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

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Title: **Evaluation of MultiScreen -MIC Plates in Chemotaxis Assays**

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ABSTRACT

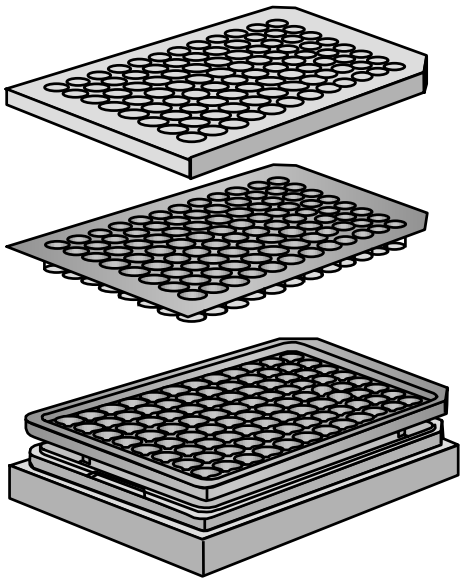
The reproducibility of Chemotaxis assays was assessed on various membrane pore sizes of MultiScreen-MIC (Migration, Invasion and Chemotaxis) plates. The chemotaxis of suspension cell lines HB-124 (hybridoma cell line) and K-562 (lymphoblastic cell line) was evaluated on 3 μm membrane pore size MultiScreen-MIC plate (Cat. MAMIC3S10). The chemotaxis profiles of highly migratory adherent cell lines MDA-MB-231 (breast cancer cell line) and HT-1080 (fibrosarcoma cell line) was evaluated on 5 μm membrane pore size MultiScreen-MIC plates (Cat. MAMIC5S10). The effect of two inhibitors (Cytochalasin D and Tamoxifen) on MDA-MB-231 cell chemotaxis 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.

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 functional 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. Chemotaxis is the movement of cells in response to a concentration gradient set up by a chemoattractant. The premise of the cell-based assays described here is that cells migrate in response to a chemical gradient (chemotaxis) set up using the MultiScreen-MIC plate.

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 and compound libraries for drug discovery. The proteins, antibodies and compound libraries can be screened to assess their ability to alter cell behavior in assays described in this application note.

MATERIALS

Cell Lines: HB-124 (spleen cells fused with Sp2/0-Ag14 myeloma cells, Cat. HB-124); K-562 (lymphoblastic cell line isolated from a chronic myelogenous leukemia patient, Cat. CCL-243); MDA-MB-231 (invasive breast cancer cells, Cat. HTB-26); HT-1080 (invasive fibrosarcoma cell line, Cat. CCL-121) were all purchased from ATCC (Manassas, VA).

Cell Culture Media: DMEM, high glucose (Cat. D5796), 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). Sodium pyruvate (Cat. S8636) which was used as additive for HT-1080 cell growth medium was also purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) (Cat. SH30070) was purchased from Hyclone (Logan, UT).

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 (3 μm, Cat. MAMIC 3S 10; 5 μm, Cat. MAMIC 5 S10) 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
Fluorescent Plate Reader (Wallac/Perkin Elmer Victor™ 2)
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

HB-124, K-562 and HT-1080 cells were cultured and propagated in DMEM, high glucose supplemented with 10% FBS, 1X HEPES, 1X NEAA, 1X L-glutamine-penicillin/streptomycin. Cells were routinely passaged twice a week at 100% confluency and incubated at 37^{o} C, 5 % CO_2. 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.

Assay set-up

(1) Chemotaxis assay with suspension cells

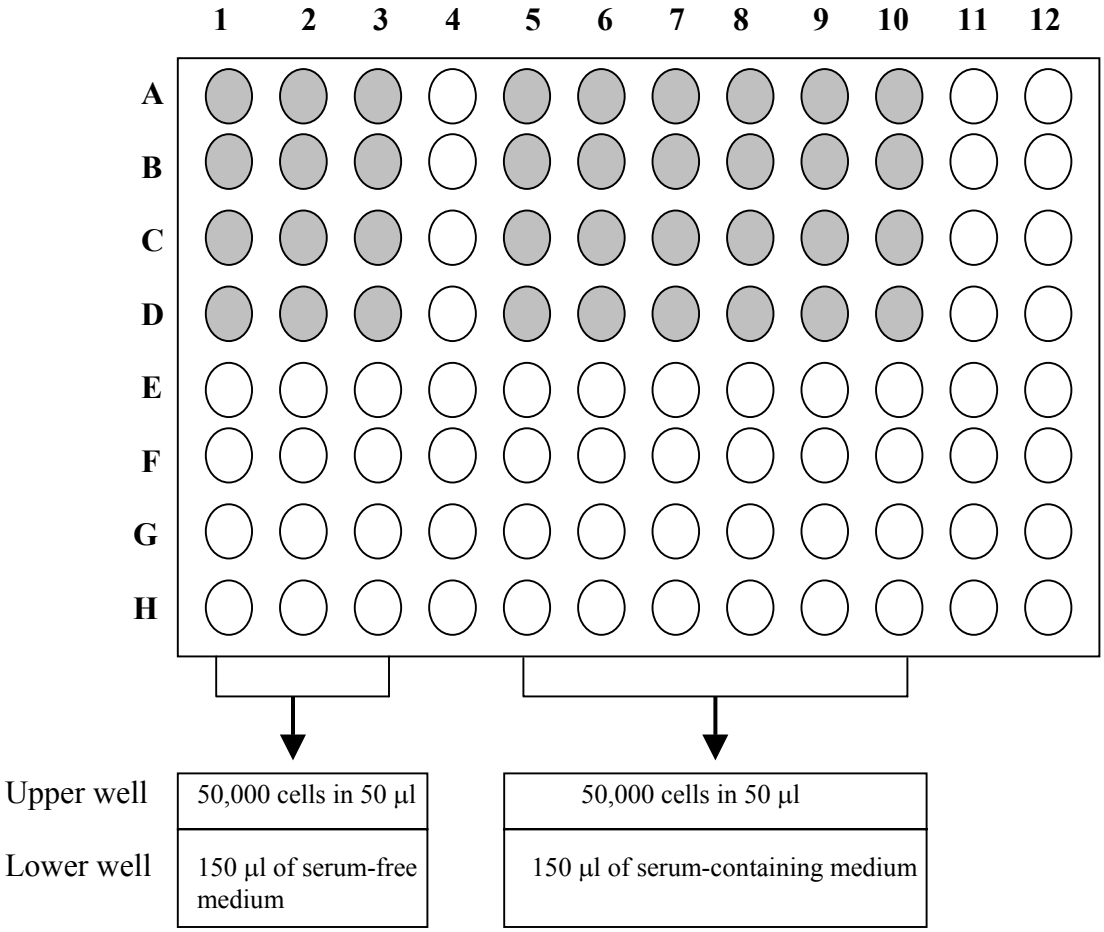
Cell expansion and preparation of suspension cells for chemotaxis assay.

