In vitro cell migration and invasion assays using Nunc Polycarbonate Cell Culture Inserts

Introduction

Cells that make up the human body are involved in a process called cellular migration in which individual cells, cell sheets, and clusters move from one location to another, usually in response to extrinsic biochemical signals [1-5]. *In vitro*, cells can migrate across flat surfaces or through microporous membranes. Some cell types can also invade a three-dimensional (3D) matrix via proteolysis of extracellular matrix (ECM) components [6]. The ability to migrate is indispensable for cells to invade, which means nonmigratory cells cannot invade a 3D matrix, whereas noninvasive cells can still migrate on a 2D substrate.

Cell migration and invasion are critical events in many physiological and pathological processes, including morphogenesis, tissue repair, cancer metastasis, and inflammation [7,8]. *In vitro* assays can help researchers study cell migration by allowing quantification of cell migratory capacity under controlled experimental conditions [8]. By analyzing the movement of cells in response to chemical or mechanical signals, researchers can understand the underlying mechanisms for a wide range of biological processes, and can use this knowledge for development of potential therapeutics interventions. The study of cellular migration or invasion is often performed using microporous membrane inserts, which Thermo Fisher Scientific offers with polycarbonate membranes available in multiple pore sizes. These tissue culture inserts are prepacked in sterile Thermo Fisher™ Nunclon™ Delta treated multi-well plates and can be paired with an optional carrier plate that provides height-adjusting grooves within each well for use with the inserts to suit individual needs [9].

The migration and invasion protocols described here outline the steps for conducting both qualitative and quantitative invasion and migration assays. Cell culture inserts with 8 µm pore diameter in a 24-well format are used with breast cancer cell lines MCF7 and MDA-MB-231. Breast cancer is a leading cause of malignancy-related deaths of women worldwide [10]. In patients with breast cancer, invasion and metastasis are primary causes of morbidity and mortality [11,12]. Cell culture inserts can help researchers study the molecular mechanisms involved in migratory and invasive properties of cancer cells, study target proteins involved in the process, and evaluate the effects of different drugs.

The migration and invasion assays described here employ a Boyden chamber–like design, with two chambers separated by a porous membrane. Migration assays are generally performed with cells plated directly on the porous membrane, while invasion assays require coating of the membrane with an ECM gel, which acts as a barrier through which cells invade. In the protocols outlined here, Gibco™ Geltrex™ matrix is used to create an ECM barrier. Target cells in serum-free medium are placed in the top chamber, and medium containing a chemoattractant is added to the bottom chamber. Cells migrate or invade from the top chamber through the uncoated or ECM-coated membrane to the underside of the membrane. Here, cell movement has been determined by two methods: (1) staining cells with crystal violet and visualizing the cells that have moved through the permeable membrane, and (2) staining live cells with Invitrogen™ Calcein AM cell-permeant dye and using a dissociation reagent to remove cells that have migrated/invaded from the membrane to allow for plate reader–based quantification (Figure 1).

The following protocols have been optimized using MCF7 and MDA-MB-231 cell lines. Results may vary depending upon the types of cells, media, seeding density, incubation time, and type of chemoattractant used, and the protocols may require optimization for other cell types.

