PROTOCOL

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IncuCyte® S3 Multi-Spheroid Assay

This protocol describes a solution for creating multi-spheroids using 96-well flat bottom plates coated with Matrigel®. 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 (Essen Cat #9600-0019)

Recommended materials

- IncuCyte® Cool Accessories (Cat # 1500-0078)
  - CoolBox 96F System (Includes x1 Block with gelpack and Coolsink 96F)
- IncuCyte® NucLight Red or Green Lentivirus Reagent (Essen Bioscience, Cat #4476 or #4475)
- IncuCyte® CytoLight Red or Green Lentivirus Reagent (Essen Bioscience, Cat #4481 or #4482)

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 manufacture's 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.
- 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.

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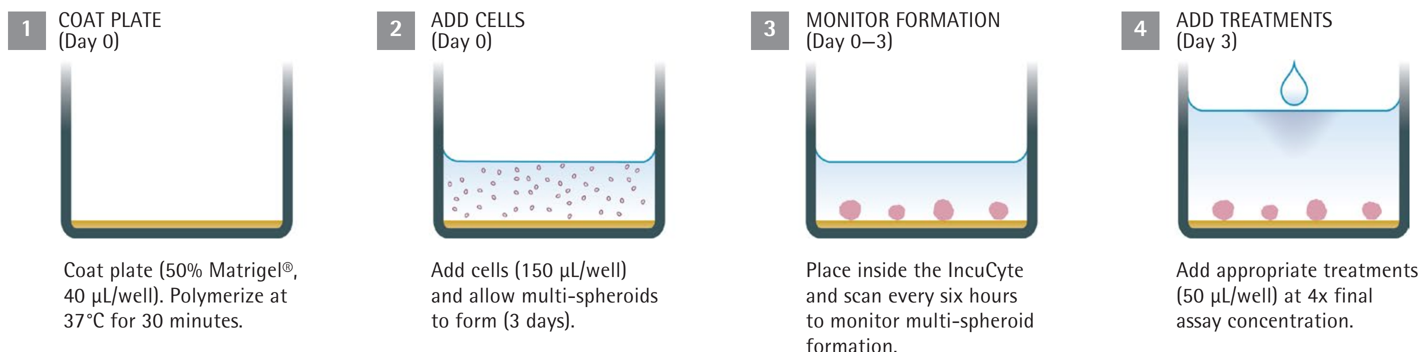
Quick Guide



Protocol

IMPORTANT:

1. In advance of multi-spheroid experiments, it is important to have stored the Cool pack accessories at the correct temperatures for at least 4h:
   a. Coolbox x 1 (block with gelpack: -20°C),
   b. Coolsink 96F x1 (4°C)
2. Keep all culture-ware and reagents coming in contact with Matrigel® on ice during the entire process.
3. Store pipette tips used for dispensing diluted Matrigel® at + 4°C.

Day 0

1 Coat plate with Matrigel®

1.1 In a cell culture hood, chill plates (10 – 15 minutes) on a pre-chilled Coolsink 96F within a Coolbox 96F box.

1.2 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®.
   a. To coat a single 96-well plate, add 2.5 mL of cold serum-free culture media to a pre-chilled polypropylene tube.
   b. 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.

1.3 Pour prepared solution into a pre-chilled sterile reagent reservoir (keep on ice).

1.4 Using pre-chilled pipette tips and reverse pipetting technique, coat the pre-chilled 96-well plate by carefully adding 40 µL of diluted Matrigel® into the center of each well.
   a. 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 µL. 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 (150 µL per well) 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 µm in diameter).
NOTE: Seeding density will need to be optimized for each cell type used. As a 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.

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

Day 0–3

3 Monitor multi-spheroid formation

3.1 Place the cell plate into the IncuCyte® live-cell analysis System and schedule 24 hour repeat scanning:
   a. Objective: 10x (96-well corning) 1 image per well
   b. Channel selection: Phase Contrast + Brightfield and "Red" or "Green" if required
   c. Scan type: Spheroid, Spheroid Type: Multi
   d. Scan interval: Every 6 hours

Day 3

4 Add treatments

4.1 Once multi-spheroids have reached desired size, remove the plate from the IncuCyte and carefully add appropriate treatments at 4x final assay concentration (50 µL per well).

4.2. Continue to monitor multi-spheroid growth (e.g. every 6 hours for 7 days).

5 Re-feed cultures (optional)

5.1 Re-feed cultures every 3 – 4 days. Remove plate from IncuCyte. Carefully remove 100 µL of media per well and replace with 100 µL of media containing test agents (1x final assay concentration).

5.2 Return plate to the IncuCyte and continue to monitor multi-spheroid growth.

Analysis Guidelines

Result: Size, number and viability/health measurements

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

Secondary metrics: Fluorescent metrics within a Brightfield Object Boundary