MultiScreen®-MIC (Migration, Invasion and Chemotaxis) Applications Note

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Title: **Evaluation of MultiScreen -MIC Plates in Invasion and Angiogenesis Assays**

Author: Lakshmi Kamath, Ph.D.
Millipore Life Science Division

ABSTRACT

The reproducibility of invasion assays was assessed on the MultiScreen-MIC (Migration, Invasion and Chemotaxis) plates. The invasion profile of the highly invasive adherent cell line MDA-MB-231 (breast cancer cell line) was evaluated on 8 µm membrane pore size MultiScreen-MIC plates (Cat. MAMIC8S10). The effect of two inhibitors (Cytochalasin D and Tamoxifen) on MDA-MB-231 cell invasion was also evaluated. These experiments were conducted on different lots and different experimental days to assess effects of these variables on assay outcome. The results presented here demonstrate that consistent inter-plate, intra-plate, inter-lot, and intra-lot data can be obtained with MultiScreen-MIC plates using the recommended protocol conditions for these assays. The suitability of these plates to assess *in vitro* tube formation (representing angiogenesis) assays using HUVEC (human umbilical vein endothelial cells) was also determined.

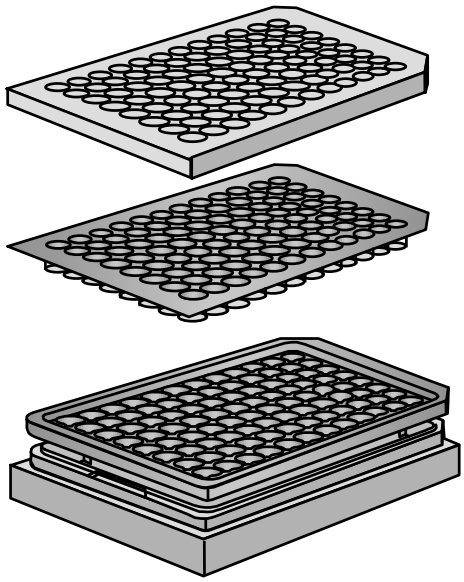
INTRODUCTION

In an effort to rapidly identify leads in the pursuit of new drugs, cell-based assays are gaining tremendous importance in pre-screening of compounds. These screening programs are increasingly focused on incorporating functional cell-based assays in primary and secondary screening stages of these compounds. These functional assays have the potential to provide valuable information early in the drug discovery process and result in better lead identification from a functional perspective. Many drugs under development in cancer drug discovery are directed at altering the metastatic properties of cancer cells such as chemotaxis and invasion. Cells like HUVEC that interact with cancer cells in invasive processes such as angiogenesis are also of interest as drug targets.

Invasion is defined as the movement of cells across a barrier, such as extracellular matrix (ECM), in response to a concentration gradient. Angiogenesis is the ability of HUVEC to form blood capillaries in response to cell stimulating factors. These blood capillaries supply the cancerous tumor with factors required for its growth. Angiogenic properties can be measured *in vitro* by the tube formation assay within a multi-dimensional matrix such as ECM. The premise of the cell based assays described here is that cells migrate in response to a chemical gradient and traverse a barrier such as in ECM (invasion) or migrate and exhibit tube formation (analogous to capillary formation *in vivo*) within a matrix such as ECM (angiogenesis)

Development of high throughput screening (HTS) cell-based assays that are designed to be able to measure effects of lead compounds on such functional processes pose a challenge. The MultiScreen-MIC plate is a 96-well sterile and disposable device designed to support such assays. These plates are available in three pore sizes (3, 5 and 8 μm) for use with a variety of suspension and adherent cell lines.

The MultiScreen-MIC plate has three components as listed in Figure 1.



(A) Lid

(B) MultiScreen-MIC filter plate

(C) MultiScreen-MIC receiver plate

Figure 1. MultiScreen-MIC plate components

These plates are particularly recommended for use in cell-based functional assays for screening protein libraries, antibody libraries screening and compound libraries for drug discovery. The proteins, antibodies and compound libraries can be screened to assess their ability to alter cell behavior in the assays described in this application note. These plates are also recommended for screening of compounds that inhibit the angiogenesis process of endothelial cells.

MATERIALS

Cell Lines: MDA-MB-231 (invasive breast cancer cells, Cat. HTB-26) was purchased from ATCC (Manassas, VA). HUVEC (Human umbilical vein endothelial cells, Cat. CC-2519) was purchased from Cambrex (Walkersville, MD).

Cell Culture Media: RPMI (Cat. R8758), culture media components MEM non-essential amino acids (NEAA) (Cat. M7145), HEPES buffer (Cat. H0887) and L-glutamine-penicillin/streptomycin solution (Cat. G1146) were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) (Cat. SH30070) was purchased from Hyclone (Logan, UT). Endothelial growth medium (EGM-2) (Cat. CC-3162) was purchased from Cambrex (Walkersville, MD).

Other: The Hema®-3 stain kit (Cat. 22122911), cotton swabs (Cat. 14-960-3P) and extracellular matrix (Matrigel®, Cat. CB40234A, manufactured by Becton Dickinson) were purchased through Fisher Scientific (Suwanee, GA). Calcein AM (Cat. C-3099) was purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA) (Cat. A4503), Trypan blue solution (Cat. T8154), 1XPBS, pH 7.4 (Cat. P3813) were purchased from Sigma (St. Louis, MO). The MultiScreen-MIC plates (8 μm, Cat. MAMIC 8S 10) were obtained from Millipore (Bedford, MA). Accessory plates (Cat. MAMC S01 10 and Cat. MAMC S 96 10) needed to set up the assay were also obtained from Millipore (Bedford, MA).

Equipment Used With Methods Described:

Hemacytometer

Zeiss® Axioplan™-2 microscope with KS300 3.0 software for cell counting and Axiovision™-2 software for cell imaging

Jouan CT422 centrifuge

METHODS

Note: Standard sterile cell culture lab practices must be followed while performing these protocols.

Cell culture

MDA-MB-231 cells were cultured and propagated in RPMI supplemented with 10% FBS, 1X HEPES, 1X NEAA, 1X L-glutamine-penicillin/streptomycin. Cells were passaged once a week at 100 % confluency and incubated at 37^{o} C, 5 % CO_2. HUVECs were propagated in EGM-2 complete medium at 37^{o} C, 5 % CO_2 for 3-5 passages as per supplier's recommendations.

Assay set-up

(1) Invasion assay with adherent cells

Cell expansion and preparation of adherent cells for invasion assay.

