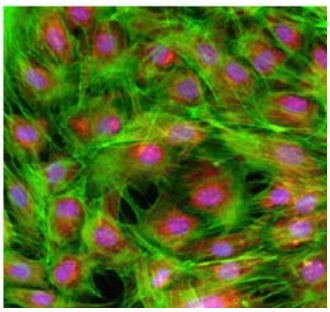
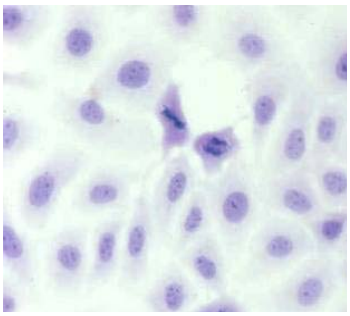
Cell Migration, Chemotaxis and Invasion Assay Protocol





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Introduction

Cell migration, the movement of cells from one area to another generally in response to a chemical signal, is central to achieving functions such as wound repair, cell differentiation, embryonic development and the metastasis of tumors. Cell invasion is similar to cell migration; however, it requires a cell to migrate through an extracellular matrix (ECM) or basement membrane extract (BME barrier by first enzymatically degrading that barrier and to then become established in a new location. Cell invasion is exhibited by both normal cells in responses such as inflammation and by tumor cells in the process of metastasis, therefore understanding the underlying mechanisms of this process are important for a wide array of biological systems.

The advent of disposable permeable supports, such as Transwell® inserts from Corning Life Sciences, provides a relatively simple in vitro approach to performing chemotaxis and cell invasion assays. Common barriers employed for invasion assays include collagen, fibronectin and laminin coatings as well as more complex extracellular or basement membrane extracts. More elaborate invasion assays establish a monolayer of endothelial cells on the permeable support in place of, or in addition to, the protein coatings or BME listed above. Similarly, cells that secrete a paracrine growth factor can be cultured in the receiver wells of the permeable support system to act as the source of

chemoattractant in either simple chemotaxis assays or for more elaborate invasion assays.

This protocol outlines the steps for conducting a cell invasion assay through a BME barrier with special notes for conducting a chemotaxis assay (similar to an invasion assay, however no BME or ECM is present). It is a generalized protocol and should be adapted to suit your needs. This protocol utilizes Corning's 96 well HTS Transwell® permeable supports, however, tables are provided with the proper volumes and amounts of pertinent materials and reagents to scale the assay for use with large permeable supports.

Materials

Cell lines:

- Noninvasive MCF-7 cells (Human breast adenocarcinoma, ATCC No. HTB-22)
- Invasive HT1080 cells (Human fibrosarcoma ATCC No. CCL-121)

Assay Plates:

- 96 well HTS Transwell Permeable Supports with 8 µm pores (Corning Cat. No. 3374)
- 96 well black receiver plate (Corning Cat. No. 3583)
- 96 well solid black microplates (Corning Cat. No. 3916)
- 6 well Transwell permeable supports with 8 µm pores (Corning Cat. No. 3428)
- 12 well Transwell Permeable Supports with 12 µm pores (Corning Cat. No. 3403)
- 24 well Transwell Permeable Supports with 8 µm pores (Corning Cat. No. 3422)
- 6 and 24 well Ultra-low Attachment plates (Corning Cat. No. 3471 and 3473)

Reagents:

- 5x Basement Membrane Extract (BME) coating solution (Trevigen Cat. No. 3455-096-02)
- 10x Coating Buffer (Trevigen Cat. No. 3455-096-03)
- 10x Cell Dissociation Solution for preparing the assay dissociating solution (Trevigen Cat. No. 3455-096-05). This solution can not be used to harvest cells.
- HyQtase Dissociation Solution for harvesting cells (Hyclone Cat. No. JQH2447)
- Calcein AM (Molecular Probes Cat. No. C3100MP) dissolved to 1.67µg/µL in DMSO
- IMDM medium with 10% FBS (Invitrogen Cat. No. 12440046 and 14037036, respectively)
- Serum-free medium (SFM), IMDM medium without serum containing 1x ITS (Invitrogen Cat No. 41400045)
- Wash Buffer – Dulbecco's Phosphate Buffered Saline (DPBS) with calcium and magnesium
- Sterile deionized water

Instruments:

- 37ºC CO_2 incubator
- Laminar flow hood
- Fluorescent plate reader with a 485nm excitation and 520nm emission filter package

Procedure

NOTE: Read entire protocol before beginning assay.

