

IncuCyte® S3 Multi-Spheroid Assay

For the quantification of multi-spheroid growth and health on a layer of Matrigel®

This protocol describes a solution for creating multi-spheroids using 96-well flat bottom plates coated with Matrigel®, and subsequent determination of cell viability, apoptosis, or cytotoxicity using IncuCyte® Cell Health Reagents. The method utilizes the IncuCyte® live-cell analysis system for image-based brightfield and fluorescence measurements of multi-spheroid size (area), number and health.

Required materials

- 96-well flat bottom TC-treated microplate (Corning Cat #3595)
- Matrigel®, protein concentration ≥ 8 mg/mL (Corning Cat #356234)
- Wet ice
- Serum-free cell culture media for Matrigel® dilutions
- Complete culture media for cell culture and assay
- Manual multi-channel pipette
- IncuCyte® S3 Spheroid Software Module, version 2018A or higher (Cat #9600-0019)
- IncuCyte® Cytotox Red or Green Reagent (Cat #4632 or #4633)
- IncuCyte® Annexin V Red or Green Reagent (Cat #4641 or #4642)
- IncuCyte® Caspase Green Reagent (Cat #4440)
- IncuCyte® NucLight Red or Green Lentivirus Reagent (Cat #4476 or #4475)
- IncuCyte® CytoLight Red or Green Lentivirus Reagent (Cat #4481 or #4482)

NOTE: Transfect cells with NucLight and CytoLight reagents prior to performing multi-spheroid experiments by following the protocols supplied with the reagents.

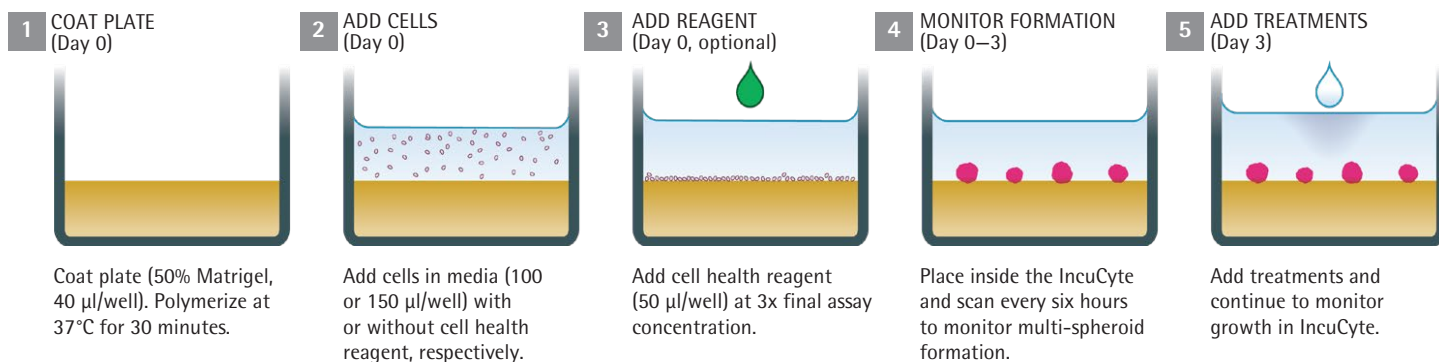
Optional Materials

- IncuCyte® Cool Accessories (Cat #4444)
 - CoolBox 96F System (Includes x2 Block with gelpack and Coolsink 96F)

General Guidelines

- All materials (e.g. culture-ware, reagents) that will come in contact with Matrigel® must be kept cold (on ice, stored at + 4°C).
- Follow manufactures guidelines for thawing and storing of 100% Matrigel®. Thaw Corning® Matrigel® overnight by submerging the vial in ice cold water placed in the rear of a refrigerator (+ 4°C). Do not allow Matrigel® to warm to room temperature at any time as this will induce polymerisation.
- We recommend sourcing a batch of Matrigel® with a concentration of ≥ 8 mg/mL.
- Following Matrigel® coating, cell seeding and after treatment 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 placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37°C for 30 minutes prior to scanning.

Quick Guide



Protocol

IMPORTANT:

- In advance of multi-spheroid experiments, it is important to have stored the Cool pack accessories at the correct temperatures for at least 4h:
 - Coolbox x 1 (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 Coat plate with Matrigel®

- In a cell culture hood, chill plates (10 – 15 minutes) on a pre-chilled Coolsink 96F within a Coolbox 96F box.
- In a cold polypropylene tube, dilute 100% Matrigel® 1:1 in cold serum-free culture media (keep all Matrigel® solutions on ice).