(a) Expand MDA-MB-231 cells into T-75 flasks for 3-5 days before experiment such that they are about 80-90 % confluent the day prior to setting up the experiment.
(b) Starve the cells overnight at 37^{o} C, 5 % CO_2 in serum free medium containing 0.2 % BSA (v/v).
(c) Thaw out extracellular matrix overnight at 4^{o} C as per manufacturer's recommendations.
(d) On the day of experiment, count the cells using the Trypan Blue cell counting procedure on the Hemacytometer (See Appendix, (A) for details)
(e) Adjust cell counts to 10^{6} cells/mL in serum free medium containing 0.2 % BSA. Also make a note of the cell viability. Cell viability of >90 % is acceptable for setting up migration experiments.

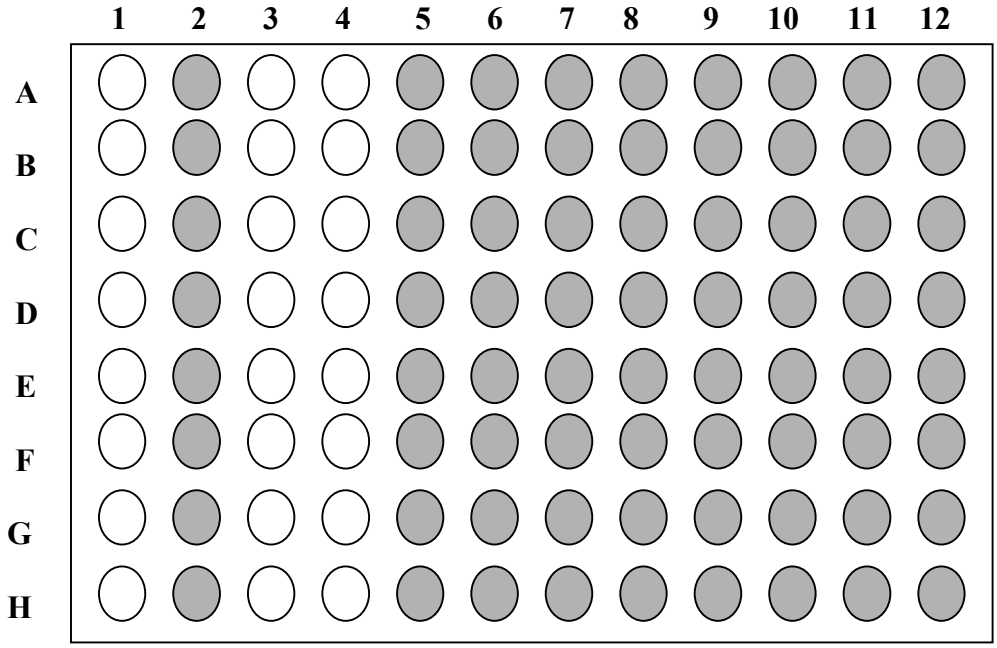
Coating procedure on MultiScreen-MIC plates for invasion assay with adherent cells.

(a) Pre-cool MultiScreen-MIC plates (Cat. MAMIC 8S 10 was used in our experiments) on ice for 30 minutes. This step can be performed prior to cell counting.

(b) Coat the membrane in the MultiScreen-MIC filter plate with 50 µL of ECM diluted to a concentration of 800 µg/mL in cold serum free medium (final ECM concentration is 40 µg/well). All dilutions of extracellular matrix need to be made using pre-cooled tubes. Keep the MultiScreen-MIC plate on ice until coating is completed. Gently dispense the extracellular matrix and use a pipette tip if needed to evenly distribute over the membrane. Avoid generating bubbles. Take care not to damage the membrane. It is critical to maintain cold conditions as per manufacturer's recommendations during coating. Use pre-cooled pipette tips and pre-cooled plates to ensure good coating. Ensure sterile practices to maintain sterility during coating procedure.

(c) Example coating template is outlined in figure 2. Templates for experiments may vary depending on format and replicates run in individual laboratories.

Figure 2. Coating template for invasion assay. Shaded wells coated in illustrated template.



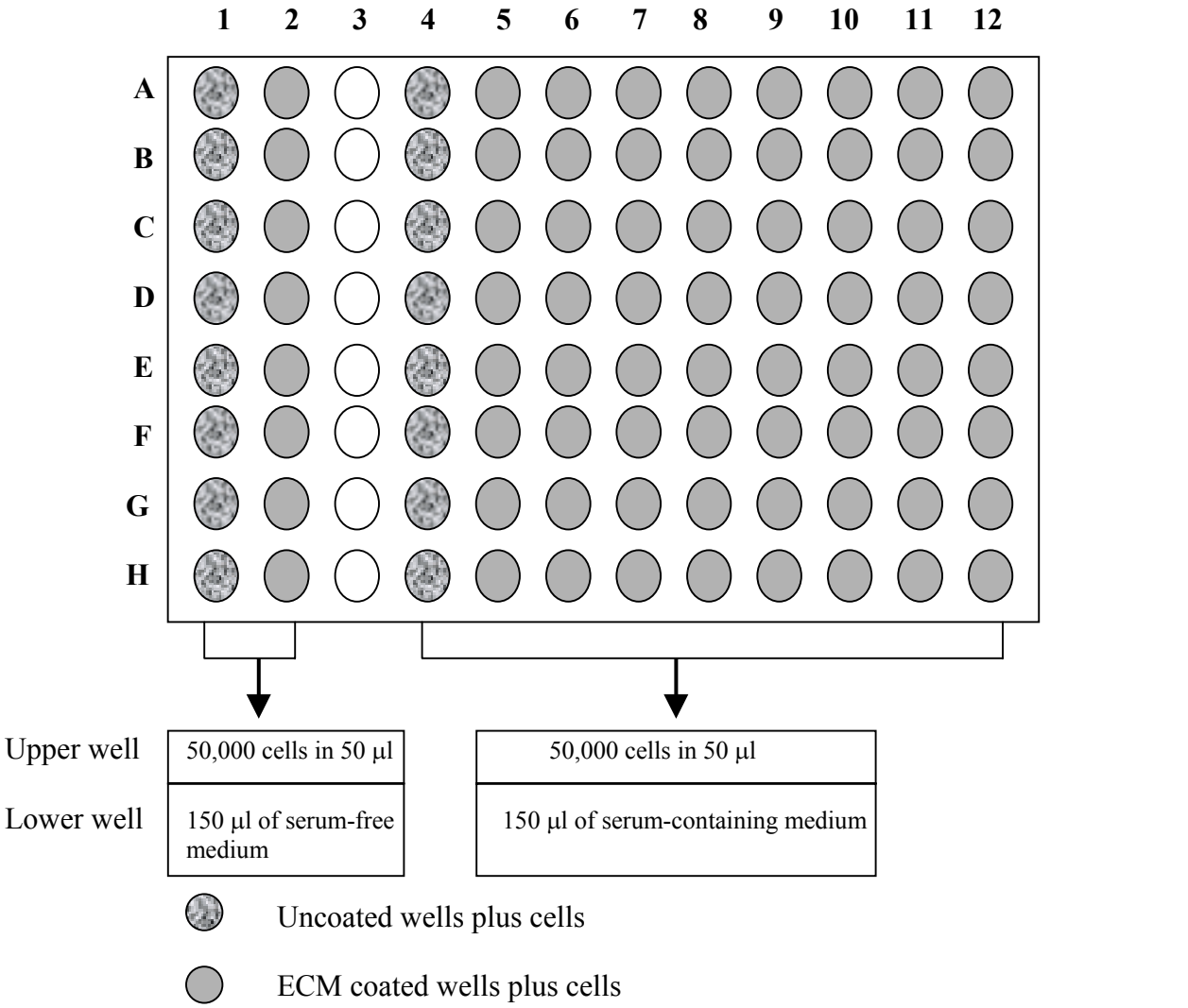
(d) Place the coated plates in the incubator at 37^{o} C for 30 minutes. Remove the plates from the incubator and place at room temperature for 1 h. This step ensures good polymerization of the extracellular matrix. Plates are now ready for setting up invasion assay.

