

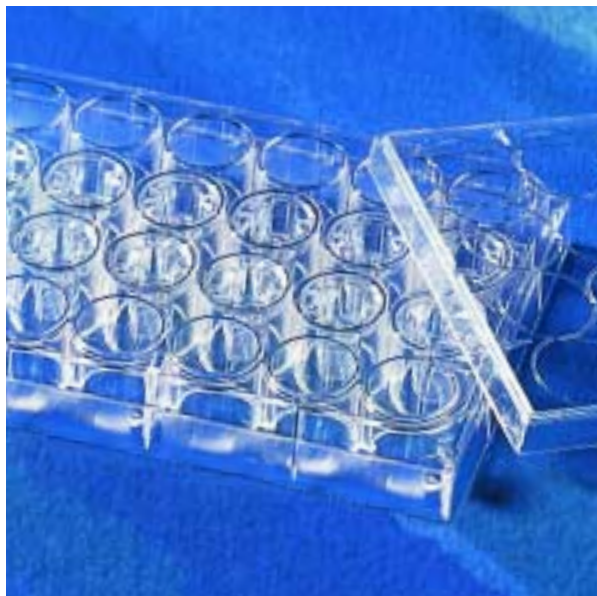
# Corning® Transwell® Permeable Support Coated with Cultrex® Basement Membrane Extract for Cell Invasion Assays

## Instructions for Use

Catalog No. 3458

For research use only.

Not for use in diagnostic procedures.



CORNING

# Corning® Transwell® Permeable Support Coated with Cultrex® Basement Membrane Extract

Catalog No. 3458  
24 Inserts

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## QUICK REFERENCE PROCEDURE

### **Transwell® Permeable Support Inserts Coated with Cultrex® Basement Membrane Extract (BME) for Cell Invasion Assays (Cat. No. 3458)**

Read through the complete Instructions for Use prior to using this kit. This page is designed to be copied and used as a checklist.

#### **Prior to Day 1**

- 1. Culture cells per manufacturer's recommendation. Adherent cells should be passaged at least one time and cultured to 80% confluence. Plan accordingly for sufficient numbers of cells per well.

#### **Day 1 or 2 (Preparation of cells and Coating of Insert Membranes)**

- 2. Twenty-four hours prior to assay, starve cells in serum-free media (0.5% FBS may be used if needed).

#### **Day 2**

- 3. Rehydrate the inserts by adding 100  $\mu$ L of warm serum free media to each insert and 500  $\mu$ L to each receiver well. Incubate at 37°C in a CO<sub>2</sub> incubator for at least 1 hour.
- 4. After incubating cells 24 hours under serum starvation, harvest and count cells.
- 5. Centrifuge cells at 250 x g for 10 min, remove supernatant, wash with sterile PBS or HBSS, and suspend at  $1 \times 10^6$  cells/mL in serum free media (0.5% FBS may be used if needed). Inhibitors may also be added to cells at this time.
- 6. After rehydration, carefully remove excess media from inserts and receiver wells. Do not puncture membrane. DO NOT ALLOW INSERTS TO DRY. Add 100  $\mu$ L of cells per well to top chamber.
- 7. Add 650  $\mu$ L of media per well to bottom chamber (with or without chemo-attractants).
- 8. Assemble chamber and incubate at 37°C in a CO<sub>2</sub> incubator for 4 to 48 hours.
- 9. If desired, assay remaining cells for standard curve (please see control section page 5).

#### **Day 3**

- 10. Carefully aspirate top chamber, and wash each well with 100  $\mu$ L of 1X wash buffer. Do not puncture membrane.
- 11. Aspirate bottom chamber, and wash each receiver well twice with 500  $\mu$ L 1X wash buffer.
- 12. Add 12  $\mu$ L of Calcein AM (page 6) solution to 10 mL of cell dissociation solution (page 3).
- 13. Add 300  $\mu$ L of Cell Dissociation Solution/Calcein AM to bottom chamber, assemble cell migration device, and incubate at 37°C in a CO<sub>2</sub> incubator for one hour.
- 14. Remove inserts, and transfer cell dissociation solution/Calcein AM with cells to a black plate. Read plate at 485 nm excitation, 520 nm emission wavelengths, respectively.
- 15. Using the standard curve(s), convert RFU to cell number and calculate percent invasion.