NOTE: To prevent incomplete gel formation, for coating we recommend using ≥ 4 mg/mL Matrigel®. As a guideline, a total volume of 5 mL diluted Matrigel® will adequately coat a single 96-well plate.

- To coat a single 96-well plate, add 2.5 mL of cold serum-free culture media to a pre-chilled polypropylene tube.
 - Using a cold serological pipette, slowly pipette 2.5 mL of 100% Matrigel® into the serum-free media and, taking care to avoid bubbles, slowly mix by pipetting the solution up and down.
- Pour prepared solution into a pre-chilled sterile reagent reservoir (keep on ice).
 - Using pre-chilled pipette tips and reverse pipetting technique, coat the pre-chilled 96-well plate by carefully adding 40 μ l of diluted Matrigel® into the center of each well.
 - While the plate is cold and Matrigel® is still liquid, gently rock the plate once within the Coolbox to ensure even coating of each well.

NOTE: To avoid cell penetration to the base of the plate, coat wells with a minimum of 40 μ L. Use of reverse pipetting technique is important to minimize bubbles.

1.5 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.

1.6 Place the plate in a 37°C incubator for 30 minutes to polymerize the Matrigel®.

2 Seed cells

2.1 Seed cells of interest (100 μ L per well if using cell health reagent, 150 μ L if not using cell health reagent) at an appropriate density on top of polymerized Matrigel® base such that by day 3, multi-spheroids have formed with the desired size (e.g. 30 – 80 μ m in diameter).

NOTE: Seeding density will need to be optimized for each cell type used. As an example and guide, we recommend seeding A549, MCF-7 and MDA-MB-231 at 1000 – 2000 cells per well or SKOV-3 at 2000 – 4000 cells per well.

3 Add Cell Health Reagent

NOTE: Annexin V (red or green) reagent requires solubilization in assay media before use. Centrifuge briefly to collect solid in bottom of vial and add 100 μ L assay media to achieve a 100% stock concentration.

3.1. Prepare Cell Health Reagent at 3x required concentration.

This concentration may require optimization for specific cell lines however as a guide we recommend the use of Cytotox reagents (red or green) at 250 nM final assay concentration, Annexin V (red or green) at 1% final assay concentration, and Caspase (green only) at 2.5 μ M final assay concentration.

- IncuCyte Cytotox reagent: Dilute the stock solution 1:1333 in complete medium to make a 750 nM (3x final assay concentration) working solution.
- IncuCyte Annexin V reagent: Dilute the stock solution 1:33 in complete medium to make a 3% (3x final assay concentration) working solution.
- IncuCyte Caspase Green reagent: Dilute the stock solution 1:666.7 in complete medium to make a 7.5 μ M (3x final assay concentration) working solution.

3.2. Add the Cell Health Reagent solution(s) on top of the cells (50 μ L total per well).

3.3. Place plate in a 37°C incubator for 30 minutes prior to scanning.

Day 0–3

4 Monitor Multi-Spheroid formation

- Place the cell plate into the IncuCyte® live-cell analysis system and schedule 24 hour repeat scanning:
 - Objective: 10x (96-well corning) 1 image per well
 - Channel selection; Phase Contrast + Brightfield and "Red" or "Green" depending on reagent used
 - Scan type: Spheroid, Spheroid Type: Multi
 - Scan interval: Every 6 hours

Day 3

5 Add Treatments

- Once multi-spheroids have reached desired size, remove the plate from the IncuCyte and carefully add appropriate treatments at:
 - No cell health reagent utilized: 4x final assay concentration (50 μ L per well)
 - With cell health reagent utilized: 11x final assay concentration (15 μ L per well).
- Continue to monitor multi-spheroid growth (e.g. every 6 hours for 7 days).

6 Re-feed cultures (optional and not recommended when using Annexin, Cytotox, or Caspase reagents)

Analysis Guidelines

Result: Size, number and viability/health measurements

Suggested Metric: Brightfield Object Area (Total, Average), Object Count

Secondary Metrics: Fluorescent metrics within a Bright field Object Boundary

- For Cell Health reagents use Mean Intensity within Brightfield Object Boundary
- For fluorescently labelled cell lines use Integrated Intensity within Brightfield Object Boundary.

Spectral Unmixing: To analyze Green reagent response in NuLight Red-labeled cells, remove 12% red from the green channel. To analyze Red reagent response in NuLight or CytoLight Green-labeled cells, there is no need for spectral unmixing.