(a) Expand HB-124 and K-562 cells into T-75 flasks for 3-5 days before the experiment such that they are about 80-90 % confluent the day prior to setting up the experiment.
(b) Using a 15 ml centrifuge tube, centrifuge the cells at 120g for 8 minutes at room temperature to pellet the cells.
(c) Resuspend the cells in serum free medium containing 0.2 % BSA (v/v).
(d) Starve the cells overnight at 37^{o} C, 5 % CO_2.
(e) On the day of experiment, count the cells using the Trypan Blue cell counting procedure on the Hemacytometer (See Appendix, (A) for details).
(f) 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 chemotaxis experiments.

Setting up chemotaxis assay on MultiScreen-MIC plates with suspension cells.

(a) Separate out the filter plate with membrane (Cat. MAMIC 3S 10) 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 chemotaxis experiment. Example template and chemoattractants is outlined in figure 2. (Templates for experiments may vary depending on format and replicates run in individual laboratories).

Figure 2. Example template for chemotaxis assay with suspension cells. Shaded wells used in illustrated template.



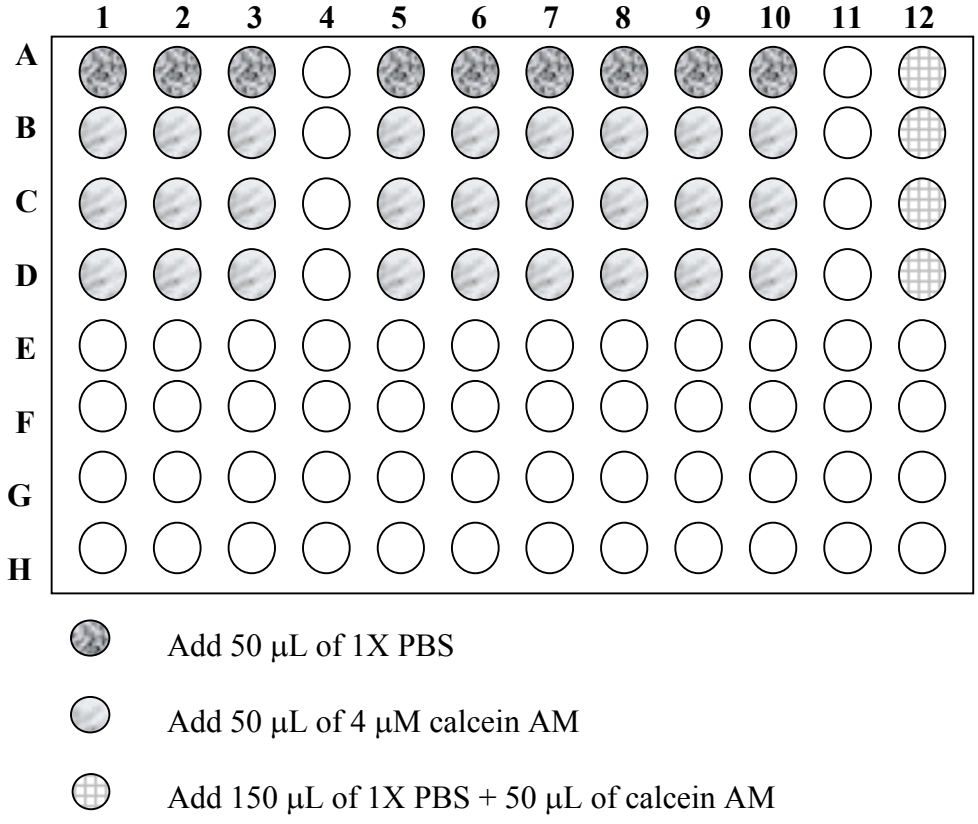
(c) Add cells to upper wells in the MultiScreen-MIC filter plate (placed in the single well tray). Ensure that cell suspension is evenly distributed across membrane. Add chemoattractants to lower wells in the MultiScreen-MIC receiver plate. Avoid generating bubbles while adding solutions to upper and lower wells.
(d) Columns 1-3 will test for unstimulated migration (basal migration) and columns 5-10 will test for stimulated migration (chemotaxis) to factors in serum containing medium.
(e) GENTLY assemble together the MultiScreen-MIC filter plate and receiver plate. DO NOT SHAKE or TILT THE PLATES, as this will disturb the concentration gradient.

(f) Incubate this MultiScreen-MIC plate assembly for 4 h at 37° C. Do not shake the plates during incubation.

Fluorescence labeling procedure for suspension cells after chemotaxis assay.

(a) During incubation, pre-warm 1XPBS, pH 7.4 to 37° C and equilibrate the Calcein AM to room temperature.
(b) Prepare a 4 μM solution of calcein AM from stock using pre-warmed 1X PBS, pH 7.4.
(c) After 4 h of incubation remove the chemotaxis assay plates, without shaking or tilting, from the incubator.
(d) Gently remove MultiScreen-MIC filter plate and discard.
(e) Add reagents to the MultiScreen-MIC receiver plate as per the example template in figure 3.

Figure 3. Example template for addition of detection reagents for chemotaxis assay with suspension cells.



(f) Wells A1–A3 will test background interference from cells and serum free medium. Wells B1-B3, C1-C3 and D1-D3 will test for basal migration. Wells A5 –A10 will test background interference from cells and serum containing medium. Wells B5-B10, C5-C10 and D5-D10 will test for stimulated migration (chemotaxis) to factors present in the serum. Column 12 will test for backgrounds from 1X PBS and calcein AM.
(g) Incubate plates for 1 h at 37° C.

(h) Read the MultiScreen-MIC receiver plates in a Wallac/Perkin Elmer Victor™ 2 Fluorescent Plate Reader at 485/535 nm in the top read mode. The receiver plate will fit in without any adapters.

Calculations for chemotaxis assay with suspension cells.

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

Table 1. Example of data output from reading a MultiScreen-MIC receiver plate after a chemotaxis assay with suspension cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100	100		300	300	300	300	300	300		25
B	2000	2000	2000		10000	10000	10000	10000	10000	10000		25
C	2000	2000	2000		10000	10000	10000	10000	10000	10000		25
D	2000	2000	2000		10000	10000	10000	10000	10000	10000		25

Average of wells A1-A3=100
Average of wells B1-B3, C1-C3, D1-D3=2000
Subtract 100 from 2000=1900
Average of wells A12-D12=25
Subtract 25 from 1900=1875. This is background chemotaxis in response to BSA.
Average of wells A5-A10=300
Average of wells B5-B10, C5-C10, D5-D10=10000
Subtract 300 from 10000=9700.
Subtract 25 from 9700=8675. This is stimulated chemotaxis in response to serum.
Calculate number of cells that values 1875 and 9700 correspond to using the equation: y=mx which is the slope of the cell standard curve generated. See Appendix, (B) for details on how to generate a standard curve for your cells and for the values used in this example.