## BACKGROUND

Transwell® Permeable Support Inserts Coated with Cultrex® BME were originally created in an effort to accelerate the screening process for compounds that influence cellular invasion through extracellular matrices, which is fundamental to angiogenesis,<sup>1</sup> embryonic development,<sup>2</sup> immune responses,<sup>3</sup> and tumor cell metastasis.<sup>4</sup> This format provides a platform for the analysis of responses to chemokines, toxins, drugs and other analytes of interest, for larger numbers of cells per well.

The Transwell permeable support inserts coated with Cultrex BME utilize a Transwell Boyden chamber design with an 8 µm polycarbonate (PC) membrane. Detection of cell invasion can be quantified using Calcein AM. After Calcein AM is internalized intracellular esterases cleave the acetomethylester (AM) moiety to generate free Calcein. Free Calcein fluoresces brightly and this fluorescence may be used to quantitate the number of cells that have migrated using a standard curve.

Because different cell lines and different treatments can result in a wide range of invasive potentials, the permissiveness of the BME has been optimized for distinguishing between a highly invasive cell line and a non-invasive cell line. Adjustments to cell concentration, chemokine concentration and incubation time may be required to optimize results.

## PRECAUTIONS AND LIMITATIONS

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of these products may not yet have been fully investigated; therefore, Corning recommends the use of gloves, lab coats, and eye protection while using these chemical reagents. Corning assumes no liability for damage resulting from handling or contact with these products.
3. Material safety data sheets are available on request.

## MATERIALS SUPPLIED

Cat. No.	Component	Quantity	Storage
3458	24 Well Cell Invasion Chamber	24 inserts	≤-20°C*

\*Store in a manual defrost freezer; for long term storage, store at -80°C.

## MATERIALS/EQUIPMENT REQUIRED BUT NOT SUPPLIED

### Equipment

- ▶ 1-20 µL, 20-200 µL, and 200-1000 µL Pipettors (Corning Cat. Nos. 4961, 4963, and 4964, respectively)
- ▶ 37°C CO<sub>2</sub> incubator
- ▶ Low speed centrifuge and tubes for cell harvesting
- ▶ Hemacytometer or other means to count cells
- ▶ 50 and 500 mL Graduated Cylinders (Corning Cat. Nos. 3022-50 and 3022-500, respectively)

- ▶ -20°C and 4°C storage
- ▶ Ice bucket
- ▶ Standard light microscope (or inverted)
- ▶ Pipetting Aid (Corning Cat. No. 4910)
- ▶ Timer
- ▶ Vortex mixer
- ▶ Fluorescent 24 or 96 well plate reader, top reader (485 nm excitation, 520 nm emission)
- ▶ Computer and graphing software, such as Microsoft® Excel®.
- ▶ 96 Well Black Microplate (2 if generating standard curve) (Corning Cat. No. 3915)

## **Reagents**

- ▶ Cell harvesting buffer; EDTA, trypsin, or other cell detachment buffer
- ▶ Tissue culture growth media, as recommended by cell supplier
- ▶ Serum-free medium, tissue culture growth medium without serum
- ▶ Chemoattractants or pharmacological agents for addition to culture medium
- ▶ Quenching medium: serum-free medium with 5% BSA
- ▶ Sterile PBS or HBSS to wash cells
- ▶ Distilled or deionized water
- ▶ Trypan blue or equivalent viability stain
- ▶ Cell Wash Buffer (Trevigen Cat. No. 3455-096-04)
- ▶ Cell Dissociation Solution (Trevigen Cat. No. 3455-096-05)
- ▶ Calcein AM (Trevigen Cat. No. 4892-010-01)

## **Disposables**

- ▶ Cell Culture Flask, 25 cm<sup>2</sup> or 75 cm<sup>2</sup> (Corning Cat. No. 430639 or 430641)
- ▶ 50 mL Tubes (Corning Cat. No. 430290)
- ▶ 1-200 µL and 200-1000 µL Pipet Tips (Corning Cat. Nos. 4823 and 4809, respectively)
- ▶ 1.5 and 10 mL Serological Pipets (Corning Cat. Nos. 4021 and 4101)
- ▶ Gloves
- ▶ 10 mL Syringe
- ▶ 0.2 µm Syringe Filter (Corning Cat. No. 431229)