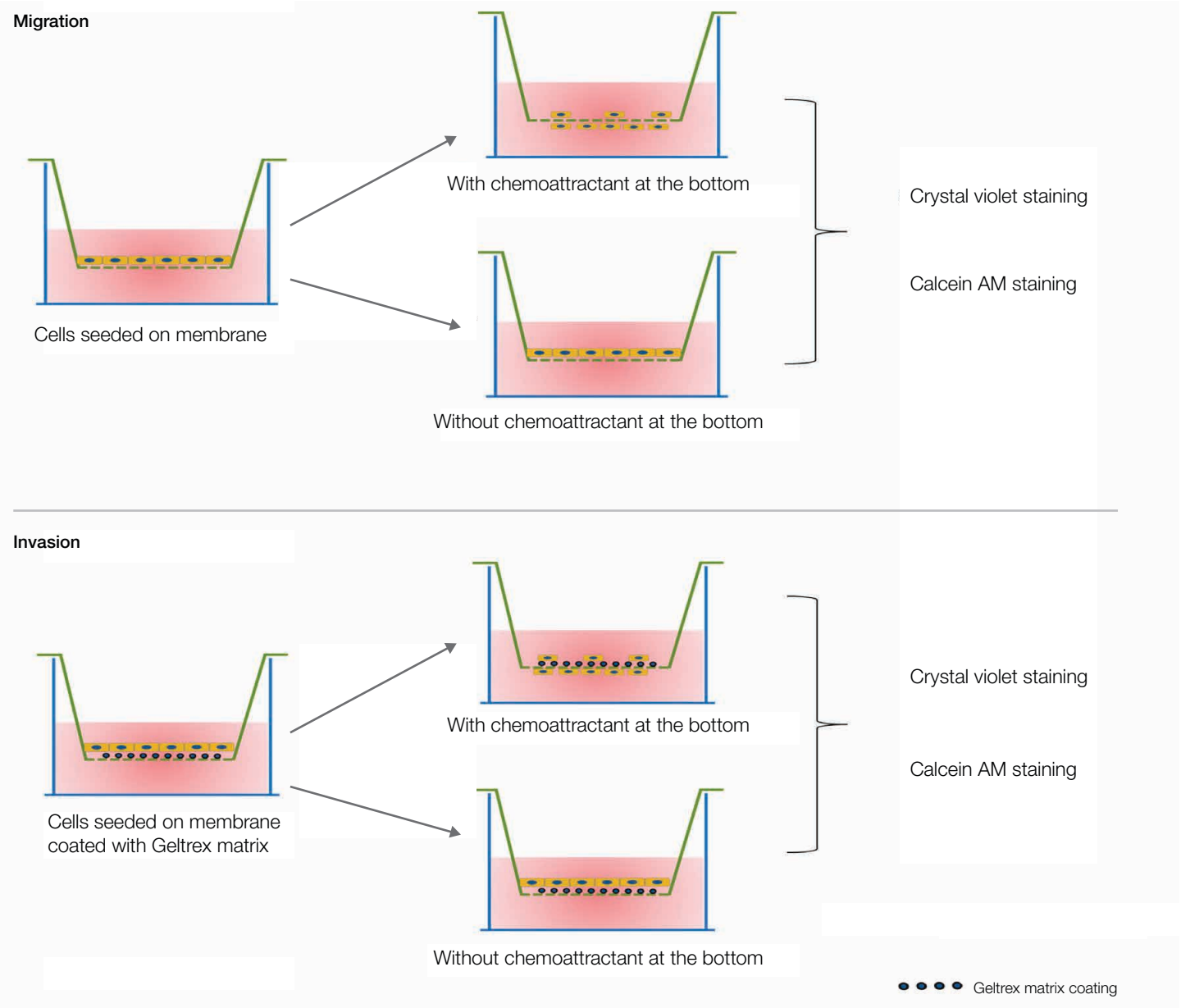

Figure 1. Representative workflow for migration and invasion assays.

Materials

Cell lines and media

- Culture MDA-MB-231, a highly invasive breast adenocarcinoma cell line, in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS)
- Culture MCF7, a noninvasive breast adenocarcinoma cell line, in the same media formulation supplemented with 0.01 mg/mL insulin

Cultures should be grown in a monolayer at 37°C in a 5% CO_2 incubator.

Note: The cell line used should be free from mycoplasma contamination.

Reagents and equipment

Product	Supplier	Cat. No.
Reagents		
Gibco™ TrypLE™ Express Enzyme (1X), phenol red	Thermo Fisher Scientific	12605010
Gibco™ PBS (phosphate-buffered saline), pH 7.4		10010049
Gibco™ DMEM, high glucose		10569-010
Gibco™ Fetal Bovine Serum		10270106
Gibco™ Penicillin-Streptomycin		15070063
Gibco™ Recombinant Human AOF Insulin		A11382II
Thermo Scientific™ Pierce™ 16% Formaldehyde (FA)		28906
Gibco™ Trypan Blue Solution, 0.4%		15250061
Calcein AM		C3100MP
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix		A1413201
Crystal violet solution, 1%	Sigma-Aldrich	V5265
Plastics		
Thermo Scientific™ Nunc™ EasYFlask™ Cell Culture Flasks	Thermo Fisher Scientific	156472
Thermo Scientific™ Nunc™ Cell Culture Inserts in Carrier Plate Systems, 8 µm pore size, 24-well format		141006
Thermo Scientific™ Nunc™ MicroWell™ 96-Well, Nunclon™ Delta Treated, Flat-Bottom Microplate, black		137103
Laboratory equipment		
Invitrogen™ Countess™ 3 Automated Cell Counter	Thermo Fisher Scientific	AMQAX2000
Invitrogen™ EVOS™ M7000 Imaging System		AMF7000
Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader		VLBL00GD0
Other		
Cotton swabs, CO_2 incubator, laminar hood, microcentrifuge		