Example: $1875 = mx \rightarrow 1875 = 1.375x \rightarrow x = 1363 \rightarrow$ number of cells migrated in response to BSA.

Use this value to calculate percent basal chemotaxis in this equation: $\frac{1363 \times 100}{50{,}000} = 2.7\%$

Example: $8675 = mx \rightarrow 8675 = 1.375x \rightarrow x = 6309 \rightarrow$ number of cells migrated in response to serum.

Use this value to calculate percent stimulated chemotaxis in this equation: $\frac{6309 \times 100}{50{,}000} = 12.6\%$

(where 50,000 is the number of cells loaded in upper wells).

Hence fold stimulated chemotaxis over background is 12.6/2.7 = 4.7 fold

(2) Chemotaxis assay with adherent cells

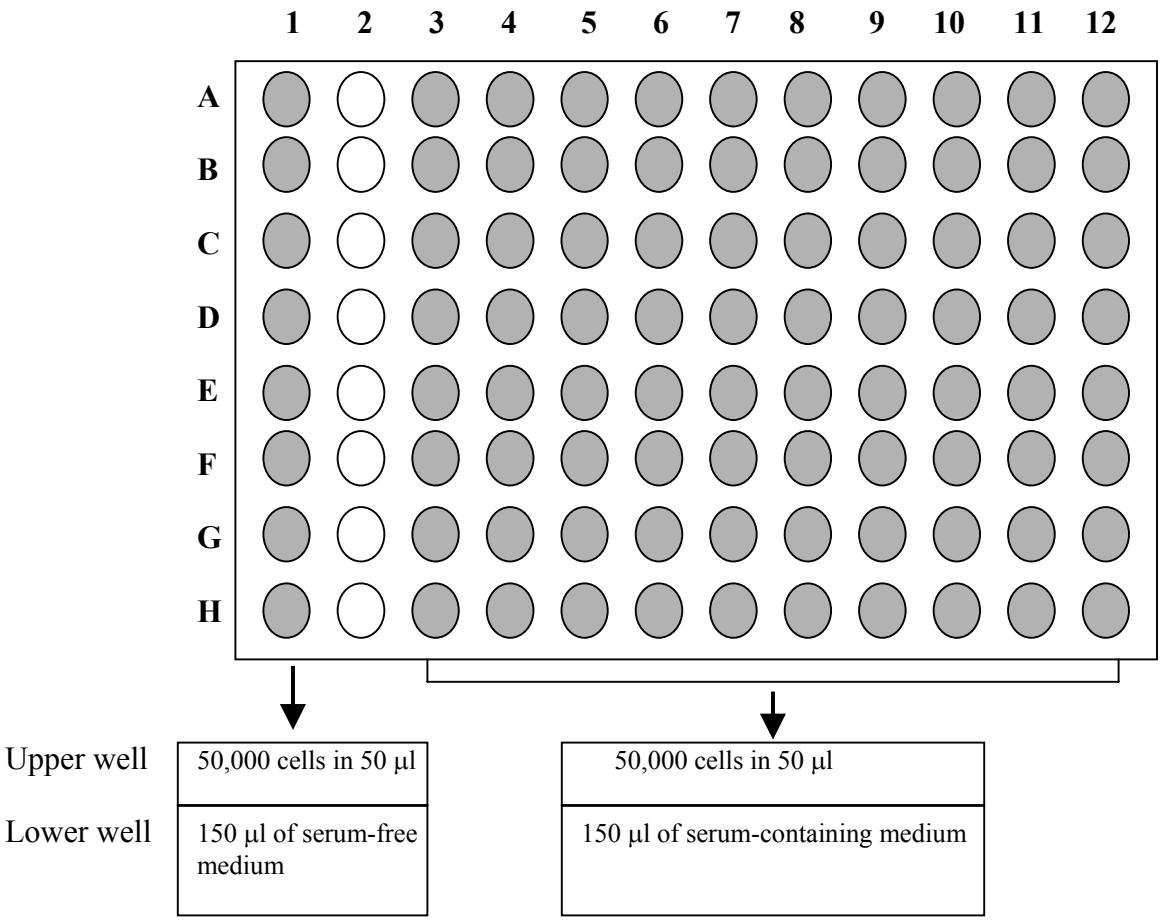
Cell expansion and preparation of adherent cells for chemotaxis assay.

(a) Expand MDA-MB-231 and HT-1080 cells into T-75 flasks for 3-5 days before the 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) On the day of experiment, count the cells using the Trypan Blue cell counting procedure on the Hemacytometer (See Appendix, (A) for details).
(d) 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 chemotaxis experiments.

Setting up chemotaxis assay on MultiScreen-MIC plates with adherent cells.

(a) Separate out MultiScreen-MIC filter plate 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 chemotaxis experiment (Cat. MAMIC 5S 10). 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 chemotaxis assay with adherent cells. Shaded wells used in the template illustrated.