<u>Setting up invasion assay and invasion inhibition assay on ECM-coated MultiScreen-MIC plates with adherent cells.</u>

(a) Separate out the MultiScreen-MIC filter plate with coated membrane and place into a sterile single well tray (Cat. MAMC S01 10) to protect the membrane prior to setting up the experiment.

(b) Set up the invasion experiment. Example template and chemoattractants is outlined in figure 3.

Figure 3. Example template for invasion assay with adherent cells. Shaded wells used in the template illustrated.

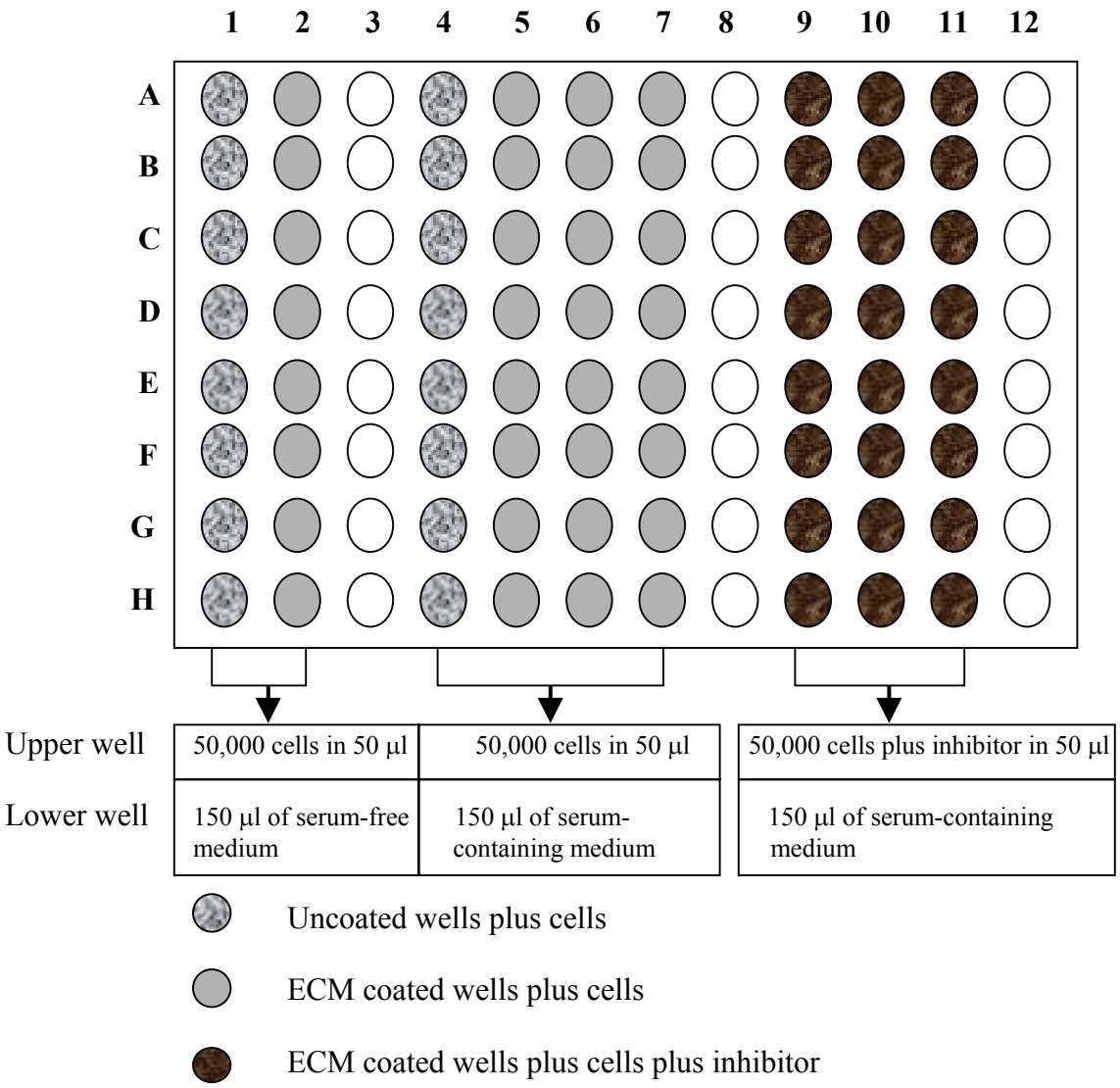


(c) Add cells to upper wells in the MultiScreen-MIC filter plate (placed in the single well tray). Ensure that cell suspension is evenly distributed across membrane. Add chemoattractants to lower wells in the MultiScreen-MIC receiver plate. Avoid generating bubbles while adding solutions to upper and lower wells.

(d) Columns 1 and 2 will test unstimulated migration in the absence (basal migration) and presence (basal invasion) of extracellular matrix respectively. Column 4 and columns 5-12 will test stimulated migration to factors in serum containing medium in the absence (chemotaxis) and presence (invasion) of extracellular matrix respectively.

(e) For invasion inhibition experiments, add inhibitors at desired concentration to cells prior to loading in upper wells. Example template and chemoattractants is outlined in figure 4. Templates for experiments may vary depending on format and replicates run in individual laboratories.

Figure 4. Example template for invasion inhibition assay with adherent cells. Shaded wells used in the template illustrated.



(f) Columns 1 and 2 will test unstimulated migration in the absence (basal migration) and presence (basal invasion) of extracellular matrix respectively. Column 4 and columns 5-7 will test stimulated migration to factors in serum containing medium in the absence (chemotaxis) or presence (invasion) of extracellular matrix. Columns 9-11 will test for invasion inhibition.