Reagent preparation

Geltrex matrix solution

Thaw the Geltrex matrix overnight at 4°C. Dilute 1 part Geltrex matrix with 4 parts of cold 1X PBS.

Formaldehyde (FA)

Dilute 16% FA to 4% using 1X PBS.

CAUTION: FA is toxic and corrosive. Avoid any direct contact. Collect and discard waste appropriately.

Crystal violet solution

Dilute the 1% crystal violet stain to 0.2% using 1X PBS.

Calcein AM stock preparation

Quickly centrifuge the microcentrifuge tube to pellet the powder before opening the tube. Add 25 µL of sterile DMSO to make a 2 mM stock solution, and pipette up and down gently to mix the solution. Store the calcein AM stock in single-use aliquots at –20°C.

Helpful tips

- The concentration of Geltrex matrix necessary for the assay is dependent on the cell line. The concentration should be optimized before use. The mixture of Geltrex matrix and PBS should be prepared fresh for each assay.
- Geltrex matrix is a stable liquid at 4°C, but it polymerizes at room temperature (RT). Always make sure to work in cold conditions while handling Geltrex matrix. Best results will be observed when pipette tips, microcentrifuge tubes, centrifuge tubes, and other materials are chilled prior to contact with Geltrex matrix–containing solutions.

Key points to consider before starting the experiment

- All cell culture work should be carried out under sterile conditions.
- Use healthy cells in the active growth phase. Cells should be subcultured for at least two passages before initiating the assay.
- The protocol suggested may need to be optimized depending on the cell type. Critical parameters include the culture medium, chemoattractant, concentration of Geltrex matrix (for invasion assays only), cell seeding density, and incubation time.
- Running each sample at least in triplicate is recommended to obtain statistically significant data.
- The choice of pore size is critical and is dependent on the size of cells being used. In this protocol, membranes with pores 8 µm in diameter are used. If the pore size is too small, there will be little migration/invasion; if the pore size is too large, spontaneous migration/invasion can occur even without chemoattractant, leading to misleading results. We recommend using the smaller pore sizes (e.g., 3 µm pore size for small cells like leukocytes, and 8 µm pore size for larger cells like endothelial cells, epithelial cells, macrophages, and fibroblasts).
- Avoid using high concentrations of solvents like DMSO that may adversely affect the polycarbonate membrane.

Controls for the migration/invasion experiment

- Use wells with no chemoattractant as a negative control.
- Include nonmigratory or noninvasive cells to confirm there is no spontaneous migration and inhibition of the targeted cells (if an inhibitor is being used in the experiment).
- When performing an invasion assay, include a control without the ECM barrier to assess the difference between migrating and invading cells.

Cells: culture, maintenance, and harvest

1. Maintain and culture the cells following the recommended protocol.
2. Replace culture medium every 2–3 days. Allow the cells to reach 70–80% confluence before setting up the assay.
3. Remove serum-containing medium from cultures and rinse using 1X PBS the day before the migration/invasion experiment. Add serum-free medium and incubate at 37°C with 5% CO_2 for 24 hr. Serum starvation can increase the sensitivity of the cells to the chemoattractant and thus increase the migratory response, as well as reduce potential variability.
4. Remove the medium from the flask by gentle aspiration, wash the cells twice with sterile 1X PBS, add enough TrypLE enzyme to completely cover the cells, and place them at 37°C for 3–5 min.
5. Add double the volume of complete medium (e.g., for 2 mL TrypLE enzyme, add 4 mL of complete medium), and collect all the liquid in a sterile centrifuge tube.
6. Centrifuge the cell suspension for 5 min at 300 x *g* at RT, then remove the supernatant and resuspend the cells in fresh serum-free medium.
7. Determine the cell count using a manual or automated cell counter.