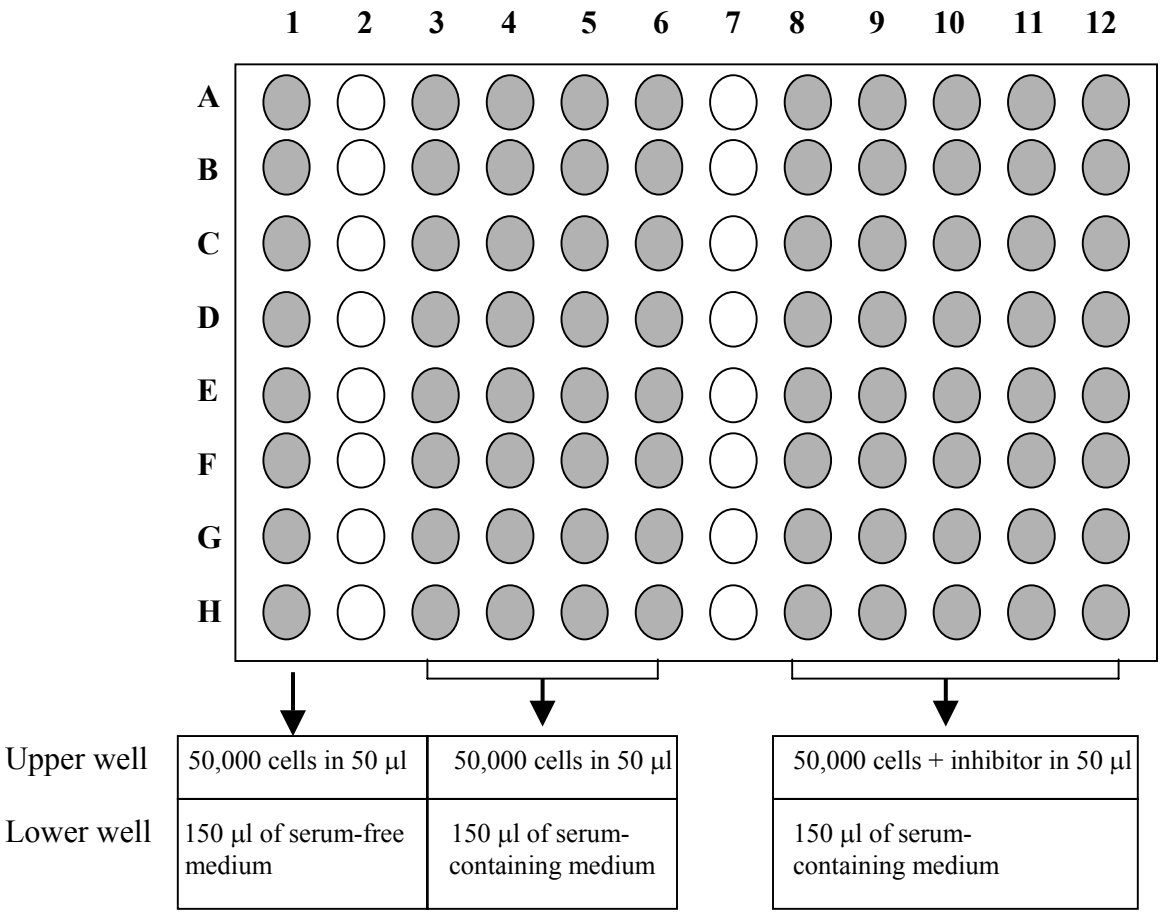


(c) Add cells to upper wells in the MultiScreen-MIC filter plate (placed in the single well tray). Ensure that cell suspension is evenly distributed across membrane. Add chemoattractants to lower wells in the MultiScreen-MIC receiver plate. Avoid generating bubbles while adding solutions to upper and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

(d) Column 1 will test for unstimulated migration (basal migration) and columns 3-12 will test stimulated migration (chemotaxis) to factors in serum containing medium.

(e) For chemotaxis inhibition experiments, add inhibitors at desired concentration to cells prior to loading in upper wells. Example template and chemoattractants is outlined in figure 5.

Figure 5. Example template for chemotaxis inhibition assay with adherent cells. Shaded wells used in the template illustrated.



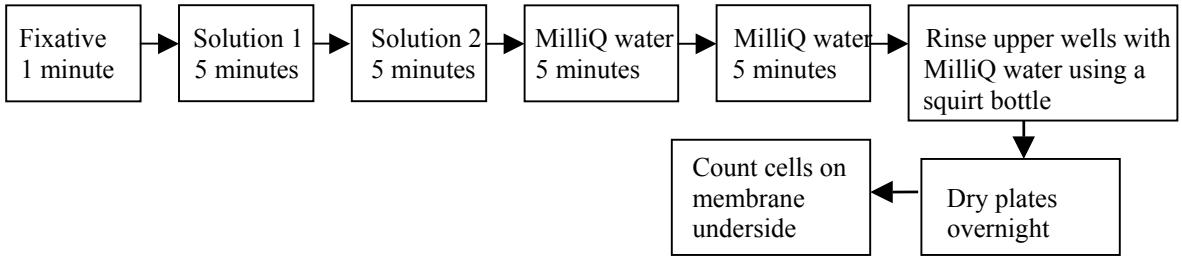
(f) Column 1 will test unstimulated migration (basal migration), columns 3-6 will test stimulated migration (chemotaxis) to factors in serum containing medium and columns 8-12 will test for inhibition of chemotaxis.
(g) GENTLY assemble the top and bottom plates together. DO NOT SHAKE or TILT THE PLATES, as this will disturb the concentration gradient.
(h) Incubate this MutiScreen-MIC plate assembly for 4 h at 37^{o} C. Do not shake plates during incubation.

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

(a) Towards the end of the incubation period, set up staining solutions from the Hema-3 stain kit in MutiScreen-MIC receiver plates (Cat. MAMC S 96 10). Dispense 150 µl of the fixative, solution 1 and solution 2 in series is separate plates. Set up two additional plates with MilliQ® water.
(b) After incubation, remove chemotaxis 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 upper wells with 1X PBS using a squirt bottle.

(e) Stain MultiScreen-MIC filter plates as illustrated in figure 6. Pass the plate through the staining solutions as indicated.

Figure 6. 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 chemotaxis assay or chemotaxis inhibition assay.

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

Table 2. Example of data output from reading a MultiScreen-MIC filter plate after a chemotaxis assay with adherent cells.

	1		3	4	5	6	7	8	9	10	11	12
A	10		800	800	800	800	800	800	800	800	800	800
B	10		800	800	800	800	800	800	800	800	800	800
C	10		800	800	800	800	800	800	800	800	800	800
D	10		800	800	800	800	800	800	800	800	800	800
E	10		800	800	800	800	800	800	800	800	800	800
F	10		800	800	800	800	800	800	800	800	800	800
G	10		800	800	800	800	800	800	800	800	800	800
H	10		800	800	800	800	800	800	800	800	800	800

-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 % of membrane surface area counted.

Table 3. Example of data output after multiplying by a factor of 9.09

	1		3	4	5	6	7	8	9	10	11	12
A	91		7272	7272	7272	7272	7272	7272	7272	7272	7272	7272
B	91		7272	7272	7272	7272	7272	7272	7272	7272	7272	7272
C	91		7272	7272	7272	7272	7272	7272	7272	7272	7272	7272
D	91		7272	7272	7272	7272	7272	7272	7272	7272	7272	7272
E	91		7272	7272	7272	7272	7272	7272	7272	7272	7272	7272
F	91		7272	7272	7272	7272	7272	7272	7272	7272	7272	7272
G	91		7272	7272	7272	7272	7272	7272	7272	7272	7272	7272
H	91		7272	7272	7272	7272	7272	7272	7272	7272	7272	7272

Average of wells A1-H1=91
Use this value to calculate percent basal chemotaxis in this equation: $\frac{91 \text{ X } 100}{50{,}000} = 0.2\%$

Average of wells A3-A12 to H3-H12 =7272
Use this value to calculate percent stimulated chemotaxis in this equation: $\frac{8000 \text{ X } 100}{50{,}000} = 14.5\%$

(where 50,000 is the number of cells loaded in upper wells).
Hence fold stimulated chemotaxis over background is 14.5/0.2 = 73 fold

Table 4. Example of data output from reading a MultiScreen-MIC filter plate after a chemotaxis inhibition assay with adherent cells.

