Cultrex™

Laminin I Cell Invasion Assay

Catalog Number: 3456-024-K (24-well Kit)
3456-096-K (96-well Kit)

Reagent kits for investigating chemotaxis, cell migration, or cell invasion.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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BACKGROUND

Cultrex™ Cell Invasion Assays 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 (1), embryonic development (2), immune responses (3), and tumor cell metastasis (4). To provide a platform for the analysis of responses to chemokines, toxins, drugs and other analytes of interest, for larger numbers of cells per well, R&D Systems offers its Laminin I Cell Invasion Assay, 24-well Kit. The modular format of the Laminin I Cell Invasion Assay, 24-well assay offers flexible utility, and sufficient insert size for informative results.

The Cultrex Laminin I Cell Invasion Assay utilizes a simplified Boyden chamber design with an 8 µm polycarbonate (PC) membrane. Detection of cell invasion or migration is quantified using Calcein AM. Calcein AM is internalized by cells, and 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 invaded or migrated using a standard curve.

Cultrex Cell Invasion Assays allow cell invasion to be evaluated on:

- Laminin I (Catalog # 3456-024-K and 3456-096-K)
- Collagen I (Catalog # 3457-024-K and 3457-096-K)
- Collagen IV (Catalog # 3458-024-K and 3458-096-K)
- Basement Membrane Extract (BME) (Catalog # 3455-024-K and 3455-096-K)

Migration can be assayed using uncoated inserts. The process of transfection itself may also alter the invasive capacity of the cells under investigation, and as a result, may require a more permissive barrier (lower amounts of Laminin I). Thus, some optimization may be required for the cell type(s) of interest.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTION

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

Note: *Components for this kit require different storage/shipping temperatures and will arrive in separate packaging.*

CATALOG #	PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
3456-024-K	10X Coating Buffer	3455-096-03	1 mL	Store 2-8 °C.
	25X Cell Wash Buffer	3455-096-04	2 vials (1.5 mL/vial)	
	10X Cell Dissociation Solution	3455-096-05	2 vials (1.5 mL/vial)	
	Cell Invasion/Migration Plates, 24-well	3455-024-01	24-well plate	
	Calcein AM	4892-010-01	50 µg	Store at ≤ -70 °C.
	Cultrex Laminin I Solution, 5X	3456-096-02	1 mL	
3456-096-K	10X Coating Buffer	3455-096-03	1 mL	Store 2-8 °C.
	25X Cell Wash Buffer	3455-096-04	2 vials (1.5 mL/vial)	
	10X Cell Dissociation Solution	3455-096-05	2 vials (1.5 mL/vial)	
	Cell Invasion/Migration Plates, 96-well	3455-096-01	96-well plate	
	Calcein AM	4892-010-01	50 µg	Store at ≤ -70 °C.
	Cultrex Laminin I Solution, 5X	3456-096-02	1 mL	

OTHER SUPPLIES REQUIRED

Reagents:

- Cell Harvesting Buffer (EDTA, trypsin, or other cell detachment buffer)
- Cell Culture Medium (as recommended by cell supplier)
- Serum-free Cell Culture Medium
- Chemoattractants or pharmacological agents for addition to culture medium
- Sterile PBS or HBSS
- Distilled water
- Trypan blue or equivalent viability stain

Equipment:

- Pipettes and pipette tips
- 37 °C CO_2 incubator
- Low speed centrifuge and tubes for cell harvesting
- Hemocytometer or other means to count cells
- 50 and 500 mL graduated cylinders
- ≤ -70 °C and 2-8 °C storage
- Ice bucket
- Standard light microscope
- Timer
- Vortex mixer
- Fluorescent 96-well plate reader, top reader \(485 nm excitation, 520 nm emission)
- Clear, Flat bottom 24 Well Plates (if generating standard curve)

Disposables:

- Cell culture flask, 25 cm^2 or 75 cm^2
- 50 mL tubes
- 1, 5 and 10 mL serological pipettes
- Gloves
- 10 mL syringe
- 0.2 µm filter

REAGENT PREPARATION

Thaw reagents completely before diluting

1X Coating Buffer - Dilute 1 mL of 10X Coating Buffer in 9 mL of sterile, distilled water to make 1X Buffer; filtration at 0.2 µm recommended. Store at 2-8 °C.