Preparation of cell culture inserts

Inserts can be used without any additional treatment for migration assays but should be coated with ECM prior to performance of invasion assays. Commonly used ECMs include collagen, laminin, fibronectin, or basement membrane extracts like Geltrex matrix. Geltrex matrix is a LDEV-free, reduced growth factor basement membrane matrix composed of laminin, collagen IV, entactin, and heparin sulfate proteoglycans.

Add the appropriate volume of the Geltrex matrix to each cell culture insert and keep in a 37°C incubator for 1 hr (refer to Table 1 for cell culture insert surface area and recommended ECM coating volume). It is recommended to coat the insert on the day of the assay.

Table 1. Suggested volume for Geltrex matrix coating.

Plate format	Culture area	ECM coating/well
6-well	Two available options: 3.14 cm^2 and 4.1 cm^2	1,000 µL
12-well	1.13 cm^2	300 µL
24-well	0.47 cm^2	100 µL

Note: To optimize to the appropriate coating concentration, try different coating concentrations along with some uncoated wells. When the coating is appropriate, there should be a statistically significant difference between the number of cells that have migrated through the uncoated membranes, compared to ECM-coated membranes.

Plating of cells on cell culture inserts

1. Determine the cell count and dilute the cell suspension to the necessary seeding density in serum-free medium (Table 2). Here, for the 24-well format, 100 µL of cell suspension containing 2 x 10^5 cells has been used.

Table 2. Suggested cell seeding concentrations.

Plate format	Number of cells per well	Seeding volume per insert	Bottom well volume
6-well	3 x 10^5 to 5 x 10^5	1–1.5 mL	2 mL
12-well	1 x 10^5 to 2.5 x 10^5	350–400 µL	1 mL
24-well	1 x 10^5 to 2 x 10^5	100–200 µL	500–650 µL

Note: Cell seeding densities have been optimized for this protocol using MCF7 and MDA-MB-231 cells. Optimized densities will help maximize migrated cells and minimize nonspecific background. If cells are plated too sparsely, quantification of cells that have migrated/invaded may be inconsistent. If too many cells are plated, cell–cell interactions may impede migration/invasion, leading to inaccurate results. Cell seeding densities should be optimized for each cell line for each plate format.

2. In some cases, you may observe small volumes of liquid remaining after the Geltrex matrix solidifies. If desired, carefully aspirate excess liquid from inserts (for invasion).
3. Seed the cell suspension from step 1 into each well of the 24-well insert with 8 µm pore size. It is recommended to keep the insert at the lowest level. Add 650 µL of chemoattractant-containing medium to the bottom well. In this work DMEM with 10% FBS is used.

Note: Include a few wells without chemoattractant (only serum-free medium) as a negative control and at least one well without cells as a blank.

4. Incubate seeded cell culture inserts for 20 hr at 37°C with 5% CO_2.

Note: The time required for cells to migrate/invade through the membrane depends on the type of cells, the concentration of chemoattractant, and the concentration of ECM used. Longer incubation may allow for migration in negative control wells, so it is important to standardize the migration time to avoid confounding results.

Crystal violet staining

1. Aspirate the medium from receiver wells and cell culture inserts.

2. Wash both the inserts and receiver wells with 1X PBS (see Table 3).

Note: Handle membranes with care, and do not allow them to dry out.

3. Gently swab the inside of each insert using cotton swabs to remove cells that have not migrated/invaded. Be thorough around the edges of the membrane.

Note: Take proper care so that the membrane is not damaged during this process. Cell removal must be gentle, with enough pressure to remove all the cells on the upper surface of the membrane without significantly deforming or tearing the membrane. If necessary, repeat the scraping with a second cotton swab.

4. Add 4% FA to the wells containing inserts and incubate for 15 min at RT (Table 3). Alternatively, cells can be fixed using 100% methanol for 20 min at RT.

Table 3. Suggested volumes of PBS, FA, and crystal violet solution.