	1		3	4	5	6		8	9	10	11	12
A	10		800	800	800	800		200	200	200	200	200
B	10		800	800	800	800		200	200	200	200	200
C	10		800	800	800	800		200	200	200	200	200
D	10		800	800	800	800		200	200	200	200	200
E	10		800	800	800	800		200	200	200	200	200
F	10		800	800	800	800		200	200	200	200	200
G	10		800	800	800	800		200	200	200	200	200
H	10		800	800	800	800		200	200	200	200	200

Table 5. Example of data output after multiplying by a factor of 9.09

	1		3	4	5	6		8	9	10	11	12
A	91		7272	7272	7272	7272		1818	1818	1818	1818	1818
B	91		7272	7272	7272	7272		1818	1818	1818	1818	1818
C	91		7272	7272	7272	7272		1818	1818	1818	1818	1818
D	91		7272	7272	7272	7272		1818	1818	1818	1818	1818
E	91		7272	7272	7272	7272		1818	1818	1818	1818	1818
F	91		7272	7272	7272	7272		1818	1818	1818	1818	1818
G	91		7272	7272	7272	7272		1818	1818	1818	1818	1818
H	91		7272	7272	7272	7272		1818	1818	1818	1818	1818

Average of wells A1-H1=91
Use this value to calculate percent basal chemotaxis in this equation: $\frac{91 \times 100}{50{,}000} = 0.2\%$

Average of wells A3-A6 to H3-H6 =7272
Use this value to calculate percent stimulated chemotaxis in this equation: $\frac{7272 \times 100}{50{,}000} = 14.5\%$

Average of wells A8-A12 to H8-H12=1818
Use this value to calculate percent stimulated chemotaxis in presence of inhibitors in this equation: $\frac{1818 \times 100}{50{,}000} = 3.6\ \%$
(where 50,000 is the number of cells loaded in upper wells).

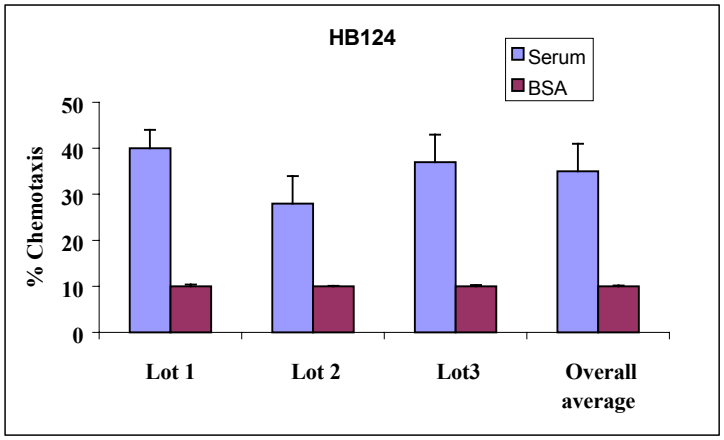
14.3 % represents percent stimulated chemotaxis in absence of inhibitors (14.5 minus 0.2 to subtract out basal chemotaxis)

3.4 % represents percent chemotaxis in presence of inhibitors (3.6 minus 0.2 to subtract out basal chemotaxis)
Normalize stimulated chemotaxis in absence of inhibitors to 100 % that is 14.3 % =100 %
Hence, normalized stimulated chemotaxis in presence of inhibitors is = $\frac{3.4 \times 100}{14.3} = 24\ \%$
Percent chemotaxis inhibition=100-24=76 percent inhibition.

RESULTS

(1) Chemotaxis assay with suspension cells.

The uniformity of chemotaxis values obtained was assessed in experiments performed on the same day using different lots. Figure 7 demonstrates values obtained in experiments performed with HB-124 and K-562 cells in response to 10 % serum or 0.2 % BSA as a chemoattractant. Inter-plate, inter-lot and intra-lot chemotaxis values for HB-124 cells are shown in Table 6.



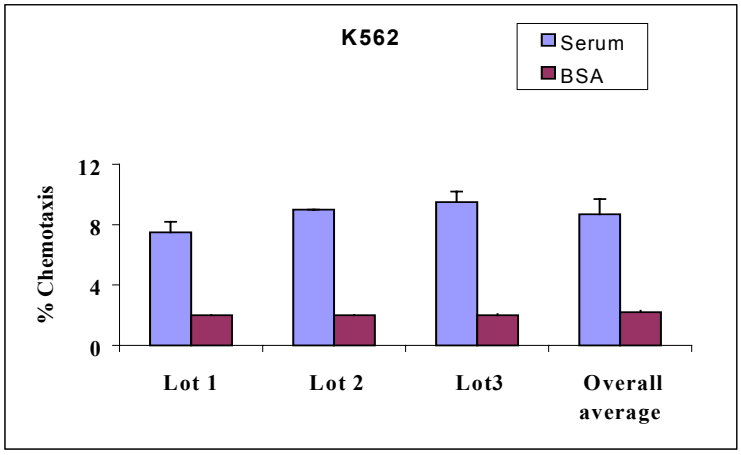


Figure 7. Percent chemotaxis exhibited by HB-124 and K-562 cells in response to 10% serum or 0.2 % BSA containing medium as chemoattractant (per lot: n=5, r=24 for HB-124; per lot: n=2, r=24 for K-562 cells, where n is the number of plates and r is the number of wells tested).

Table 6. Reproducibility of chemotaxis assays with HB-124 cells.

	Inter-plate (3 lots)	Inter-lot (3 lots)	Intra-lot		
			Lot1	Lot 2	Lot 3
Average percent stimulated chemotaxis	35 ± 7	35 ± 6	40 ± 4	28 ± 6	37 ± 6
Range of chemotaxis values within each lot	-	-	36-41	21-35	31-46

5 plates per lot, with 24 replicates per plate, were tested. Three lots were tested.

(2) Chemotaxis assay with adherent cells.

Chemotaxis of MDA-MB-231 cells in response to 0.2 % BSA and 10 % serum is shown in figures 8A and 8B respectively. Data presented in Figure 9 and Table 5 represent assays performed on two experimental days using three different lots. Inter-plate, inter-lot and intra-plate values obtained with MDA-MB-231 cells are shown in Table 5 and that obtained with HT-1080 are shown in Table 6.

