



Protocol

IncuCyte® S3 3D Spheroid Invasion Assay

For the quantification of tumor spheroid invasion into surrounding Matrigel®

This protocol provides an overview for the measurement of 3D Tumor Single Spheroid cell invasion into surrounding extracellular matrix (Matrigel®). This method utilizes the IncuCyte® Live-Cell Analysis System

for kinetic, image-based brightfield and fluorescence measurements of spheroid invasion (invading cells and whole spheroid areas).

Required materials

- 96-well round bottom, ultra-low attachment plate (e.g., Corning® Cat. No. 7007, S-BIO Cat. No. MS-9096UZ, BRAND Cat. No. 781900)
- Matrigel®, protein concentration ≥ 8 mg/mL (Corning Cat. No. 356234)
- Wet Ice
- Complete culture media for cell culture and assay
- Manual multi-channel pipette
- IncuCyte® S3 Spheroid Software Module, version 2019B (Essen Bioscience Cat. No. 9600-0019)

Optional materials

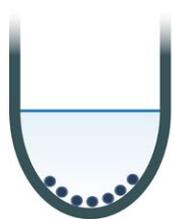
- IncuCyte® Cool Accessories (Cat. No. 4444)
 - CoolBox 96F System (Includes x2 Block with gelpack and Coolsink 96F)
- IncuCyte® NuLight Red or Green Lentivirus Reagent (Essen Bioscience Cat. No. 4476 or 4475)
- IncuCyte® CytoLight Red or Green Lentivirus Reagent (Essen Bioscience Cat. No. 4481 or 4482)

General guidelines

- Follow manufactures guidelines for thawing and storing of 100% Matrigel®. Thaw Corning® Matrigel® overnight by submerging the vial in ice cold water in the rear of a refrigerator (+ 4° C). Do not allow Matrigel® to warm to room temperature at any time as this will induce polymerization.
- All materials (e.g. culture-ware, reagents) that will come in contact with Matrigel® must be kept cold (on ice, stored at + 4° C).
- We recommend sourcing a batch of Matrigel® with a concentration of ≥ 8 mg/mL.
- Following cell seeding, treatments and Matrigel® addition, remove bubbles from all wells by gently squeezing a wash bottle containing 70–100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.
- After seeding, place the plate in the IncuCyte live-cell analysis system, allow the plate to warm to 37° C for 30 mins prior to scanning.
- After treatment, place plate in the IncuCyte® live-cell analysis system and leave for at least 1 hour to allow condensation to dissipate prior to scanning.

Quick guide

1. Seed cells (day 0)



Seed cells into 96W Ultra Low Attachment plate. Centrifuge.

2. Spheroid formation (day 0–3)



Place plate inside IncuCyte and monitor spheroid formation for 3 days.

3. Add treatments (day 0)



Add treatments to plate (10 μ L/well) at 20x final assay concentration.

4. Add Matrigel on top (day 3)



Add Matrigel on top (90 μ L/well) at 50% final assay concentration. Polymerize at 37° C for 30 minutes.

5. Monitor spheroid invasion (day 3–10)



Monitor spheroid invasion for up to 10 days.

Protocol

IMPORTANT:

- In advance of experiments it is important to have stored the Cool pack accessories at the correct temperatures for at least 4 h:
 - Coolbox x2 (block with gelpack: -20° C)
 - Coolsink 96F x1 (4° C)
- Keep all culture-ware and reagents coming in contact with Matrigel® on ice during the entire process.
- Store pipette tips used for dispensing diluted Matrigel® at +4° C

Day 0:

1. Seed cells

- Seed cells of interest (100 μ L per well) at an appropriate density into a 96-well ULA plate such that by day 3, single spheroids have formed with the desired size (e.g. 200–500 μ m in diameter). Seeding density will need to be optimized for each cell line used, however it is usually in the range of 1000–5000 cells per well.

NOTE: Some cell lines may require the addition of a basement membrane extract, typically 2.5% v/v Matrigel® to promote tight spheroid formation.

- Centrifuge the ULA plate (125 g, 10 minutes) at room temperature (20–25° C).