## ASSAY PROTOCOL

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

### A. Cell Harvesting

1. Subject cells may be prepared for investigation as desired. The following procedure is suggested and should be optimized to suit the cell type(s) of interest.
2. Cells should be passaged 2 or 3 times prior to use in the assay, and adherent cells need to be 80% confluent. Each chamber can accommodate  $1 \times 10^5$  to  $5 \times 10^5$  cells, depending upon cell type. A 25 cm<sup>2</sup> or 75 cm<sup>2</sup> flask will yield approximately  $3 \times 10^6$  or  $9 \times 10^6$  cells, respectively. Plan to have enough cells for a standard curve, if used, controls and cell invasion assay.
3. Starve cells by incubating 18 to 24 hours in serum-free medium (see Materials Required But Not Supplied) prior to assay (0.5% FBS may be used if needed).  
Prior to harvest, visually inspect cells, and record cell health, relative number, and morphology.
4. Wash cells two times with sterile PBS without divalent cations. Use 5 mL per wash for a 25 cm<sup>2</sup> flask and 10 mL per wash for a 75 cm<sup>2</sup> flask.
5. Harvest cells. For 25 cm<sup>2</sup> flask or 75 cm<sup>2</sup> flask, add 1 mL or 2 mL, respectively, of cell harvesting buffer (see Materials Required But Not Supplied), and incubate at 37°C for 5 to 15 minutes (until cells have dissociated from bottom of flask).
6. Transfer cells to a 15 mL centrifuge tube and add 5 mL of quenching medium (see Materials Required But Not Supplied).
7. Centrifuge cells at 250 x g for 10 minutes to pellet, remove quenching medium; and resuspend cells in 2 mL of serum-free medium (0.5% FBS may be used if needed). Cells may need to be gently pipetted up and down with serological pipet to break up clumps.
8. Count cells and dilute to  $1 \times 10^6$  cells per mL in serum-free medium (0.5% FBS may be used if needed).

### B. Conversion of Relative Fluorescence Units (RFU) into Cell Number

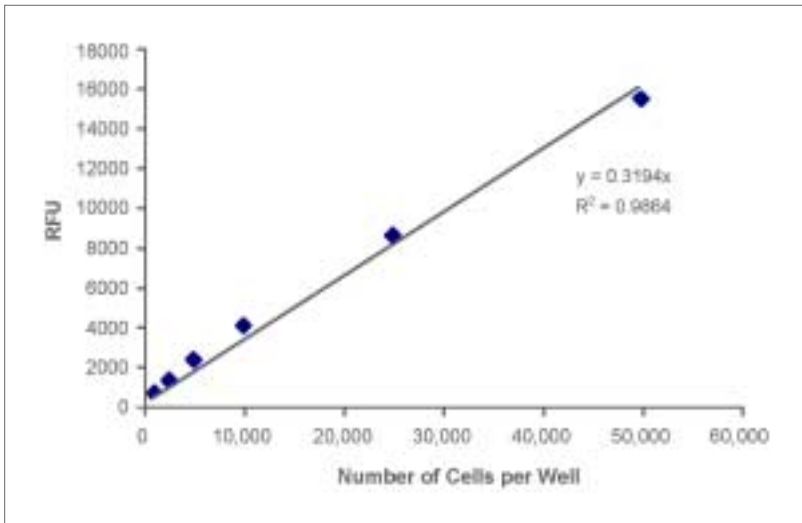
Many investigators express their results relative to untreated cells. In order to convert relative fluorescence units into number of cells, standard curves are recommended. It is not necessary to use inserts in order to do this. If used, a separate standard curve may be run for each cell type and assay condition. Control and experimental replicates should be performed in triplicate.

1. For a standard curve, a serial dilution can be made from cells resuspended in 1X cell dissociation solution. Add 50  $\mu$ L of each dilution, as well as a blank, in triplicate to a black microplate.
2. Add 24  $\mu$ L of Calcein AM solution to 10 mL of 1X cell dissociation solution, cap tube, and invert to mix.
3. Add 50  $\mu$ L of 1X cell dissociation solution/Calcein AM to each well of standard curve microplate, and incubate for one hour.
4. Read supernatants at 485 nm excitation, 520 nm emission (see Table 1 for sample data) to obtain RFU values.