Grow enough cells in advance to accommodate the different cell concentrations required to set up assay (Table 2). A separate flask should be set up at a lower concentration in order to run standard curve on day of assay (day 3 of protocol).

Protocol Overview:

- **Day 1** – Starve cells and coat Transwell inserts with Basement Membrane Extract (BME)
- **Day 2** – Plate cells in Transwell inserts and stimulate with FBS attractant; optional setup of standard curve.
- **Day 3** – Detect cells that pass through membrane and standard curve.

Day 1

1. Cell Maintenance

NOTE: Using the cell requirements from Table 2, prepare enough cell cultures one day prior in order to accommodate the number of cells required to set up the assay.

a. Cultures should be below 80% confluence, this is important so that cells are properly starved without becoming confluent during the 24 hr starvation period.
b. Remove serum containing medium from cultures.
c. Thoroughly but gently rinse cultures with PBS to remove all serum.
d. Replace culture medium with serum-free IMDM medium (SFM). Return cultures to incubator for 24 hours.

2. Basement Membrane Extract (BME) Coating

The concentration of BME necessary for the assay is dependent on the cell line. We recommend testing various BME concentrations (0.1x to 1x, for example) to find the optimal concentration for a particular cell line. The optimal concentration will provide the biggest difference between the invasive and noninvasive cell lines in response to a chemoattractant

NOTE: In order to properly assess cell invasion, it is necessary to set up a few wells without an ECM barrier to determine the percent of cells that would exhibit chemotaxis. This will become important for analyzing the data in Step 6). See Table 3 for typical layout for a 96 well plate inserts.

a. Under sterile conditions prepare 1x Coating Buffer working solution by diluting 10x Coating Buffer stock with sterile filtered deionized water.
b. Thaw out 5x BME stock solutions by gently swirling vial in a 37°C water bath. Once thawed dilute immediately or keep vial on ice until ready to use; BME becomes viscous at warmer temperatures.
c. Dilute the 5x BME stock to desired working concentration using 1x Coating Buffer.
d. Coat Transwell inserts with BME solution (See Note at left, and Table 1 on page 4)
e. Incubate plate overnight in 5% CO_2 incubator at 37°C.

Table 1. Recommended Basement Matrix Extract Coating Volumes

Assay plate format	Number of inserts	Well bottom area (cm^2)	BME coating/well (mL)	Total BME coating/plate (mL)
HTS TW-96	96	0.143	0.050	**5**
24 well	12*	0.3	0.100	**2**
12 well	12	1.1	0.3	**4**
6 well	6	4.5	1	**6.5**
*Corning® Transwell® 6.5mm diameter inserts are packed 12 per 24 well plate.				

Day 2

3. Plating cells for the invasion assay and standard curve

This protocol uses HTS-96 Transwell plates for running a cell invasion assay. The protocol can be adapted for assays using larger permeable supports or fewer wells. As part of the procedure, inserts are setup without a BME barrier (ot differentiate cell invasion from chemotaxis, see Introduction) as a control to calculate total invasion.