Day 0–3:

2. Monitor spheroid formation

- Place the cell plate into the IncuCyte® live-Cell Analysis System and schedule 24 hour repeat scanning:
 - Objective: 4x (96-well) 1 image per well
 - Channel selection; Phase Contrast + Brightfield and "Red" or "Green" if required
 - Scan type: Spheroid, Spheroid Type: Single
 - Scan interval: Every 6 hours

Day 3:

3. Add treatments

- Once spheroids have reached desired size, remove the plate from the IncuCyte® and carefully add appropriate treatments (e.g. inhibitors) at 20x final assay concentration (10 μ L per well).
 - Add media alone to control wells.

4. Add Matrigel®

- Place the plate containing spheroids on a pre-chilled Coolsink 96F within a Coolbox 96F box for approximately 5–10 minutes.
- In a cold polypropylene tube, dilute Matrigel® stock at 2x final assay concentration (e.g. prepare 25–100% or 4.5–9 mg/mL Matrigel for a final assay concentration of 12.5–50% or 2.25–4.5 mg/mL) in complete culture media.

NOTE: Using a cold serological pipette, slowly pipette Matrigel® into complete culture media and taking care to avoid bubbles, slowly mix by pipetting the solution up and down.

- Pour diluted Matrigel® solution into a pre-chilled sterile reagent reservoir (keep on ice).

- Using pre-chilled pipette tips and reverse pipetting technique, slowly add 90 μ L of diluted Matrigel® to appropriate wells.

NOTE:

- When adding Matrigel to wells, aim the tip towards the inside wall of the well. This step is critical to obtain optimal imaging and ensure spheroids remain in the center of the well.
- Cell types that require the addition of a basement membrane extract to promote spheroid formation may not remain in center of well post treatment, which may affect imaging capabilities.

- Remove any bubbles using a wash bottle containing 70–100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.

Optional: To ensure spheroids are centrally located or to remove excess bubbles, spin assay plate (300g, 3', 4° C).

- Place plate in a 37° C incubator for 30 mins to polymerize the Matrigel®.
- Post polymerization remove plate from incubator and gently add 50 μ L/well of complete culture media containing test material (e.g. inhibitors) or media alone at 1x final concentration.
- Return plate to the IncuCyte® to monitor spheroid invasion (e.g. every 6 hours for 7 days).

NOTE:

- Allow plate to warm up for at least 1 hour to allow condensation to dissipate prior to imaging.
- Wiping the underside of the plate using 'lint free wipes' can help reduce condensation.

Analysis guidelines

1. Create a new Analysis Definition

- a. In the Analysis Wizard window select 'Spheroid Invasion' Analysis Type.
- b. Select representative images of both invasive and non-invasive phenotypes (control and treated).
- c. Adjust the Whole Spheroid Brightfield Sensitivity slider to accurately determine the boundary of the whole spheroid (spheroid body and invasive phenotype).
- d. Evaluate the Whole Spheroid Mask and refine filter parameters accordingly. 'Preview All' to ensure parameters set appropriately mask all representative images within the collection.
- e. Adjust the Invading Cells Brightfield Sensitivity slider to delineate the invasive cell area from that of the non-invasive spheroid body.
- f. Evaluate the Invading Cells Mask and refine filter parameters accordingly. 'Preview All' to ensure parameters set appropriately mask all representative images within the collection.
- g. Once satisfied with all parameters (for both Whole Spheroid and Invading Cells), complete the Launch Wizard analysis by selecting the scan times and wells to be analyzed.

NOTE: If your experiment is in progress you will have an option to check 'Analyze Future Scans' to perform real-time analysis.

2. Data Interpretation (Single Spheroid Invasion)

Once the Analysis Job is complete the following primary metrics are provided:

- a. Largest Whole Spheroid Brightfield Area. This metric represents the area of the largest brightfield whole spheroid within the image (well) and is recommended for tracking whole spheroid size over time.
- b. Largest Invading Cells Brightfield Area. This metric represents the area of the largest invading cells in the image (well) and is recommended for tracking invasive capacity over time.
- c. Fluorescence within Brightfield Boundary (Whole Spheroid or Invading Cells) metrics are available for tracking changes in intensity over time.

A complete suite of cell health applications is available to fit your experimental needs. Find more information at www.sartorius.com/incucyte

For additional product or technical information, please email us at AskAScientist@sartorius.com

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