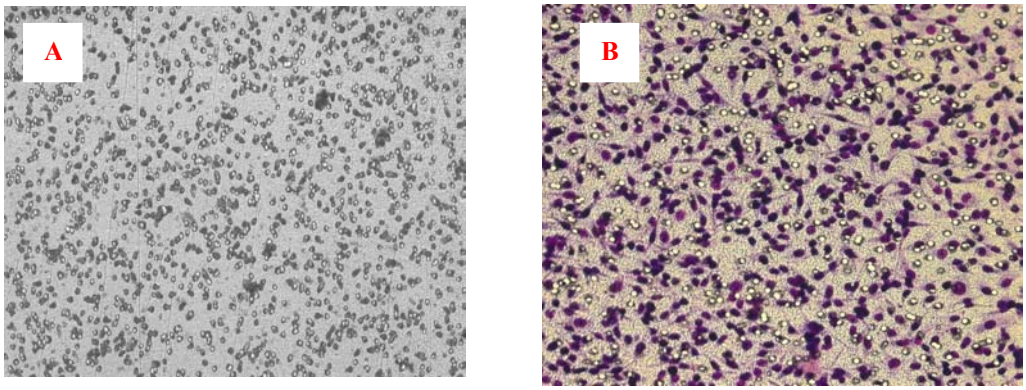
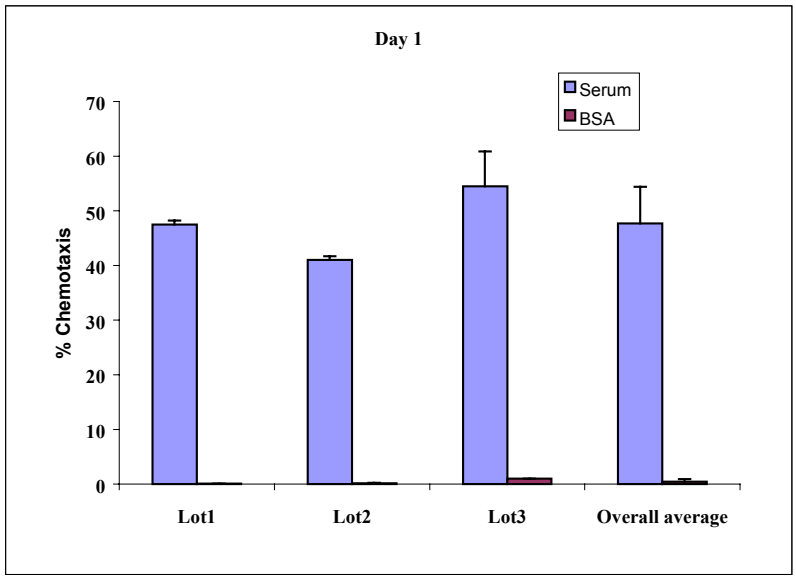


Figure 8. Chemotaxis of highly migratory MDA-MB-231 cells in response to (A) 0.2 % BSA containing medium and (B) 10 % serum-containing medium as a chemoattractant on 5 µm MultiScreen-MIC plate. Cells were stained with Hema-3 stain kit.



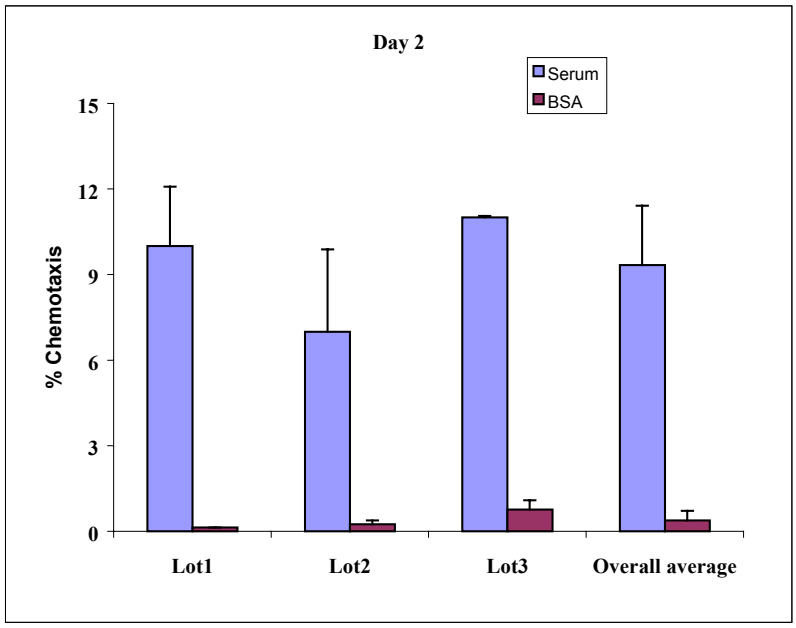


Figure 9. Percent chemotaxis exhibited by MDA-MB-231 cells in response to 10% serum or 0.2 % BSA containing medium as chemoattractant (per lot: n=2, r=80 on Day 1; per lot: n=3, r=40 or 56 on Day 2, where n is the number of plates and r is the number of wells tested).

Table 7. Reproducibility of chemotaxis assays with MDA-MB-231 cells.

	Inter-plate (3 lots)		**Inter-lot (3 lots)**		**Intra-plate**			**Intra-plate**		
					Day 1			Day 2		
	Day 1	Day 2	Day 1	Day 2	Lot1	Lot 2	Lot 3	Lot1	Lot 2	Lot 3
Average percent stimulated chemotaxis	48 ± 7	9 ± 2	48 ± 7	9 ± 2	48	41	55	10	7	11
Plate 1	-	-	-	-	47 ± 18	37 ± 13	53 ± 20	9 ± 3	4 ± 2	12 ± 7
Plate 2	-	-	-	-	48 ± 19	45 ± 14	56 ± 29	9 ± 4	9 ± 7	10 ± 7
Plate 3	-	-	-	-	-	-	-	12 ± 7	8 ± 2	11 ± 7

2 plates per lot, with 80 replicates per plate, were tested on Day 1. Three lots were tested.
3 plates per lot, with 40 or 56 replicates per plate, were tested on Day 2. Three lots were tested.

Table 8. Reproducibility of chemotaxis assays with HT-1080 cells.

	Inter-plate (3 lots)		**Intra-plate (3 lots)**	
	Day 1	**Day 2**	**Day 1**	**Day 2**
Percent stimulated chemotaxis	82 ± 6	20 ± 2	82	20
Plate 1, Lot 1	-	-	76 ± 14	21 ± 9
Plate 2, Lot 2	-	-	82 ± 8	18 ± 10
Plate 3, Lot 3	-	-	87 ± 11	21 ± 9

1 plate per lot, with 80 replicates per plate, was tested on Day 1 and Day 2.
Three lots each were tested on Day 1 and Day 2.

(3) Chemotaxis inhibition assay with adherent cells.

Chemotaxis inhibition experiments were performed to assess the performance of MultiScreen-MIC plates in drug discovery screening type assays and to assess repeatability of inhibition response. Figure 10 illustrates inhibition profiles obtained with Cytochalasin D and Tamoxifen on two days using three different lots. Table 9 shows lot to lot reproducibility of chemotaxis inhibition response of MDA-MB-231 cells to Cytochalasin D and Tamoxifen.