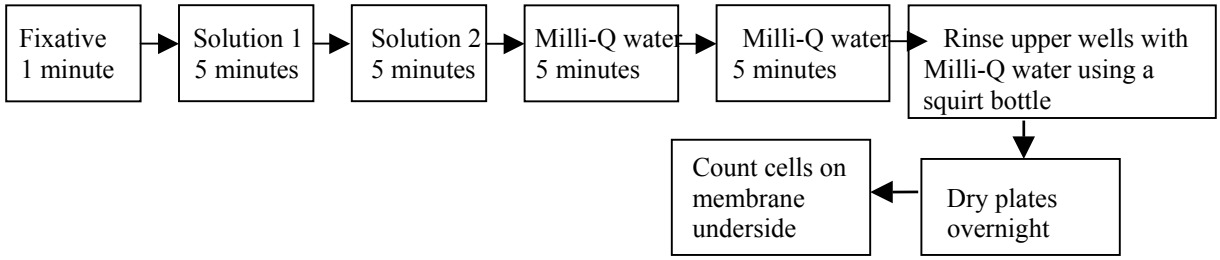
(g) GENTLY assemble the top and bottom plates together. DO NOT SHAKE or TILT THE PLATE, as this will disturb the concentration gradient.

(h) Incubate this MultiScreen-MIC plate assembly for 24 h at 37^{o} C. Do not shake plates during incubation.

Staining procedure for adherent cell invasion assay and invasion inhibition assay plates.

(a) Towards the end of the incubation period, set up staining solutions from the Hema-3 stain kit in the MultiScreen-MIC receiver plate (Cat. MAMC S 96 10). Dispense 150 µl of the fixative, solution 1 and solution 2 in series in separate plates. Set up two additional plates with Milli-Q® water.
(b) After incubation, remove plates without shaking or tilting from the incubator.
(c) Remove unmigrated cells in upper wells with a cotton swab. Use gentle pressure to effectively remove cells from the wells. Take care not to puncture the membrane.
(d) After swabbing all wells, gently rinse the wells with PBS using a squirt bottle.
(e) Stain MultiScreen-MIC filter plates as illustrated in figure 5. Pass the plate through the staining solutions as indicated.

Figure 5. Staining procedure to detect adherent cells on membrane underside.



(f) Optional: Quickly swab upper wells in filter plate with cotton swabs to remove any residual dye prior to drying plates. This minimizes dye interference when counting.

Image analysis of stained invasion assay or invasion inhibition assay plates.

(a) The experimental plates in our experiments were counted using a Zeiss Axioplan-2 microscope equipped with KS300 3.0 automated cell counting software. 11% percent of membrane surface area was counted and the cell number was extrapolated to the entire membrane (0.3 cm^2 surface area) as indicated in the calculation. (Plates can also be manually counted using a standard microscope with sufficient magnification to view the cells. Place the filter plate on a slide to avoid damage to membrane. If objectives are above the stage, simply flip the plate so membrane side is facing up towards the objective. Calculate the area of counting field in your microscope and count a sufficient number of fields in the membrane corresponding to 5-10 % of total membrane surface area).

Calculations for adherent cell invasion assay or invasion inhibition assay.

(a) Calculate results as follows. ***Values shown in data tables are not representative of actual data***.

Table 1. Example of data output from reading a MultiScreen-MIC filter plate after an invasion assay with adherent cells.

	1	2		4	5	6	7	8	9	10	11	12
A	55	2		2000	500	500	500	500	500	500	500	500
B	55	2		2000	500	500	500	500	500	500	500	500
C	55	2		2000	500	500	500	500	500	500	500	500
D	55	2		2000	500	500	500	500	500	500	500	500
E	55	2		2000	500	500	500	500	500	500	500	500
F	55	2		2000	500	500	500	500	500	500	500	500
G	55	2		2000	500	500	500	500	500	500	500	500
H	55	3		2000	500	500	500	500	500	500	500	500

-Counts were multiplied by a multiplication factor of 9.09 to reflect migrated cells in entire membrane. (User will have a different multiplication factor depending on area of counting field and percent of membrane surface area counted).

Table 2. Example of data output after multiplying by a factor of 9.09

	1	2		4	5	6	7	8	9	10	11	12
A	500	18		18180	4545	4545	4545	4545	4545	4545	4545	4545
B	500	18		18180	4545	4545	4545	4545	4545	4545	4545	4545
C	500	18		18180	4545	4545	4545	4545	4545	4545	4545	4545
D	500	18		18180	4545	4545	4545	4545	4545	4545	4545	4545
E	500	18		18180	4545	4545	4545	4545	4545	4545	4545	4545
F	500	18		18180	4545	4545	4545	4545	4545	4545	4545	4545
G	500	18		18180	4545	4545	4545	4545	4545	4545	4545	4545
H	500	18		18180	4545	4545	4545	4545	4545	4545	4545	4545

Average of wells A1-H1=500.
Average of wells A2-H2=18
Use these values to calculate percent basal invasion in this equation:

$$\frac{18 \times 100}{500} = 3.6\ \%$$

3. 6 % is basal invasion in response to serum-free medium and is calculated relative to basal migration in response to serum-free medium.

Average of wells A4-H4=18180
Average of wells A5-A12 to H5-H12=4545
Use these values to calculate percent stimulated invasion in this equation:

$$\frac{4545 \times 100}{18180} = 25\ \%$$

25 % is stimulated invasion in response to serum-containing medium and is calculated relative to stimulated chemotaxis in response to serum-containing medium.

Hence fold stimulated invasion over background is 25/3.6= 7 fold

Table 3. Example of data output from reading a MultiScreen-MIC filter plate after an invasion inhibition assay with adherent cells.

	1	2		4	5	6	7		9	10	11	
A	55	2		2000	500	500	500		80	80	80	
B	55	2		2000	500	500	500		80	80	80	
C	55	2		2000	500	500	500		80	80	80	
D	55	2		2000	500	500	500		80	80	80	
E	55	2		2000	500	500	500		80	80	80	
F	55	2		2000	500	500	500		80	80	80	
G	55	2		2000	500	500	500		80	80	80	
H	55	3		2000	500	500	500		80	80	80	

-Counts were multiplied by a multiplication factor of 9.09 to reflect migrated cells in entire membrane. (User will have a different multiplication factor depending on area of counting field and percent of membrane surface area counted).

Table 4. Example of data output after multiplying by a factor of 9.09

	1	2		4	5	6	7		9	10	11	
A	500	18		18180	4545	4545	4545		727	727	727	
B	500	18		18180	4545	4545	4545		727	727	727	
C	500	18		18180	4545	4545	4545		727	727	727	
D	500	18		18180	4545	4545	4545		727	727	727	
E	500	18		18180	4545	4545	4545		727	727	727	
F	500	18		18180	4545	4545	4545		727	727	727	
G	500	18		18180	4545	4545	4545		727	727	727	
H	500	18		18180	4545	4545	4545		727	727	727	

Average of wells A1-H1=500
Average of wells A2-H2=18
Use this value to calculate percent basal invasion in this equation:

$$\frac{18 \times 100}{500} = 3.6\ \%$$

3.6 % is basal invasion in response to serum-free medium and is calculated relative to basal migration in response to serum-free medium.

