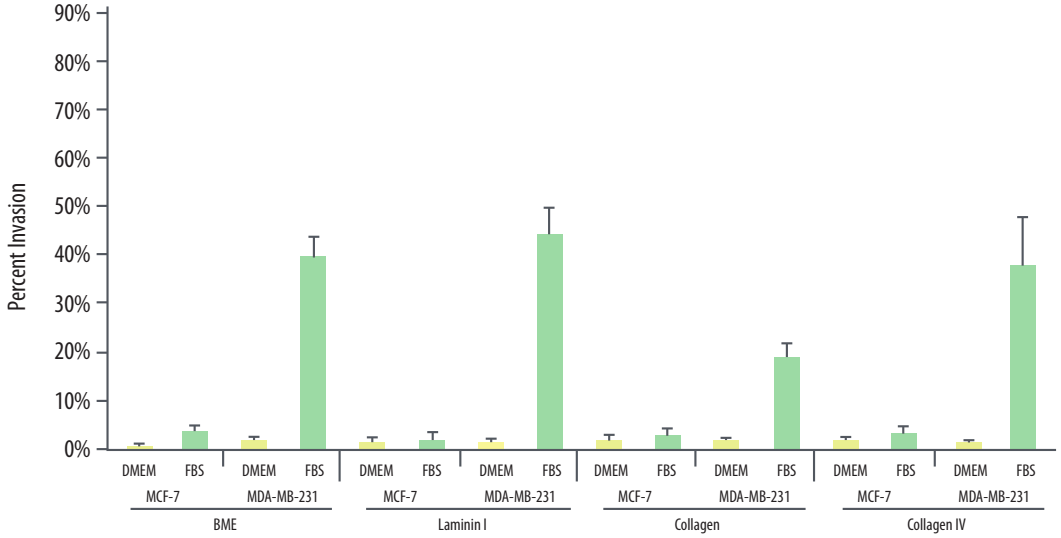

Figure 2. FBS Stimulates Migration of MDA-MB-231 Human Breast Cancer Cells. The MCF-7 and MDA-MD-231 human breast cancer cell lines were treated with 10% fetal bovine serum (FBS). The migration of untreated (yellow bars) and treated (green bars) NIH-3T3 and HT-1080 cells against different extracellular matrix components, including Cultrex BME, Laminin I, Collagen I, Collagen IV, were quantified using the Cultrex Cell Invasion Assay Kits (R&D Systems, Catalog # 3455-096-K, 3456-096-K, 3457-096-K, 3458-096-K, respectively). Data from four experiments was quantified for both non-invasive (MCF-7) and invasive (MDA-MD-231) cell types.

TROUBLESHOOTING

Problem	Cause	Action
No signal	Cells did not penetrate barrier/Barrier may not be permissive enough for cell type.	Use more diluted coating solution.
	Cell type may be noninvasive or chemo-attractant may be insufficient.	Select for more invasive subpopulations by subculturing/ Optimize chemo-attractant concentrations using a dilution series.
	Number of cells is not enough to degrade matrix.	Increase number of cells or increase time of the assay.
	The FBS used lacks the appropriate chemo-attractant at the expected concentration.	Screening of FBS lots may be necessary for optimal results.
	Cells did not invade or migrate.	Cell type may be non-invasive chemoattractant may be insufficient.
	Cells may have died as a result of treatment/toxic test agent was used.	Test cells for viability in treatment regimen.
High	Insufficient washing - agents in media, FBS, and/ or chemo-attractants may react with Calcein AM.	Re-assay, and make sure to wash well.
	Contamination - proteases released by bacteria or mold may degrade the BME and 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	Inconsistent pipetting.	Calibrate pipettors, and monitor pipette tips for air bubbles.
	Membrane punctured with pipette tips.	Disregard data from wells that are punctured; re-assay if necessary.

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