5. Average values for each condition; then subtract background from each value (Table 1).
6. Plot standard curve RFU values vs. number of cells (see Fig. 1).
7. Insert a trend line (best fit) and use the equation  $y = mx + b$  for each cell line in calculating number of cells that invaded (Fig. 1).

**Table 1. Sample Data for Standard Curve (actual results may vary)**

Cells/Well	Wells				Avg.	Background = 254
	1	2	3			
50,000	15710	15415	16135	15663	- Bg. = 15409	
25,000	9118	8702	8644	8821	- Bg. = 8567	
10,000	4454	4257	4091	4267	- Bg. = 4013	
5,000	2609	2541	2599	2583	- Bg. = 2329	
1,000	930	922	881	911	- Bg. = 657	



**Figure 1.** Standard Curve for a Cell Invasion Assay. HT-1080 cells were harvested, diluted, incubated for one hour with Calcein AM, and assayed for fluorescence (page 4). The trend line and line equation are included on the graph. A separate standard curve for each tested cell line is recommended.

## C. Cell Invasion Assay

### Prior to Day 1:

Culture cells to be assayed to 80% confluence. Plan accordingly for sufficient numbers of cells per insert. 24 hours prior to assay, cells may need to be serum starved in order to allow ligands to bind to free receptors. This step may be omitted, depending on the cell types under investigation.

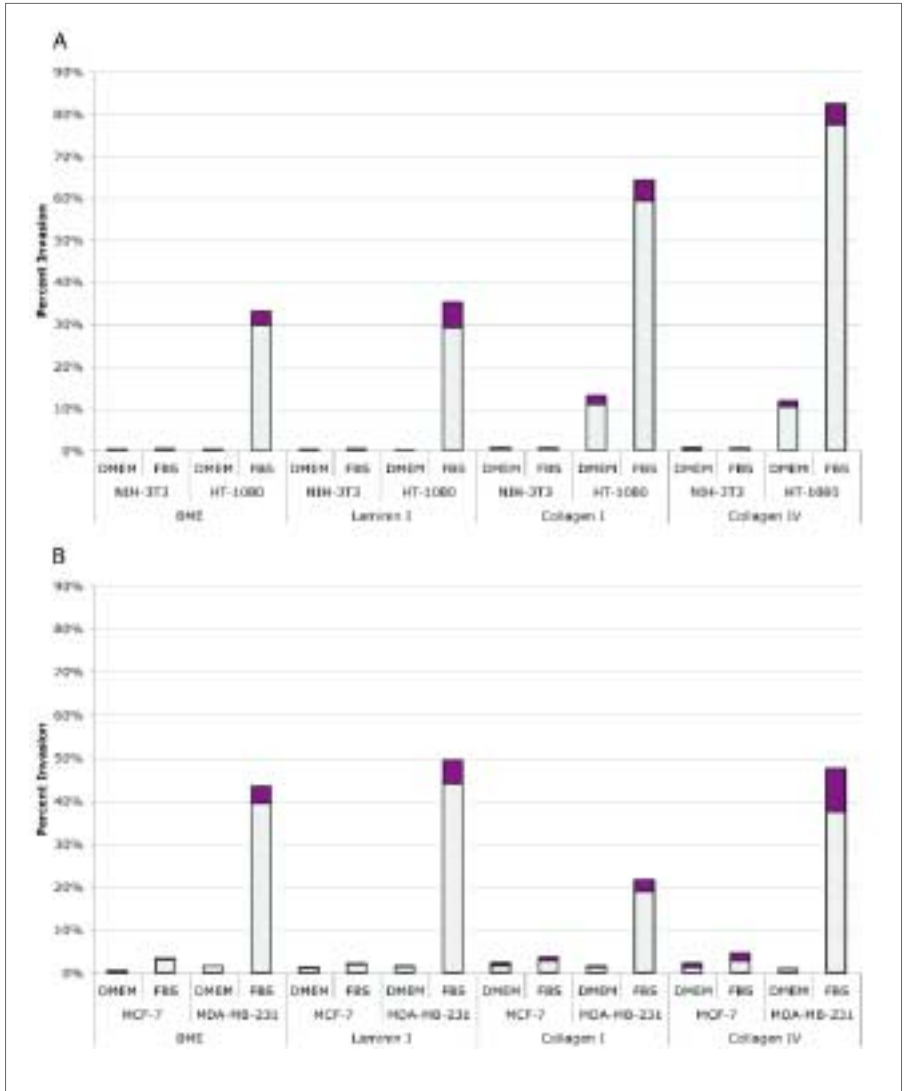
### Day 1:

1. Rehydrate the inserts by adding 100  $\mu\text{L}$  of warm serum free media to each insert and 500  $\mu\text{L}$  to each receiver well, and incubating at 37°C in a CO<sub>2</sub> incubator for at least 1 hour.
2. After serum starvation, if used, harvest cells, dilute to working concentration (1 x 10<sup>6</sup> cells/mL recommended) in serum-free medium.
3. After rehydration, remove excess media from inserts and receiver wells. Do not puncture or disrupt the BME coating.
4. Add 100  $\mu\text{L}$  of cells per well to each top chamber.
5. **Note:** An alternative method of seeding would be to add cells directly to re-hydration media so that removal is not necessary.
6. Add 650  $\mu\text{L}$  of test medium to bottom chambers (with or without drugs, chemokines, etc.).
7. Incubate at 37°C in CO<sub>2</sub> incubator; incubation times may be varied 4 to 48 hours.

### Day 1-3:

8. Carefully aspirate top and bottom chambers and wash each insert with 100  $\mu\text{L}$  of warm 1X wash buffer (item 1, page 3) and each receiver well with 400  $\mu\text{L}$  of wash buffer. Do not puncture membrane.  
**Note:** Always aspirate receiver well before aspirating the insert and always fill the insert before filling the receiver well.
9. Add 12  $\mu\text{L}$  of Calcein AM solution to 10 mL of 1X Cell Dissociation Solution (page 3).
10. Add 300  $\mu\text{L}$  of cell dissociation solution/Calcein AM to the bottom chamber of each well and incubate at 37°C in a CO<sub>2</sub> incubator for 60 minutes.
11. Disassemble chambers, and read assay chamber solutions (bottom) at 485 nm excitation, 520 nm emission using same parameters (time and gain) as standard curve, or controls. The cell dissociation solution/Calcein AM with cells needs to be transferred to a black plate to be read.  
**Note:** Remember to keep the volume being read equal to the final volume of the standard curve you ran earlier. This means that you can have 2 or 3 replicate wells being read from the same insert.
12. Compare experimental data to controls, and convert RFU into cell number (page 5) to determine the number of cells that have migrated, invaded, or failed to migrate or invade according to experimental design.

## EXAMPLE RESULTS



**Figure 2.** Quantitation of the ability of fibroblastic cell lines (A) and breast cancer cell lines (B) to cross a barrier consisting of an 8-micron polyester filter occluded with different extracellular components over a 24-hour period in response to 10% FBS. Samples were run in quadruplicate for non-invasive cell types MCF-7 and NIH-3T3, and invasive cell types HT-1080 and MDA-MB-231. Light area represents average invasion, and dark area represents standard deviation.

## TROUBLESHOOTING

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
No signal	Cells did not traverse the barrier.	Cell type may be non-invasive or chemoattractant may be insufficient.  There is inherent variability in FBS from lot to lot; this can affect the assay if used.
	Cells may have died as a result of treatment.	Test cells for viability in treatment regimen.
High background	Insufficient Washing - agents in media, FBS, and/or chemoattractant may react with Calcein AM.	Retest, and make sure to wash well.
	Contamination - proteases released by bacteria or mold may activate Calcein AM.	Start a new culture from seed stocks, and retest. If seed stock is contaminated, then it may be prudent to get new cells.
Well-to-well variability	Inconsistent pipetting	Calibrate pipettors, and monitor pipet tips for air bubbles.
	Puncture membrane with pipet tips	Disregard data from wells that are punctured; retest if necessary.

## REFERENCES

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**The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.**

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