Average of wells A4-H4=18180
Average of wells A5-A7 to H5-H7=4545
Use this value to calculate percent stimulated invasion in this equation:

$$\frac{4545 \times 100}{18180} = 25\ \%$$

Average of wells A9-A11 to H9-H11=727
Use this value to calculate percent invasion in presence of inhibitors in this equation:

$$\frac{727 \times 100}{18180} = 4\ \%$$

4 % is invasion in response to serum-containing medium in presence of inhibitors and is calculated relative to stimulated chemotaxis in response to serum-containing medium.

Normalize stimulated invasion in absence of inhibitors to 100 % that is 25 % =100 %
Hence, normalized stimulated invasion in presence of inhibitors is=$\frac{4 \text{ X } 100}{25}$ = 8 %

Percent invasion inhibition = 100-8 = 92 %.

(2) Angiogenesis (tube formation) assay setup with HUVEC cells

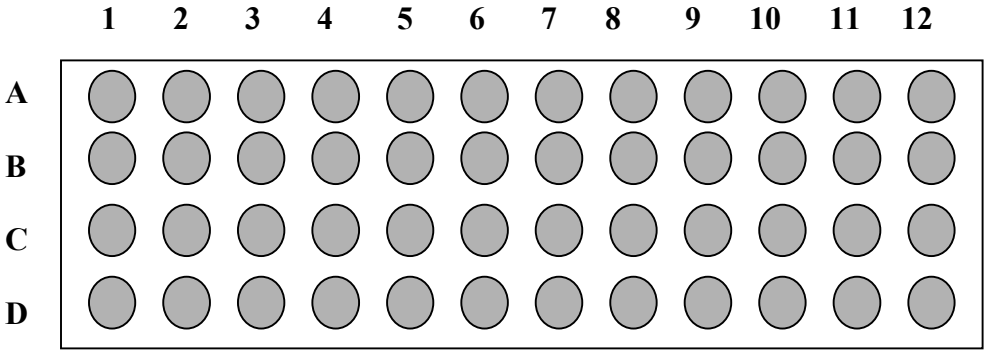
Cell expansion and preparation of HUVEC cells for angiogenesis assay.

(a) Expand HUVEC cells into T-75 flasks for 5-7 days before experiment such that they are about 80-90 % confluent the day prior to setting up the experiment.
(b) Starve the cells overnight at 37^{o} C, 5 % CO_2 in serum free medium containing 0.2 % BSA (v/v).
(c) Thaw out extracellular matrix overnight at 4^{o} C as per manufacturer's recommendations.
(d) On the day of experiment, count the cells using the Trypan Blue cell counting procedure on the Hemacytometer (See Appendix, (A) for details).
(e) Adjust cell counts to 10^5 cells/mL in 1X PBS.
(f) Also make a note of the cell viability. Cell viability of >90 % is acceptable for setting up angiogenesis experiments.

Coating procedure on MultiScreen-MIC plates for angiogenesis assay with HUVEC cells.

(a) Pre-cool MultiScreen-MIC plates (Cat. MAMIC 5S 10 was used in these experiments) on ice for 30 minutes. This step can be performed prior to cell counting.
(b) Coat the membrane in the MultiScreen-MIC filter plate with 50 µL of ECM diluted to a concentration of 8 mg/mL in cold serum free medium (final ECM concentration is 400 µg/well). All dilutions of extracellular matrix need to be made using pre-cooled tubes. Keep the MultiScreen-MIC plate on ice until coating is completed. Gently dispense the extracellular matrix and use pipette tip if needed to evenly distribute over the membrane. Avoid generating bubbles. Take care not to damage the membrane. It is critical to maintain cold conditions as per manufacturer's recommendations during coating. Use pre-cooled pipette tips and pre-cooled plates to ensure good coating. Ensure sterile practices to maintain sterility during coating procedure.
(c) Example coating template used is outlined in figure 6. Templates for experiments may vary depending on format and replicates run in individual laboratories.

Figure 6. Coating template for angiogenesis assay. Shaded wells coated in illustrated template.



(d) Place the coated plates in the incubator at 37° C for 30 minutes. Remove from incubator and place at room temperature for 1 h. This step ensures good polymerization of the extracellular matrix. Plates are now ready for setting up angiogenesis assay.

Setting up angiogenesis assay on ECM-coated MultiScreen-MIC plates with HUVEC cells.

(a) Separate out the filter plate with coated membrane and place into a sterile single well tray (Cat. MAMC S01 10) to protect the membrane prior to setting up the experiment.
(b) Set up the angiogenesis experiment. Add 10,000 cells/100 µl (in PBS) to coated wells.
(c) Allow cells to adhere to extracellular matrix for 30 minutes at 37°C. Replace 1X PBS with EGM-2 basal medium in column 1 and EGM-2 complete medium in columns 2-12.
(d) Column 1 will be control wells and columns 2-12 will be test wells.
(e) Incubate plates for 24 h at 37°C.

Staining procedure for angiogenesis assay plates.

(a) Stain upper wells using the Hema-3 stain kit taking care not to disrupt the ECM in the wells as illustrated in figure 7.

Figure 7. Staining procedure to detect *in vitro* tube formation (angiogenesis) exhibited by HUVEC on ECM in upper wells.