Plate format	Volume in insert	Volume in bottom plate
6-well	1 mL	2 mL
12-well	500 µL	1 mL
24-well	200 µL	500 µL

5. Wash both the top and bottom of the membrane twice with sterile 1X PBS to remove debris, unattached cells, and excess FA. Be gentle with all washes to avoid detaching cells. Samples can be stored in 1X PBS at 4°C for up to 1 month.

Note: Using the Nunc Carrier Plate makes it easy to remove all the inserts at once while removing the residual liquid from receiver plate/inserts, and eliminates the need to individually pick each insert using tweezers. This saves time and reduces the risk of tearing the membranes with sharp tweezers.

6. Add the 0.2% crystal violet stain solution (refer to "Crystal violet solution") to each well containing an insert, making sure the membrane is completely covered with stain, and allow to rest for 10 minutes (see Table 3 for recommended volumes). After treatment with crystal violet, the membrane may appear blue.

7. Carefully remove the staining solution, and wash three times thoroughly with distilled water or 1X PBS.

8. Visualize the inserts via microscopy. Here, the EVOS M7000 Imaging System was used.

Analysis

The crystal violet–stained cells that have migrated/invaded should be visualized via microscopy. Cells that have penetrated the filter should appear violet, and multiple fields should be captured under 10X objective lens.

For quantitative analysis, the stained cells can also be manually counted while viewing the cells under the microscope. Counts from multiple fields within a well should be averaged. For an accurate count, it is important to consider enough fields at the center and edge of the insert. Here, only qualitative analysis was performed. Figure 2 shows crystal violet staining of MCF7 and MDA-MB-231 cells for both migration and invasion assays.

Data interpretation

- There should be little to no migration/invasion of cells without chemoattractant for both MCF7 and MDA-MB-231 cells (Figure 2).
- There should be a noticeable difference in numbers of cells moved between coating (cell invasion) and without coating (cell migration) when using chemoattractant. Whereas MDA-MB-231 cells showed both migration and invasion due to its metastatic nature, MCF7 cells showed minimal migration and invasion, demonstrating the noninvasive nature of MCF7 cells (Figure 2).

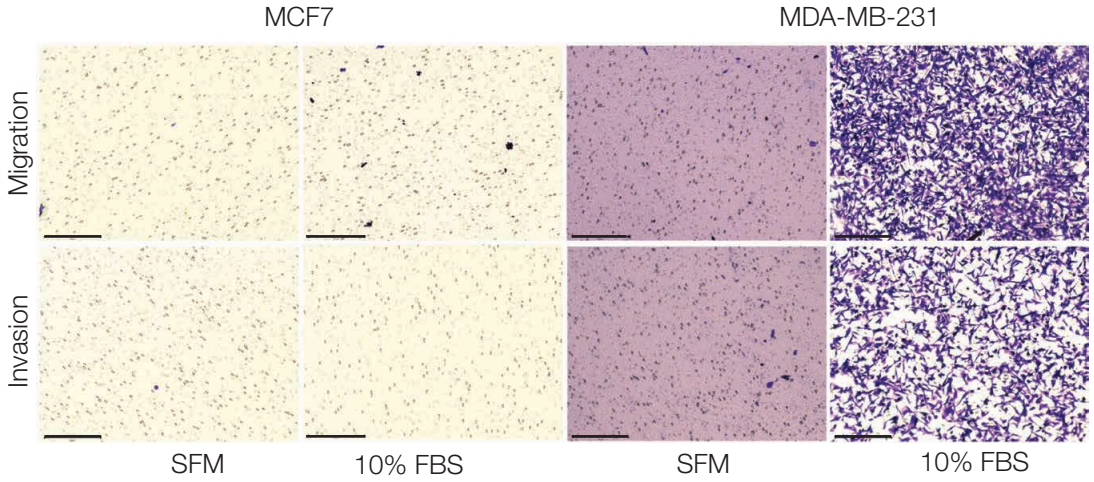

Figure 2. Representative images of migration and invasion of MCF7 and MDA-MB-231 cells stained with crystal violet. Images were taken at 10x magnification using an EVOS M7000 Imaging System. SFM = serum-free medium, scale bar = 275 µm.

Live-cell calcein AM staining for migration/invasion

Calcein AM staining is used to quantitatively determine migration/invasion of MCF7 and MDA-MB-231 cells. This method is fast, reduces variability, and can be performed for high-throughput readouts.