Cultrex Laminin I Solution, 5X - Dilute 1 mL Cultrex Laminin I Solution, 5X in 4 mL 1X Coating Buffer on ice to make a 1X solution.

1X Cell Wash Buffer - Dilute 3 mL of 25X Cell Wash Buffer in 72 mL of sterile, distilled water to make 1X solution.

1X Cell Dissociation Solution - Dilute 3 mL of 10X Cell Dissociation Solution in 27 mL of sterile, distilled water to make a 1X solution.

Calcein-AM Solution - Centrifuge microtube momentarily to pellet powder before opening tube, and add 30 µL of sterile DMSO to make working solution. Pipet up and down to mix, and store solution at < -20 °C.

Quenching Medium - Prepare Serum-free Cell Culture Medium containing 5% BSA.

ASSAY PROTOCOL

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

Cell Harvesting

Subject cells may be prepared for investigation as desired. The following procedure is suggested and may be optimized to suit the cell type(s) of interest.

1. 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 1×10^5 - 5×10^5 cells depending upon cell type. A 25 cm^2 or 75 cm^2 flask will yield approximately 3×10^6 or 9×10^6 cells, respectively. Plan to have enough cells for a standard curve, if used, controls and cell invasion assay.

2. Starve cells by incubating 18-24 hours in Serum-free Cell Culture Medium prior to assay (0.5% FBS may be used if needed).

3. Prior to harvest, visually inspect cells, and record cell health, relative number, and morphology.

4. Wash cells two times with sterile PBS or HBSS. Use 5 mL per wash for a 25 cm^2 flask and 10 mL per wash for a 75 cm^2 flask.

5. Harvest cells. For 25 cm^2 flask or 75 cm^2 flask, add 1 mL or 2 mL, respectively, of Cell Harvesting Buffer, and incubate at 37 °C for 5-15 minutes (until cells have dissociated from bottom of flask).

6. Transfer cells to a 15 mL conical tube, and add 5 mL of Quenching Medium.

7. Centrifuge cells at 250 x g for 10 minutes to pellet, remove Quenching Medium, and resuspend cells in 2 mL of Serum-Free Cell Culture 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 x 10^6 cells/mL in Serum-Free Cell Culture Medium (0.5% FBS may be used if needed).

Controls

Many investigators express their results relative to untreated cells. In order to convert relative fluorescence units into number of cells, standard curves are recommended (please see table 1 below). 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.

Fluorescence Determination

1. Determine the saturation range for your cells (*e.g.* 50,000 to 100,000 cells), beyond which, additional invasion is difficult to detect.

2. For a standard curve, a serial dilution series is aliquotted as appropriate, whereas untreated controls are allowed to invade coated or uncoated membranes in triplicate wells.

3. Add 12 µL of Calcein AM Solution to 12 mL of 1X Cell Dissociation Solution, cap tube, and invert to mix.

4. Add 500 µL of 1X Cell Dissociation Solution/Calcein AM to each well, and incubate for one hour; omit cells (and inserts) from at least three wells to calculate background.

5. Read supernatants at 485 nm excitation, 520 nm emission, and then subtract the average background value (see Table 1 for sample data) to obtain relative fluorescence units (RFU).

6. Calculate the average values for each condition (see Table 1).

7. If desired, plot standard curve RFU values vs. number of cells (Figure. 1).

8. Insert a trend line (best fit) and use the line equation for each test in calculating number of cells that invaded or migrated (Figure 1).