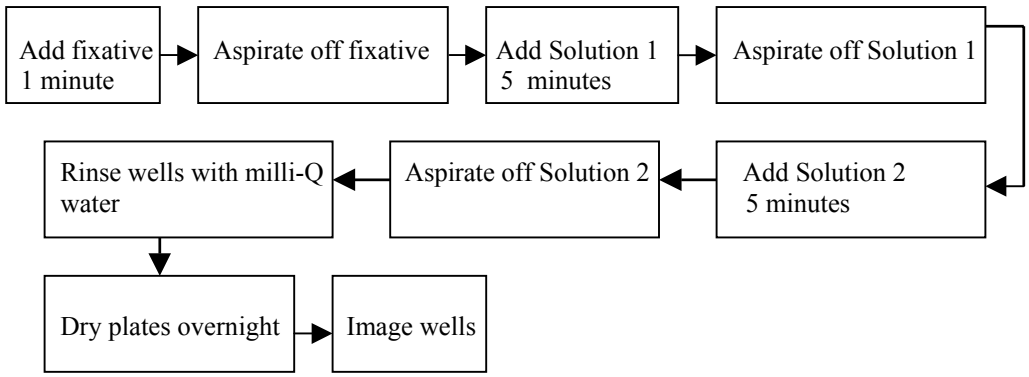


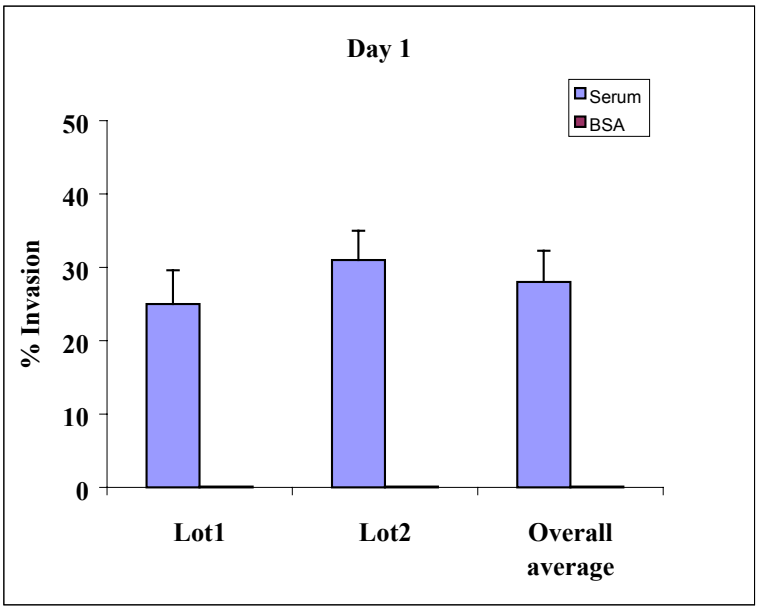
Image analysis of stained angiogenesis assay plates.

(a) The experimental plates in our experiments were imaged on a Zeiss Axioplan-2 microscope equipped with Zeiss Axiovision-2 software.

RESULTS

(1) Invasion assay with adherent cells.

The uniformity of invasion values obtained was assessed in experiments performed on the same day using different lots. Invasion values were also determined in assays performed on two days using two different lots. Figure 8 demonstrates values obtained in experiments performed with MDA-MB-231 in response to 10 % serum or 0.2 % BSA as a chemoattractant. Inter-plate, inter-lot and intra-plate invasion values are shown in Table 5. The contribution of coating to data variability is shown in Table 6.



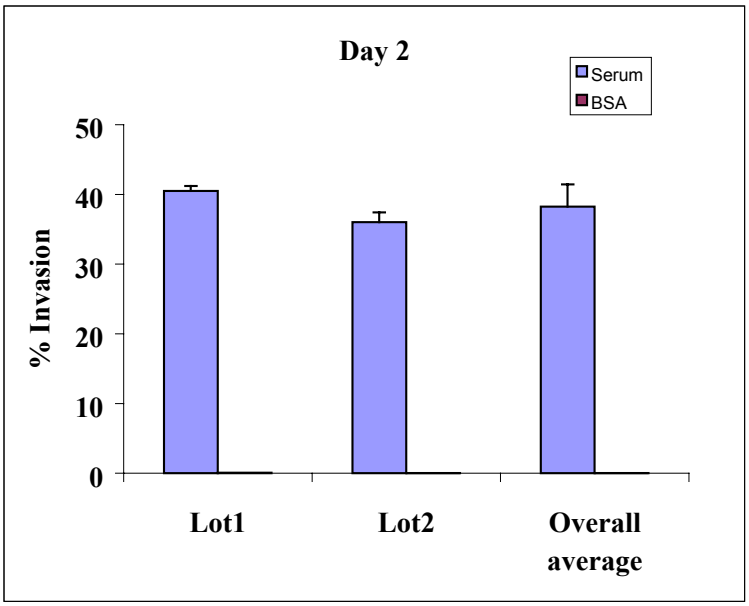


Figure 8**.** Invasion profiles of MDA-MB-231 on 8 μm MultiScreen-MIC plates in response to 10% serum or 0.2 % BSA containing medium as a chemoattractant (n=3 for lots 1 and 2, r=64 on Day 1; n=2 for lots 1 and 2, r=64 on Day 2, where n is the number of plates and r is the number of wells tested).

Table 5. Reproducibility of invasion assays with MDA-MB-231 cells.

	Inter-plate (2 lots)		**Inter-lot (2 lots)**		**Intra-plate**		**Intra-plate**	
					Day 1		Day 2	
	Day 1	Day 2	Day 1	Day 2	Lot 1	Lot 2	Lot 1	Lot 2
Average percent stimulated invasion	28 ± 4	38 ± 3	28 ± 4	38 ± 3	25	31	41	36
Plate 1	-	-	-	-	20 ± 7	27 ± 11	40 ± 15	35 ± 19
Plate 2	-	-	-	-	26 ± 9	31 ± 13	41 ± 22	37 ± 20
Plate 3	-	-	-	-	29 ± 10	35 ± 15	-	-

3 plates per lot, with 64 replicates per plate, were tested on Day 1. Two lots were tested
2 plates per lot, with 64 replicates per plate, were tested on Day 2. Two lots were tested.

Table 6. Contribution of coating to variability in invasion assays with MDA-MB-231 cells

	Inter-plate (2 lots)		**Intra-plate**		**Intra-plate**	
			Day 1		**Day 2**	
	Day 1	**Day 2**	**Lot 1**	**Lot 2**	**Lot 1**	**Lot 2**
Percent stimulated chemotaxis in absence of extracellular coating	71 ± 11	72 ± 10	72	69	76	69
Plate 1	-	-	85 ± 10	65 ± 9	86 ± 8	72 ± 6
Plate 2	-	-	57 ± 7	80 ± 11	65 ± 6	65 ± 9
Plate 3	-	-	74 ± 13	62 ± 7	-	-

3 plates per lot, with 64 replicates per plate, were tested on Day 1. Two lots were tested
2 plates per lot, with 64 replicates per plate, were tested on Day 2. Two lots were tested.

(2) Invasion inhibition assay with adherent cells.

Invasion inhibition experiments were performed to assess the suitability of MultiScreen-MIC plates in drug discovery screening assays and to assess reproducibility of inhibition response. Figure 9 demonstrates invasion inhibition profiles obtained with 20 µM Cytochalasin D and 50 µM Tamoxifen on two days using at least two different lots. Table 7 demonstrates reproducibility of inhibition response and Table 8 demonstrates the contribution of coating to data variability.