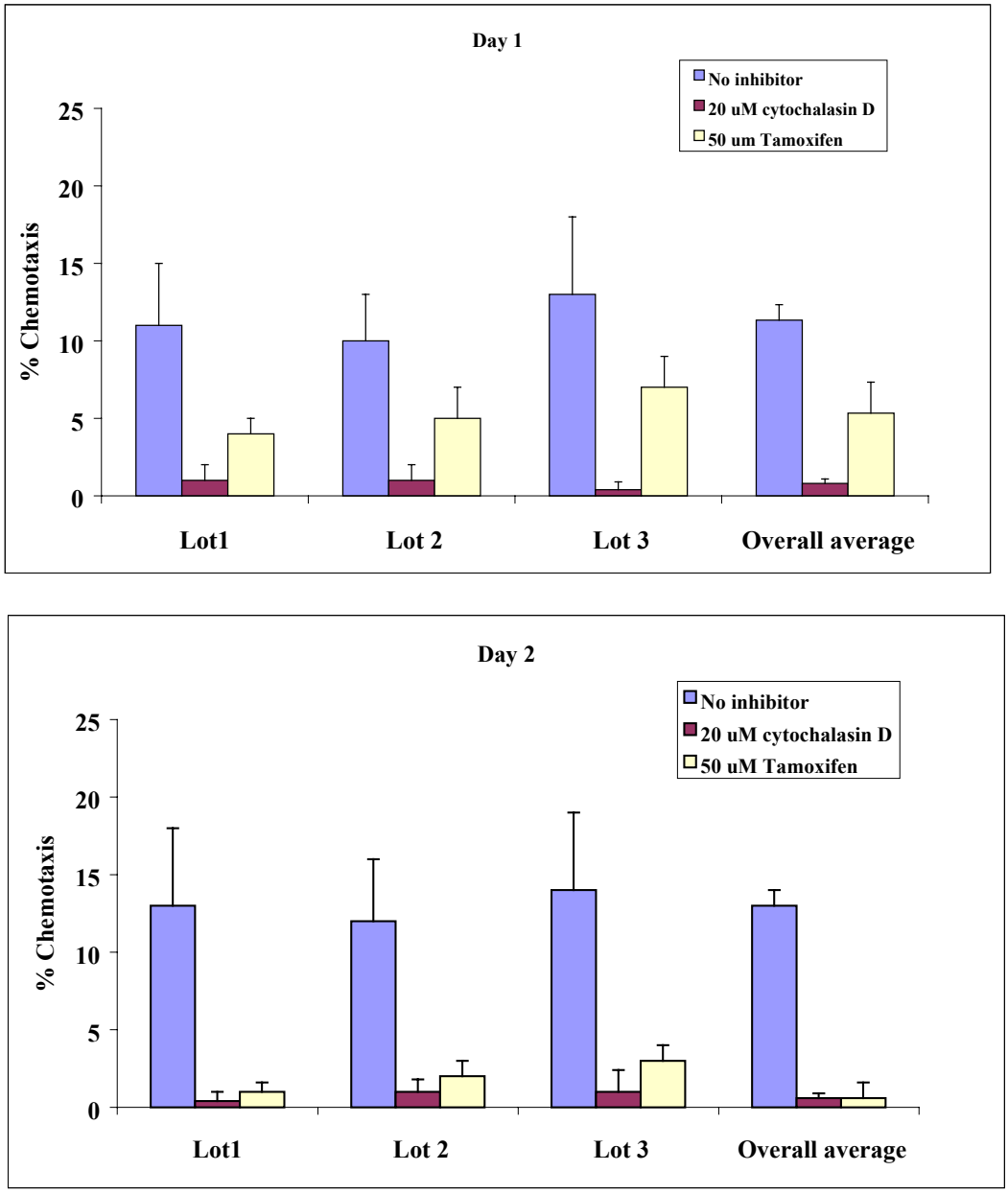


Figure 10. Chemotaxis inhibition profiles of MDA-MB-231 cells on 5 µm MultiScreen-MIC plates in response to 10% serum or 0.2 % BSA serum containing medium as chemoattractant in the presence or absence of inhibitors (per lot: n=1, r=14 per condition tested, on each experimental day, where n is the number of plates and r is the number of wells tested). Average background % chemotaxis values in the absence of inhibitors ranged from 0.3 to 0.4 on day 1 and 0.1 to 0.7 on day 2 indicating robust stimulated chemotaxis response.

Table 9. Reproducibility of MDA-MB-231 cells in chemotaxis 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 3	Lot 1	Lot 2	Lot 3
Percent chemotaxis inhibition	93	95	97	60	55	50	97	95	93	91	79	82

1 plate per lot, with 14 replicates per plate, was tested on Day 1 and Day 2.

DISCUSSION

Percent chemotaxis values in the range of 36-41 for lot 1, 21-35 for lot 2 and 31-46 for lot 3 (Figure 7 and Table 6) were obtained in chemotaxis experiments performed with HB-124 cells in response to 10 percent serum. Intra-lot standard deviations were in the range of 4-6 for the three lots tested. Overall average was 35 with a standard deviation of 6 percent. Data was consistent and reproducible within a range of 15 percent for the three lots tested. Percent chemotaxis values ranged from 8-10 in chemotaxis experiments with K-562 cells in response to 10 percent serum. Standard deviation was 0.1 to 0.7 for the three lots tested. Overall average was 8.7 with a standard deviation of 1 percent. Data was consistent and reproducible within a range of 2 percent for the three lots tested.

Excellent chemotaxis of MDA-MB-231 cells was observed in response to 10 percent serum as shown in Figure 8B. These cells exhibited consistent chemotaxis values in response to 10 percent serum on lots tested on the same experimental day (Figure 9 and Table 7). Chemotaxis values of 41-55 were obtained on Day 1 and 7-11 on Day 2. Inter-plate and inter-lot standard deviations were consistent on lots tested on the same experimental day. Intra-plate standard deviation values are higher compared to suspension cells and were in the range of 13-29 for assays performed on Day 1 and 2-7 for assay performed on Day 2 for all lots tested. This variability can be attributed to the additional processing steps involved in performing the assay with adherent cells and with the detection method used. The influence of assay variability on different experimental days is also evident as shown by different percent chemotaxis values obtained in assays performed on two different days. This assay variability is also seen with HT-1080 cells as shown in Table 8. Higher values were obtained on Day 1 compared to Day 2. These variables need to be taken into consideration when interpreting assay results across different days.

Consistent and reproducible inhibition results were obtained on lots tested on the same day. Percent inhibition obtained with Cytochalasin D (Table 9) was in the range of 93-97 on Day1 and Day 2. Cell variability in inhibition response to Tamoxifen on Day 1 (50-60 percent inhibition) versus Day 2 (79-91 percent inhibition) was observed. Percent inhibition obtained with Tamoxifen was greater on Day 2 when compared to Day 1. Such variations need to factored in when comparing responses obtained across different days.

The results discussed here thus demonstrate the utility of these plates for chemotaxis and chemotaxis inhibition assays with suspension cells and adherent cells.

GENERAL PROTOCOL CONSIDERATIONS

Chemotaxis 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 a negative chemoattractant and a negative cell line (Latter is optional. It is good practice for e.g. to run a non-migratory 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.

REFERENCES

(1) Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. 1987 Jun 15; 47(12):3239-45.

(2) Kamath L, Meydani A, Foss F, Kuliopulos A. Signaling from protease-activated receptor-1 inhibits migration and invasion of breast cancer cells. Cancer Res. 2001 Aug 1; 61(15): 5933-40.

(3) http://www.probes.com/handbook/sections/1506.html
Molecular Probes Technical note titled "Section 15.6 - Probes for Cell Adhesion, Chemotaxis, Multidrug Resistance and Glutathione"

OTHER MultiScreen-MIC PRODUCT LITERATURE

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

Applications Note, Literature piece number: AN1675EN00
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. Live cells will be clear and dead cells will be blue.
-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.