Calcein AM standard curve

To convert relative fluorescence units (RFU) to a number of cells, standard curves are generated. A separate standard curve should be run for each cell type. Both control and experimental conditions should be run at least in triplicate for accurate results.

Steps to generate a standard curve

1. Determine the saturation range of cells for an appropriate standard curve. Here, the assay is performed in a 24-well plate, and the optimal range was found to be from 3,000 to 200,000 cells/well (refer to Table 4). Cell numbers should be adjusted to the well size of your plate.
2. Harvest cells from the culture flask and resuspend cells in cell dissociation reagent (here TrypLE enzyme is used) such that there are 4 x 10^6 cells/mL. Add 100 µL of this cell suspension per well (Table 4).
3. Add 20 µL of the calcein AM stock solution (refer to "Calcein AM stock preparation") to 10 mL of TrypLE enzyme, and gently mix to form the calcein AM/TrypLE enzyme solution. Add 100 µL of this calcein AM/TrypLE enzyme solution to wells containing 100 µL of cell suspension.
4. Make serial dilutions of the remaining cell suspension using TrypLE enzyme, and add 100 µL of the calcein AM/TrypLE enzyme solution to meet all the conditions (refer to Table 4).

5. To measure the background, include a few wells containing 100 µL of calcein AM/TrypLE enzyme solution without cells.
6. Incubate the plate for 60 min at RT in dark.
7. Transfer the content of these wells to a 96-well black plate and measure the fluorescence at 485 nm excitation and 520 nm emission using the Varioskan plate reader.

Analysis of calcein AM standard curve

1. Take the average RFU for each condition and subtract the background (see sample data in Table 5).
2. Plot standard curve of corrected RFU vs. number of cells. Sample standard curves can be seen in Figure 3.
3. Determine the equation for the line of best fit; this equation is used to determine the number of cells present in each sample well.

Table 4. Sample calculations for a calcein AM standard curve using a 24-well plate.

Cells/well	Replicates	Total number of cells required	Volume of calcein AM/TrypLE enzyme solution added to each well
200,000	3	600,000	100 µL
100,000	3	300,000	100 µL
50,000	3	150,000	100 µL
25,000	3	75,000	100 µL
12,000	3	36,000	100 µL
6,000	3	18,000	100 µL
3,000	3	9,000	100 µL
Background	3	0	100 µL

Table 5. Sample data for a calcein AM standard curve. Results may vary depending on the cell line.

Number of cells/well	RFU 1	RFU 2	RFU 3	Average	Corrected RFU (average minus background)
200,000	197.90	192.00	200.21	196.7	195.5
100,000	101.20	116.90	110.32	109.5	108.3
50,000	89.88	79.90	82.43	84.1	82.9
25,000	45.21	40.49	43.34	43.0	41.8
12,000	20.82	24.07	23.56	22.8	21.6
6,000	16.70	17.96	17.12	17.3	16.1
3,000	9.01	8.06	10.72	9.3	8.1
Background	1.21	1.24	1.131	1.2	0.0