NOTE: The migration response using trypsin with these cell lines in this protocol was lower and more variable than when using the HyQtase solution. Consider trying different dissociating agents and methods if you want to optimize your protocol.

a. Harvest cells using HyQtase solution or other suitable cell dissociation solution. (See Note)

b. Quench dissociation solution with serum-free media or serum-free trypsin inhibitor.

c. Spin cells down to remove cell dissociation solution and resuspend in serum-free medium.

d. Determine cell concentration and dilute cell suspension to necessary seeding concentration with serum free medium (Table 2, below).

e. Aspirate excess BME from inserts and plate cells.

f. Leave at least 1 well without cells as a blank to subtract for background (Table 3).

g. Set up some receiver wells with serum-free medium (no chemoattractant) with the remaining wells receiving medium with serum (plus chemoattractant) as shown in Table 3 on page 5.

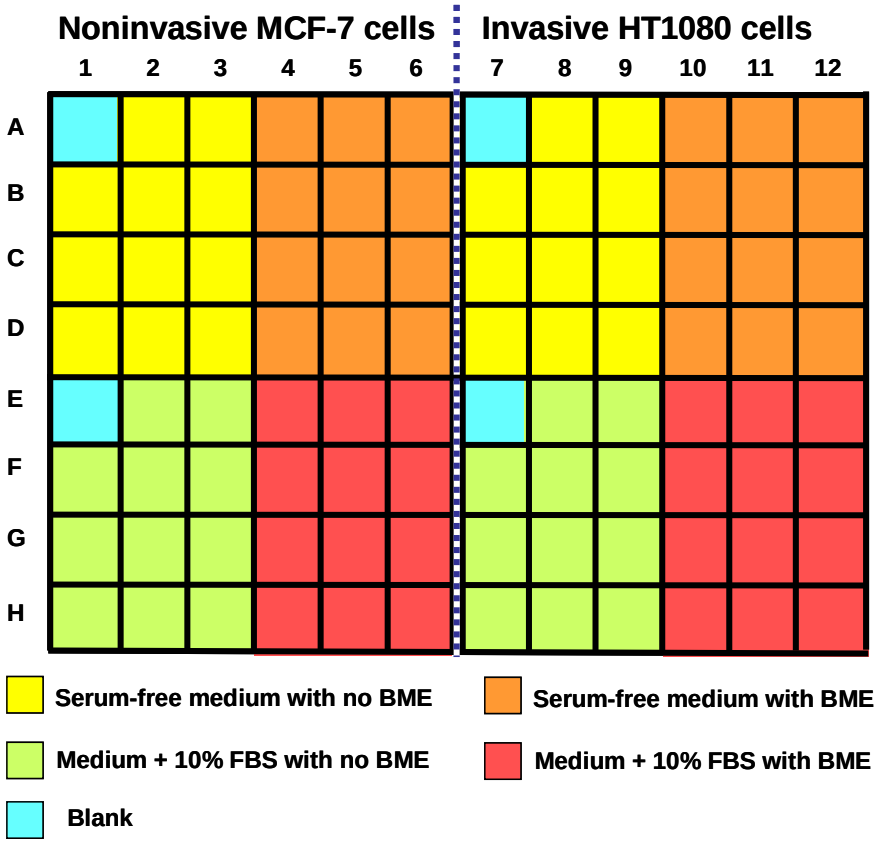
h. Incubate cultures for 12 to 24 hours depending on the invasiveness of the cell lines used.

Table 2. Recommended Cell Seeding Concentrations and Volumes

Assay plate format	Cells/well ($x10^5$)*	Seeding volume/ insert (mL)	Reservoir volume (mL/well)
HTS TW-96	0.5	0.05	0.150
24 well	1.0 to 2.0	0.100	0.65
12 well	1.0 to 2.0	0.385	1.0
6 well	3.0 to 4.0	1.5	2.0

***** These cell seeding densities have been optimized for this protocol utilizing MCF-7 and HT-1080 cells. For best results we recommend optimizing cell seeding densities using your cell lines and conditions.