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

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.

(B) Protocol for generating a standard cell curve using Calcein AM

-Centrifuge cells for 8 minutes at 120 g.
-Resuspend cells in 1X PBS, pH 7.4
-Make a dilution range of cells as indicated in Table 8 using 1X PBS, pH 7.4

Table 8. Cell standard curve dilution

Cell dilution range	**Cell stock needed**
100 cells/100 µl	10^3/mL
500 cells/100 µl	5 X 10^3/mL
1000 cells/100 µl	10^4/mL
2500 cells/100 µl	2.5 X 10^4/mL
5000 cells/100 µl	5 X 10^4/mL
7500 cells/100 µl	7.5 X 10^4/mL
10000 cells/100 µl	10^5/mL
20000 cells/100 µl	2 X 10^5/mL
30000 cells/100 µl	3 X 10^5/mL
40000 cells/100 µl	4 X 10^5/mL

-Add 100 µl of each cell dilutions in ascending order to wells A1-H1 to A10-H10 to a MultiScreen-MIC receiver plate (MAMC S96 10) as indicated in the template.

Cell number per 100 µl ⟶

	1	2	3	4	5	6	7	8	9	10
A	100	500	1000	2500	5000	7500	10000	20000	30000	40000
B	100	500	1000	2500	5000	7500	10000	20000	30000	40000
C	100	500	1000	2500	5000	7500	10000	20000	30000	40000
D	100	500	1000	2500	5000	7500	10000	20000	30000	40000
E	100	500	1000	2500	5000	7500	10000	20000	30000	40000
F	100	500	1000	2500	5000	7500	10000	20000	30000	40000
G	100	500	1000	2500	5000	7500	10000	20000	30000	40000
H	100	500	1000	2500	5000	7500	10000	20000	30000	40000

To these wells add reagents as indicated:
-Add 100 µl of 1X PBS to wells from A1-C1 to A10-C10.
-Add 50 µl of 1X PBS to wells fromD1-H1 to D10-H10.
-Add 150 µl of 1X PBS to wells from A12-H12.
-Reagent additions are illustrated in template.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS		150 µl PBS
B	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS		150 µl PBS
C	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS	100 µl PBS		150 µl PBS
D	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS		150 µl PBS
E	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS		150 µl PBS
F	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS		150 µl PBS
G	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS		150 µl PBS
H	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS	50 µl PBS		150 µl PBS

-Add 50 µl of a 4 µM working stock of calcein AM made in 1X PBS to wells D1-H1 to D10-H10 and A12-H12.
-Incubate MultiScreen-MIC receiver plate with a lid for 1 h at 37^{o} C.
-Read plates at A 485/535 nm in the top read mode in a Wallac Victor 2 fluorescent plate reader.
-Calculate results using example data from data output table illustrated.

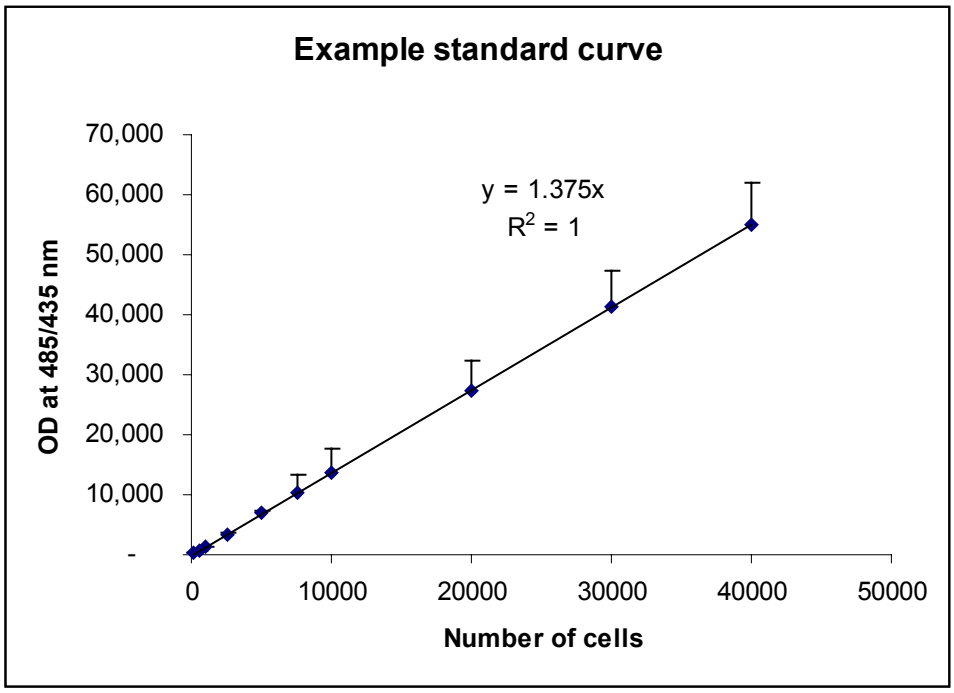
	1	2	3	4	5	6	7	8	9	10	11	12
A	100	150	200	250	300	350	400	450	500	550		50
B	100	150	200	250	300	350	400	450	500	550		50
C	100	150	200	250	300	350	400	450	500	550		50
D	425	888	1625	3738	7225	10713	14200	28000	41800	55600		50
E	425	888	1625	3738	7225	10713	14200	28000	41800	55600		50
F	425	888	1625	3738	7225	10713	14200	28000	41800	55600		50
G	425	888	1625	3738	7225	10713	14200	28000	41800	55600		50
H	425	888	1625	3738	7225	10713	14200	28000	41800	55600		50

-Subtract the average value of wells A-C from average values of wells D-H for each of the columns. This will subtract interference from cells.
-Deduct average value of column 12 from these values. This will subtract interference from calcein AM and PBS.
-Example data output after subtractions is illustrated.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D	275	688	1375	3438	6875	10313	13750	27500	41250	55000		
E	275	688	1375	3438	6875	10313	13750	27500	41250	55000		
F	275	688	1375	3438	6875	10313	13750	27500	41250	55000		
G	275	688	1375	3438	6875	10313	13750	27500	41250	55000		
H	275	688	1375	3438	6875	10313	13750	27500	41250	55000		

-Plot cell dilution range (X axis) versus average calcein AM values (Y axis) to generate a standard curve.
-Example data used to plot standard curve is illustrated.

Cell number	OD at 485/535 nm	Standard deviation
100	275	25
500	688	50
1000	1375	100
2500	3438	250
5000	6875	500
7500	10313	3000
10000	13750	4000
20000	27500	5000
30000	41250	6000
40000	55000	7000



-Use the slope of this standard curve to calculate cell numbers that migrated through in test plate.
-Slope equation is y=mx. Example: In the standard curve illustrated y=1.375x

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