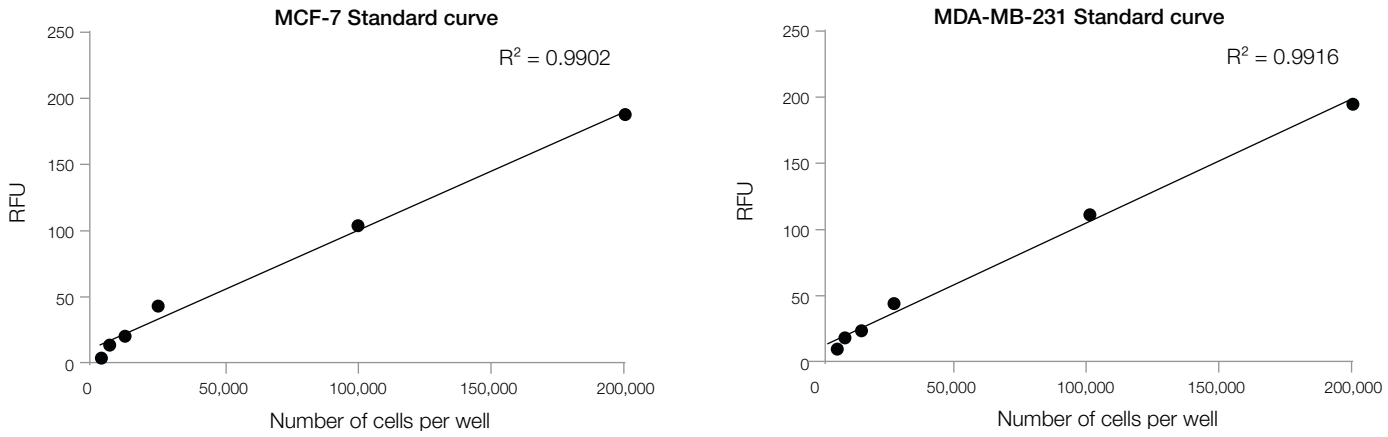

Figure 3. Standard curve for MCF7 and MDA-MB-231 cells stained with calcein AM. Relative fluorescence intensity (RFU) is measured using a Varioskan LUX Multimode Microplate Reader.

Calcein AM assay

1. After cell migration/invasion is complete, carefully lift the insert tray and remove the medium from the bottom compartment of each well of the 24-well plate and wash each well with 200 µL 1X PBS. Place the insert tray back into the plate.
2. To prepare the calcein AM/TrypLE enzyme solution, add 10 µL of calcein AM (refer to "Calcein AM stock preparation") to 10 mL of TrypLE enzyme.
3. Add 200 µL of the above solution to each bottom chamber of the well, such that the bottom of the insert is in contact with this calcein AM/TrypLE enzyme solution.
4. Shake the plate gently in forward and side directions after 30 minutes to ensure detachment of cells that have invaded/migrated, and place back at 37°C with 5% CO_2 for an additional 30 minutes.
5. Remove the insert tray and transfer the calcein AM/TrypLE enzyme solution (now containing the detached migratory/invading cells) from each bottom well to a black 96-well plate.
6. Measure the fluorescence at 485 nm excitation and 520 nm emission using the Varioskan plate reader.
7. Use the standard curve to interpolate the number of cells that have migrated or invaded.

Assay results

1. Once the RFU values for the sample are obtained, subtract the background values (see Tables 6 and 7 for representative data for migration and invasion, respectively) to obtain corrected RFUs.
2. Interpolate the sample values using the standard curves. This gives the number of cells that have migrated/invaded through the membrane.
3. To evaluate percent invasion and percent migration, the number of cells that have invaded/migrated is divided by the number of initial cells seeded, and multiplied by 100 (refer to Tables 8 and 9 for representative data for migration and invasion, respectively).
4. The percent of migration (Figure 4) and percent of invasion (Figure 5) have been represented in the form of bar graphs for both MCF7 and MDA-MB-231 cells.

Table 6. Representative results of a migration assay for MCF7 and MDA-MB-231 cells. Actual results may vary depending on the cell lines.

Cell line	Condition	RFU1	RFU2	RFU3	Average RFU	Corrected RFU
MCF7	SFM	10.18	10.49	10.21	10.29	9.25
	10% FBS	22.77	22.61	22.10	22.49	21.45
MDA-MB-231	SFM	24.42	23.65	24.36	24.14	23.10
	10% FBS	158.96	168.76	160.52	162.74	161.70

Table 7. Representative results of an invasion assay on MCF7 and MDA-MB-231 cells. Actual results may vary depending on the cell lines.

Cell line	Condition	RFU1	RFU2	RFU3	Average RFU	Corrected RFU
MCF7	SFM	7.4	7.53	7.91	7.61	6.57
	10% FBS	19.83	20.51	19.59	20.17	19.13
MDA-MB-231	SFM	21.11	22.6	22.42	22.04	21.01
	10% FBS	126.46	112.26	119.53	119.41	118.37

Table 8. Representative percent migration of MCF7 and MDA-MB-231 cells. Actual results may vary depending on the cell lines.

Cell line	Condition	Corrected RFU	Interpolated values (cell number)	RFU3
MCF7	SFM	9.25	1,282	0.64%
	10% FBS	21.45	11,697	5.84%
MDA-MB-231	SFM	23.10	12,581	6.29%
	10% FBS	161.70	162,216	81.11%

Table 9. Representative percent invasion of MCF7 and MDA-MB-231 cells. Actual results may vary depending on the cell lines.