Table 3. An example of a 96 well HTS Transwell plate layout for testing two cell lines



Day 3

4. Standard Curve Preparation

NOTE: The standard curve can be run on day 2 using cells harvested for seeding plates. It can also be run on the day of assay using a separate flask set up during the preparation of the cells for the permeable supports; these cells need to be starved for 24 hours prior to running assay (from day 2 of study).

a. A separate standard curve is required for each cell line and each assay (see Note at left).

b. Harvest cells and collect in a tube.

c. Prepare 1x Cell Dissociation Solution (1x CDS) by diluting 10x Cell Dissociation Solution with sterile filtered deionized water.

d. Thaw out Calcein AM vial (50 µg) and add 30 µL of tissue culture grade DMSO to it to prepare the working solution.

e. Pellet harvested cells and resuspend in 1x CDS.

f. Create a serial dilution of cell suspension (2 mL/dilution) starting with the highest number of cells plated per well and ending with no cells -1x CDS only. See Table 4 (page 6) for recommended cell dilutions for preparing the standard curve.

g. Add 50 µL from each dilution in triplicate to a well from a 96 well solid black microplate (Corning Cat. No. 3583 if testing a 96 well HTS plate or Corning Cat. No. 3916 for all others).

Table 4. Recommended cells/well for generating a standard curve in a 96 well plate

Cells/well Desired	Cells/mL Required (assuming 50 µL/well)
50,000	1,000,000
25,000	500,000
10,000	200,000
5,000	100,000
2,500	50,000
1,000	20,000
500	10,000
0	0

h. Dilute Calcein AM solution with 1x CDS at a ratio of 2.4 µL of Calcein AM solution to 1 mL of 1x CDS.

i. Add 50 µL of the Calcein AM/CDS mixture to each well of the standard curve.

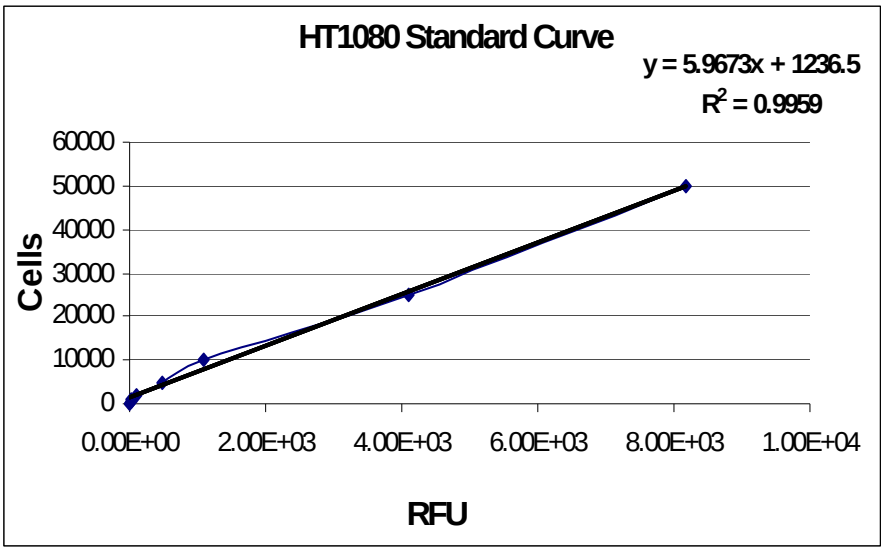
j. Incubate microplate for 1 hour in the dark at room temperature then read using a fluorescent top reader at 485nm excitation and 520nm emission.

k. Average the relative fluorescence units (RFU) values for each concentration then subtract background (no cells).

l. Plot a standard curve of cell concentration versus RFU .

m. Insert linear trend (set intercept to zero) and solve for cell concentration using **y = mx + b**, where y = cell number and x = RFU reading for test sample. See Graph 1 (below) for an example of a standard curve.

Graph 1. Example of the standard curve for the HT1080 cells.



5. Assay

a. Prepare 1x Cell Dissociation Solution (CDS) by diluting 10x Cell Dissociation Solution with sterile filter deionized water.

b. Thaw one Calcein AM vial (50 µg) and add 30 µL of tissue culture grade DMSO to prepare a working solution.

c. Combine 1x CDS with Calcein AM solution at a ratio of 1.2 µL of Calcein AM solution for every 1 mL of 1x CDS. Store solution in dark until ready to use. See Table 5 (below) for recommended volumes.