ASSAY PROTOCOL *CONTINUED*

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

Average Cells/Well	Wells					
	1	2	3	Average	Background=254	
50,000	15,710	15,145	16,135	15663	- Bg. =	15409
25,000	9118	8702	8644	8821	- Bg. =	8567
10,000	4454	4257	4091	4267	- Bg. =	4013
5000	2609	2541	2599	2583	- Bg. =	2329
1000	930	922	881	911	- Bg. =	657

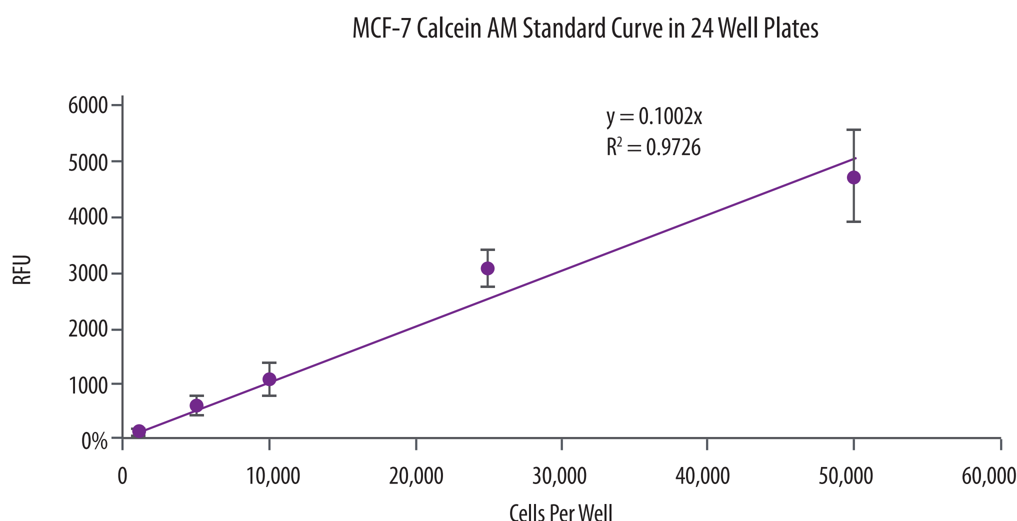

Figure 1. Standard Curve for a Cell Invasion Assay. MCF-7 cells were harvested, diluted, incubated for one hour with Calcein AM, and assayed for fluorescence. The trend line and line equation are included on the graph.

Cell Invasion Assay

1. Culture cells to be assayed to 80% confluence. Plan accordingly for sufficient numbers of cells per assay chamber.
2. 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.
3. In the hood, working on ice, add 1 mL of Cultrex Laminin I Solution, 5X to 4 mL of 1X Coating Buffer in a sterile 15 mL conical tube, and label "Laminin I Coat". Cap tube and gently invert to mix. Less invasive cell types will need less Laminin in order to traverse the coated membrane. Some optimization with varying amounts of Laminin may be required.

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4. Add Laminin I Coat into each well (100 µL/well for the 24-well Assay, 50 µL/well for the 96-well Assay). Aliquot 100 µL of Laminin I coat per insert. Place lid on chamber, and visually assess each insert. The coating buffer should be evenly distributed across each insert. If necessary, the plate may be tapped lightly to evenly disperse the coating buffer.

5. Incubate coated chambers at 37 °C for 4 hours or overnight.

6. After serum starvation, if used, load invading cells into top chamber and, to compensate for background, omit cells from at least three wells (no inserts are needed). Alternatively, set aside uncoated inserts if migration is to be detected and compared with invasion.

7. Centrifuge cells at 250 x g for 10 minutes, remove supernatant, wash with 1X Cell Wash Buffer, and suspend at 1 x 10^6 cells/mL in Serum Free Cell Culture Medium (0.5% FBS may be used if needed) or assay concentration of choice. Inhibitors may also be added to cells at this time.

8. Carefully aspirate top chamber. **DO NOT ALLOW TOP OR BOTTOM CHAMBERS TO DRY**.

9. Add cells with or without inhibitors/stimulants) to the top chamber (100 µL of cell suspension/chamber for the 24-well assay, 50 µL of cell suspension/chamber for the 96-well assay). To compensate for background, omit cells from at least three wells.
   Note: *It is important to avoid cell clumping and overcrowding.*

10. Add Cell Culture Media (with or without inhibitors) to bottom chambers (with or without chemoattractants). Assemble chambers.