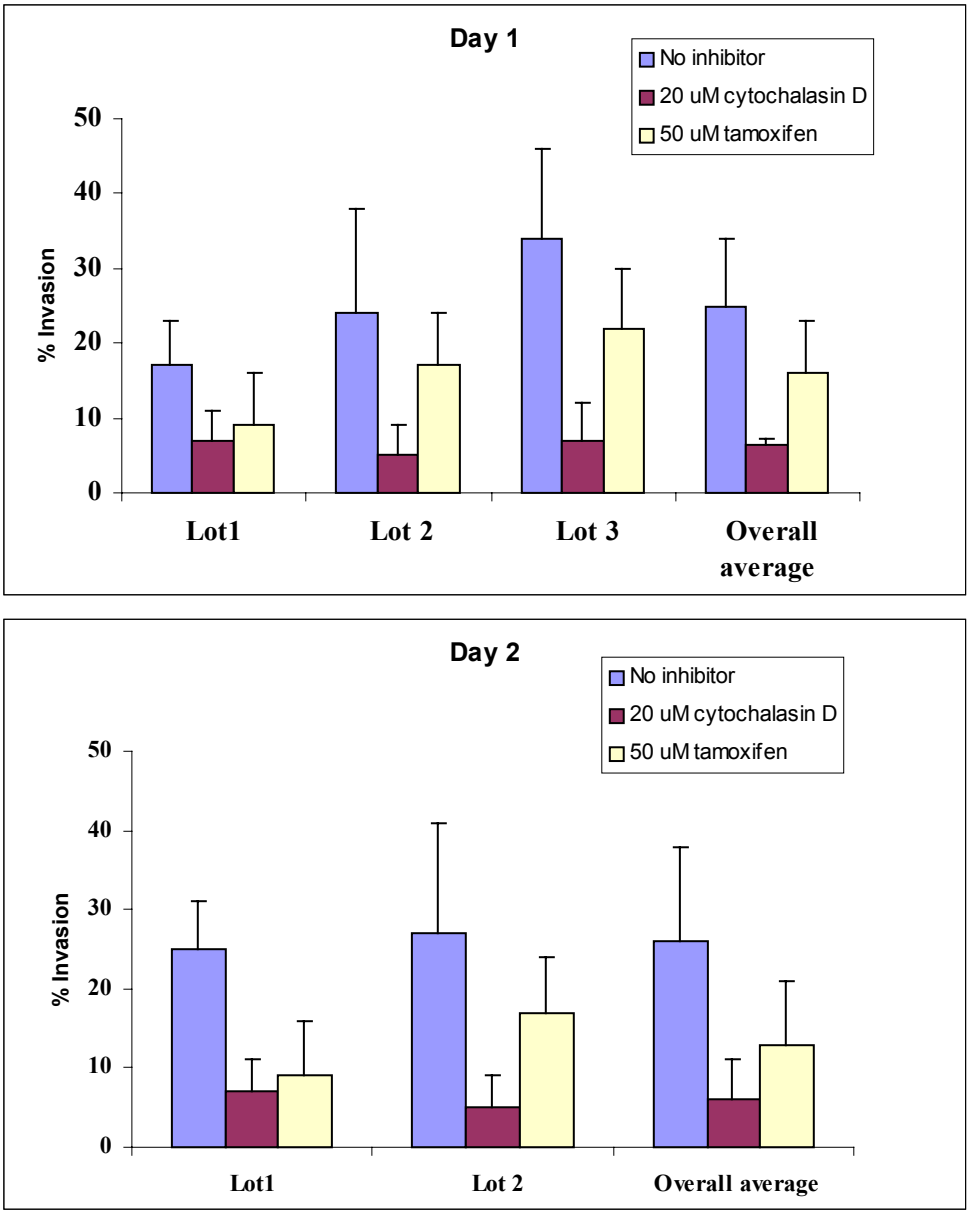


Figure 9. Invasion inhibition profile of MDA-MB-231 cells on 8 µm MultiScreen-MIC plates in response to 10% serum or 0.2 % BSA containing medium a chemoattractant in the presence and absence of inhibitors (per lot: n=1, r=14 per condition tested, on each experimental day, where n is the number of plates and r is the number of wells tested). Average background % invasion values in the absence of inhibitors ranged from 0.02 to 0.08 on day 1 and 0.01 to 0.2 on day 2 indicating robust stimulated invasion response.

Table 7. Reproducibility exhibited by MDA-MB-231 cells in invasion inhibition assays.

Day	**Day 1**						**Day 2**			
Inhibitor	**20 µM Cytochalasin D**			**50 µM Tamoxifen**			**20 µM Cytochalasin D**		**50 µM Tamoxifen**	
Lot	**Lot 1**	**Lot 2**	**Lot 3**	**Lot 1**	**Lot 2**	**Lot 3**	**Lot 1**	**Lot 2**	**Lot 1**	**Lot 2**
Percent inhibition of invasion	61	78	79	47	31	36	83	90	32	40

1 plate per lot, with 14 replicates per plate, was tested on Day 1. Three lots were tested
1 plate per lot, with 14 replicates per plate, was tested on Day 2. Two lots were tested.

Table 8. Contribution of coating to variability in invasion inhibition assays with MDA-MB-231 cells.

	Inter-plate (3 lots)		**Intra-plate**			**Intra-plate**	
			Day 1			Day 2	
	Day 1	Day 2	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2
Percent stimulated chemotaxis in absence of extracellular coating	73 ± 6	83 ± 21	67 ± 15	72 ± 14	79 ± 17	67 ± 12	98 ± 27

1 plate per lot, with 14 replicates per plate, was tested on Day 1. Three lots were tested
1 plate per lot, with 14 replicates per plate, was tested on Day 2. Two lots were tested.

(3) Angiogenesis assay with HUVEC cells.

Figure 10 demonstrates *in vitro* tube formation (angiogenesis) exhibited by HUVEC cells on Multi-Screen MIC plates.

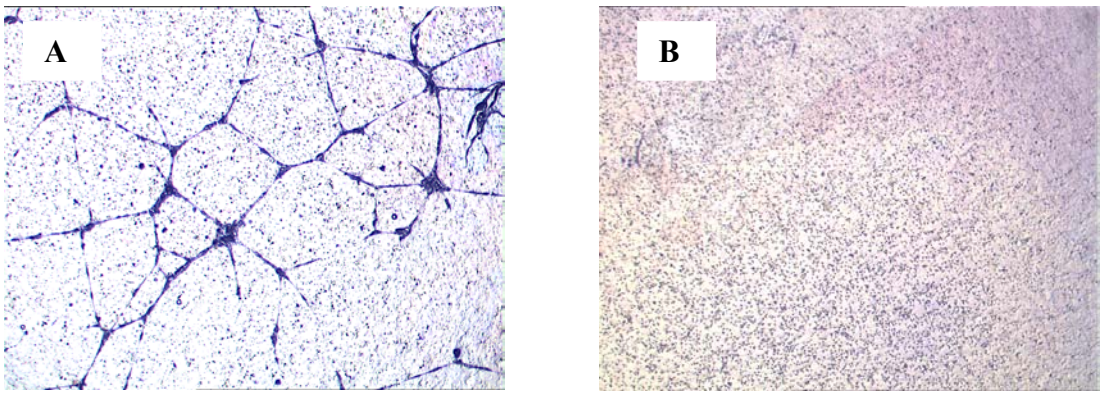


Figure 10. *In vitro* tube formation exhibited by HUVEC cells on 5 µm MultiScreen-MIC plates coated with extracellular matrix (400 µg/well) in response to EGM-2 medium as a chemoattractant. Panel A demonstrates angiogenesis in response to EGM-2 over 24 h. Panel B represents unstimulated cells.