Table 5. Recommended Calcein AM/1x CDS Solution Volumes

Assay plate format	Recommended receiver plate	Calcein AM/1x CDS solution		Multiplication factor
		mL/well	mL/plate	
HTS TW-96	96 well black receiver plate	0.1	10	1
24 well	24 well plate	0.35	4.5	3.5
12 well	12 well plate	0.5	6.5	5
6 well	6 well plate	0.8	5	8

NOTE: Cells that pass through membrane attach to the bottom of membrane or fall to the bottom of the well and attach. In our experience the loss of any cells due to aspiration in step d is negligible.

d. Aspirate medium from assay/receiver plate and Transwell inserts.

e. Wash apical/inserts once and receiver wells twice with Wash Buffer. (Table 6)

Table 6. Wash Buffer Volumes

Assay plate format	Inserts (mL/insert)	Receiver Plate (mL/well)
HTS TW-96	0.1	0.2
24 well	0.2	0.4
12 well	0.5	1.0
6 well	1.0	2.0

f. Add Calcein AM/1x CDS solution to each well of receiver plate (Table 5).

g. Place inserts into receiver plate filled with Calcein AM/1x CDS solution. Make sure there is no trapped air between bottom of insert and solution in the bottom well. Incubate plate at 37° in a 5% CO_2 incubator for 30 minutes.

h. After 30 minutes incubation, gently tap sides of microplate and then place back in incubator for 30 additional minutes.

i. Remove insert tray from 96 well receiver plate and gently shake plate to mix cell solution. Then place receiver plate directly into plate reader for reading (fluorescent top reader with filters set to 485 nm for excitation and 520 nm for emission).

NOTE: For all other receiver plate formats remove inserts from receiver wells. Mix cell solution well then transfer 100 µL/well into a 96 well solid black microplate (Corning Cat. No. 3916) in triplicate. Then read microplate.

6. Data Analysis – for simplicity we have omitted the calculations for MCF-7 cells.

a. Prepare data from plate reader. See Table 7a for data.

Table 7a. Example of raw RFU data from the plate reader for HT1080 cells using 96 well HTS Transwell Plate (MCF-7 data not shown).

No BME, No Serum		No BME + 10% Serum		With BME, No Serum			With BME + 10% Serum		
196		218		205	195		216	208	
454	348	8518	8569	269	374	300	6391	5284	5051
373	341	8451	7347	289	422	273	8401	8128	6830
394		6776		255	292	258	6522	4459	5635
				418	371	586	7159	6279	6863
				429	438	293	6876	7577	6199
				297			4941		

NOTE: If multiple readings are derived from an individual well (24, 12 and 6 well formats) then those readings should be averaged first then have background subtracted.

b. Subtract background from assay readings. The background is uncoated well with no cells plated (Table 7b; see Note on left).

Table 7b. Example of data From Table 7b after the background (blank) is subtracted.

No BME, No Serum		No BME + 10% Serum		With BME, No Serum			With BME + 10% Serum		
0		0		0	0	200*	0	0	212*
258	152	8300	8351	69	174	100	6197	5027	4839
177	145	8233	7128	89	222	73	8189	7916	6618
198		6558		55	92	58	6310	4247	5426
				218	171	386	6947	6067	6651
				229	238	93	6664	7364	5987
				97			4747		

* = average of multiple blank wells from table 7a, use for background subtract of test wells.

c. Convert RFU values to cell numbers using equation from standard curve (Step 4m). See Table 7c below.

Table 7c. Cell numbers of each well using standard curve calculations.