Cell line	Condition	Corrected RFU	Interpolated values (cell number)	RFU3
MCF7	SFM	6.57	332	0.16%
	10% FBS	19.13	7,092	3.54%
MDA-MB-231	SFM	21.01	9,137	4.56%
	10% FBS	118.37	125,071	62.53%

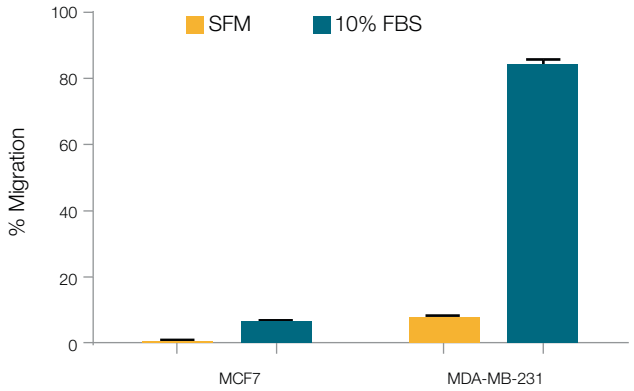

Figure 4. Relative quantification of migration properties of breast cancer cell lines across an 8 µm pore size filter, over a 20 hr period in response to 10% FBS. Two independent experiments were performed, and samples were run in triplicates.

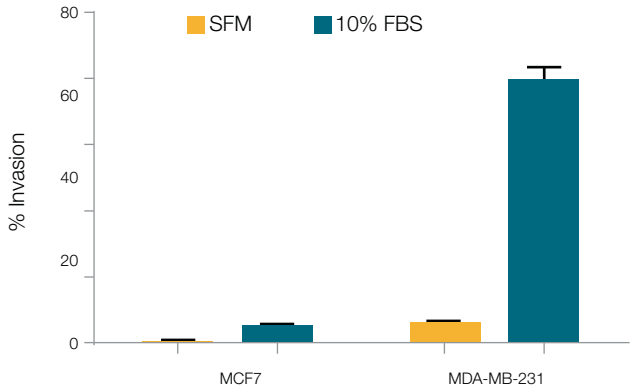

Figure 5. Relative quantification of invasive properties of breast cancer cell lines across an 8 µm pore size filter coated with Geltrex matrix, over a 20 hr period in response to 10% FBS. Two independent experiments were performed, and samples were run in triplicates.

Troubleshooting

Solutions to common problems regarding the cell migration/invasion assays are addressed below.

Problem	Solution
Improper attachment of cells on insert	• Pre-incubate permeable supports in desired medium prior to seeding
Variation between replicates	• Use calibrated pipettes and avoid air bubbles • Damage to the membrane caused by tweezers or cotton swabs can contribute to unexpected variation • Handle the membrane carefully • Be careful during wash steps
High background signal	• Ensure that cells are free from contamination • Wash the membrane properly • Use fresh stain solutions • Decrease the number of cells per well • Reduce incubation time with stain
Limited signal in wells where cells were expected to migrate/invade	• Include a migratory/invasive cell line as a positive experimental control • Check ECM concentration used (for invasion assay) • Standardize the concentration of chemoattractant used • Confirm that the pore size of insert is appropriate for the cell type used • Standardize the number of cells per well • Incubate the cells for longer duration • Increase the concentration of stain used

Conclusions

The robust protocols outlined here demonstrate the compatibility of Nunc Cell Culture Inserts for invasion and migration studies. These inserts are simple to use and can be utilized to perform both qualitative and quantitative assays. Nunc Cell Culture Inserts can be used not only to study cancer biology but also for other applications, including air/liquid interface models, transport studies, tissue engineering, and many more.

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Ordering information

Plate	Surface area of insert	Carrier plate?	Cat. No. by membrane pore size		
			0.4 µm	3 µm	8 µm
6-well	3.1 cm^2	No	140640	140642	140644
	4.1 cm^2	No	140660	140663	140668
12-well	1.13 cm^2	No	140652	140654	140656
		Yes	141078	141080	141082
24-well	0.47 cm^2	No	140620	140627	140629
		Yes	141002	141004	141006

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