DISCUSSION

Excellent invasion with MDA-MB-231 cells was observed as shown in Figure 8. Data presented in Figure 8 and Table 5 represent assays performed on two days using two different lots. Consistent invasion values in response to 10 % serum-containing medium were obtained in experiments performed with MDA-MB-231 cells across lots tested on the same experimental day. Consistent inter-plate and inter-lot values were obtained on lots tested on same experimental day as shown in Table 5. Intra-plate standard deviations were in the range of 7-15 for assays performed on Day 1 and 15-22 for assays performed on Day 2 reflecting assay variability on different experimental days. In addition to processing steps involved in performing the assay with adherent cells and with detection method used, user needs to account for coating technique variability if any. Percent chemotaxis obtained in absence of coating is shown in Table 6. These values indicate that coating did not significantly contribute to standard deviations observed on Day 1 but contributed to standard deviations observed on Day 2. All these variables need to be taken into consideration by the user when interpreting assay results.

Excellent invasion inhibition response was obtained in invasion inhibition assays with MDA-MB-231 cells. Figure 9 demonstrates invasion inhibition profiles obtained with 20 μM Cytochalasin D and 50 μM Tamoxifen on two days using three different lots. Consistent and reproducible inhibition results were obtained on lots tested on the same day. Percent inhibition obtained with Tamoxifen was consistent on Day 1 and Day 2 compared to Cytochalasin D (Table 7). Percent chemotaxis obtained in absence of coating is shown in Table 8. These values indicate that coating did not significantly contribute to standard deviations observed on Day 1 and Day 2. All these variables need to be taken into consideration by the user when interpreting assay results.

Excellent *in vitro* angiogenesis formation with HUVEC cells (figure 10) was observed using MultiScreen-MIC plates demonstrating the utility of the device for this application. Results were reproduced in experiments performed on at least two different days.

The results discussed here thus demonstrate the utility of these plates for invasion, invasion inhibition and *in vitro* tubule formation (angiogenesis assays) with adherent cells.

GENERAL CONSIDERATIONS

Invasion and Angiogenesis assays are complex assays but provide a lot of information on cell behavior and can be classified as high-content assays. The user needs to be aware of several variables that can influence the outcome of the assay and hence interpretation of assay results. As with any assay the user needs to invest some time in understanding the nature of the cell line in use and optimizing the assay to obtain good results. Some of the “watchouts” are discussed in this section.

The user is strongly advised to check out the MultiScreen-MIC website, http://www.millipore.com/multiscreenMIC, as it has a comprehensive FAQ (frequently asked question) section that provides additional information.

1. Cell type and pore size determination: It is best to obtain as much information as possible on the cell line being used through a comprehensive literature search before performing the assay. This will provide a starting point for assay parameters including choice of pore size. Typically lower pore sizes are optimal for suspension cells and higher pore sizes are optimal for adherent cells. When unsure choose one pore sizes higher and one pore size lower than cell size to assess which pore size gives better results. This is especially true for adherent cells.

2. Assay standardization: Run standardization experiments to optimize cell density, time of assay, chemoattractant concentrations and cell to pore ratio to determine optimal assay conditions. Run appropriate controls such as negative chemoattractant and a negative cell line (latter is optional. It is good practice for e.g. to run a non-invasive cell line as an assay control while optimizing assay parameters).

3. Cell variability: Cell variability is an inherent part of cell-based assays. It is best to interpret results in relation to controls run on the same experimental day for any given assay.

4. Detection method: Choice of detection method will influence variability obtained in assay results. Detection methods that employ imaging are the most sensitive giving an accurate outcome of the experiment. But they also contribute most to the standard deviation. With imaging techniques, consistency of trends in response rates rather than absolute values assume importance when comparing assays performed across different days.

5. Other Tips and Technique:
   Dispense cells evenly across membrane.
   For assays with adherent cells, scrub gently to avoid membrane warping. Change swabs frequently.
   For assays with adherent cells, rinse plates well with water after staining procedure to remove any stain deposits that might interfere with imaging/counting.
   If automated software is used to count cells, use the same counting parameters for all plates run on the same experimental day. If counting parameters differ between plates then account for software variability when interpreting results.

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OTHER MultiScreen-MIC PRODUCT LITERATURE

You can find the following literature pieces on our website
http://www.millipore.com/multiscreenMIC

Application Note, Literature piece number: AN1060EN00
Poster, Literature piece number: PS1651EN00
Poster, Literature piece number: PS0912EN00
Poster, Literature piece number: PS0913EN00
User guide, Literature piece number: P36448
Datasheet, Literature piece number: PF2627EN00

These literature pieces can be ordered via our website or by calling 1-800-645-5476. You can also call the Millipore Technical Support at 1-800-645-5476 for further assistance.

APPENDIX

(A) Trypan Blue cell counting procedure on the Hemacytometer

-Mix 50 µl of Trypan Blue with 50 µl of cells in a fresh microcentrifuge tube.
-Pipette 10 µl the cell-dye mix onto the haemocytometer at the 2 notched junctions with the cover slip on.
-Count total cells (alive and dead) in the four corner sections around the center grid.
-Divide live number by 4 for the average and then multiply the number by 2 to account for the dilution with Trypan Blue. This number is the cell concentration x 10^4/ml.
-Example: a total count of **250** cells in the 4 sections yields: (250/4 = 62.5, 62.5 x 2 =**125**) 125 x 10^4 cells /ml or **1.25 x 10^6 cells/ml.** These are the **cells that you have.** Repeat with the other side of the hemacytometer. Average the two counts to find out your cell concentration.
-Dilute cells to desired concentration with serum free medium containing 0.2 % BSA
-To calculate cell viability use the following equation.

$$\frac{\text{\# live cells in 4 quadrants}}{\text{(\# dead cells + \# live cells in 4 quadrants)}} \times 100 = \%\ \text{“live" OR Viable cells}$$

Example:

$$\frac{250\ \text{live cells}}{10\ \text{dead cells} + 250\ \text{live cells}} \times 100 = 96.2\ \%\ \text{“live” or viable cells.}$$

-Cell viability values greater than 90 % indicate healthy cells.

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