No BME, No Serum		No BME + 10% Serum		With BME, No Serum			With BME + 10% Serum		
Solve for cell number (From Graph 1): y = 5.9673x + 1236.5									
2776	2144	50765	51069	1648	2275	1833	38216	31234	30112
2293	2102	50365	43771	1768	2561	1672	50103	48474	40728
2418		40370		1565	1785	1583	38890	26580	33615
				2537	2257	3540	42691	37440	40925
				2603	2657	1791	41003	45180	36963
				1815			29563		

d. Determine the total number of cells passing through the membrane of each well by taking the cell numbers generated in step 7c and multiplying

it by the multiplication factor from Table 5 on page 7. See Table 7d below.

Table 7d. Percent invasion for each well.

No BME, No Serum		No BME + 10% Serum		With BME, No Serum			With BME + 10% Serum		
Calculate % Invasion = (Cell Concentration/Initial cell seed) x Multiplication factor (Table 5)									
5.6%	4.3%	101.5%	102.1%	3.3%	4.5%	3.7%	76.4%	62.5%	60.2%
4.6%	4.2%	100.7%	87.5%	3.5%	5.1%	3.3%	100.2%	96.9%	81.5%
4.8%				3.1%	3.6%	3.2%	77.8%	53.2%	67.2%
				5.1%	4.5%	7.1%	85.4%	74.9%	81.9%
				5.2%	5.3%	3.6%	82.0%	90.4%	73.9%
				3.6%			59.1%		

e. Determine percent invasion from each well by dividing number of cells (from Table 7c) by the initial number of plated cells (from Table 2). Calculate average % invasion for all wells of each condition. (See Table 7e, on right) and plot results (Graph 2, below).

Table 7e. Calculation of average percent invasion and chemotaxis.

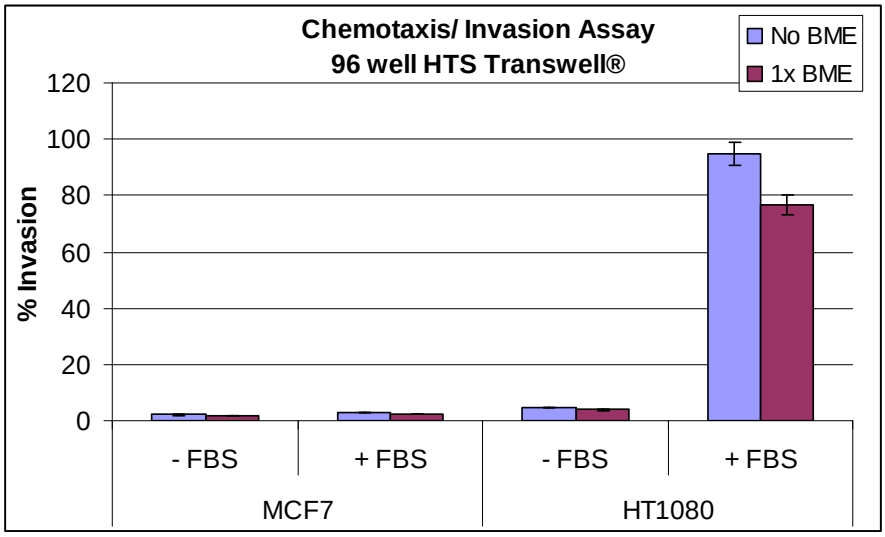
Average % Invasion HT1080

No BME	**1x BME**	
4.7%	4.4%	**- FBS**
94.5%	74.3%	**+ FBS**
STD % Invasion HT1080		
No BME	**1x BME**	
0.5%	1.2%	**- FBS**
9.8%	13.8%	**+ FBS**

7. Data interpretation

a. There should be little or no migration/movement of noninvasive cell lines with or without chemoattractant (serum).

Graph 2. Plot of Data from Table 7e with inclusion of MCF-7 data



b. There should be less then 10% movement of invasive cells without the FBS as a chemoattractant (serum-free medium conditions)

c. The difference in percent invasion should be 20% or more between BME coating (cell invasion) and no coating (cell migration) when using FBS as a chemoattractant.

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CLS-AN-0615/08Rev1