11. Incubate at 37 °C in CO_2 incubator; incubation times may be varied (4-48 hours).

12. **After incubation, carefully aspirate top chamber, without puncturing the membrane.**

13. Carefully aspirate top chamber and wash each well with Cell Wash Buffer. Do not puncture membrane.

    a) 24-well Assay: use 100 µL of 1X Cell Wash Buffer.

    b) 96-well Assay: use 200 µL of 1X Cell Wash Buffer.

14. Aspirate bottom chamber, and wash each well with Cell Wash Buffer.

    a) 24-well Assay: use 500 µL of 1X Cell Wash Buffer.

    b) 96-well Assay: use 200 µL of 1X Cell Wash Buffer.

15. Prepare Calcein AM/Dissociation Solution.

    a) 24-well Assay - Add 12 µL Calcein AM Solution to 12 mL of 1X Cell Dissociation Solution.

    b) 96-well Assay - Add 12 µL Calcein AM Solution to 10 mL of 1X Cell Dissociation Solution.

16. Add of Cell Dissociation Solution/Calcein AM to the bottom chamber of each well, reassemble the chambers, and incubate at 37 °C in a CO_2 incubator for 60 minutes.

    a) 24-well Assay: 500 µL/well of Calcein AM/Dissociation Solution.

    b) 96-well Assay: 100 µL/well of Calcein AM/Dissociation Solution.

17. 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.

18. Compare data to standard curve or controls to determine the number of cells that have migrated, or invaded, or failed to migrate or invade according to experimental design.

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DATA EXAMPLE

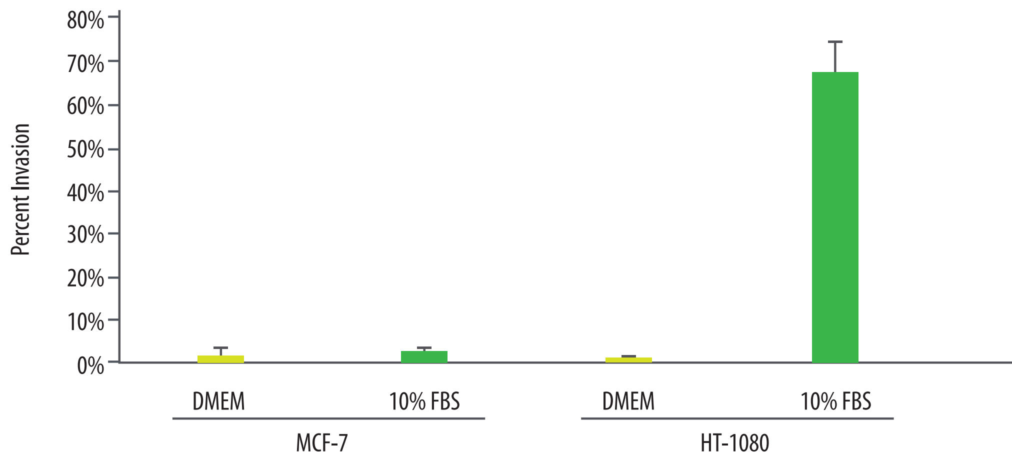

Figure 2. Example results for MCF7 and HT1080 cells. Triplicate wells containing 50,000 cells/well were used, and invasion towards 10% FBS was measured as described above.

TROUBLESHOOTING

Problem	Cause	Action
No signal	Cells did not invade or migrate.	Cell type may be non-invasive or chemoattractant may be insufficient.
		There is inherent variability in FBS from lot to lot; this can affect 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 chemo-attractant may react with Calcein AM.	Re-assay, 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 re-assay. 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 of membrane with pipette tips.	Disregard data from wells that are punctured; re-assay if necessary.

REFERENCES

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