SARTORIUS

Protocol

Incucyte® 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. No. 3595)
- Matrigel®, protein concentration ≥ 8 mg/mL (Corning Cat. No. 356234)
- Wet ice
- Serum-free cell culture media for Matrigel® dilutions
- Complete culture media for cell culture and assay
- Manual multi-channel pipette
- Incucyte® Spheroid Analysis Software Module, version 2018A or newer (Cat. No. 9600-0019)

Optional Materials

- Incucyte® Cool Accessories (Cat. No. 4444)
  - CoolBox 96F System (Includes x2 Block with gelpack and CoolSink 96F)
- Incucyte® Cytotox Red or Green Dye (Cat. No. 4632 or 4633)
- Incucyte® Annexin V Red or Green Dye (Cat. No. 4641 or 4642)
- Incucyte® Caspase 3/7 Green Dye (Cat. No. 4440)
- Incucyte® Nuclight Green, Red, Orange or NIR Lentivirus (Cat. No. 4475, 4476, 4771 or 4805)
- Incucyte® Cytolight Red or Green Lentivirus (Cat. No. 4481 or 4482)

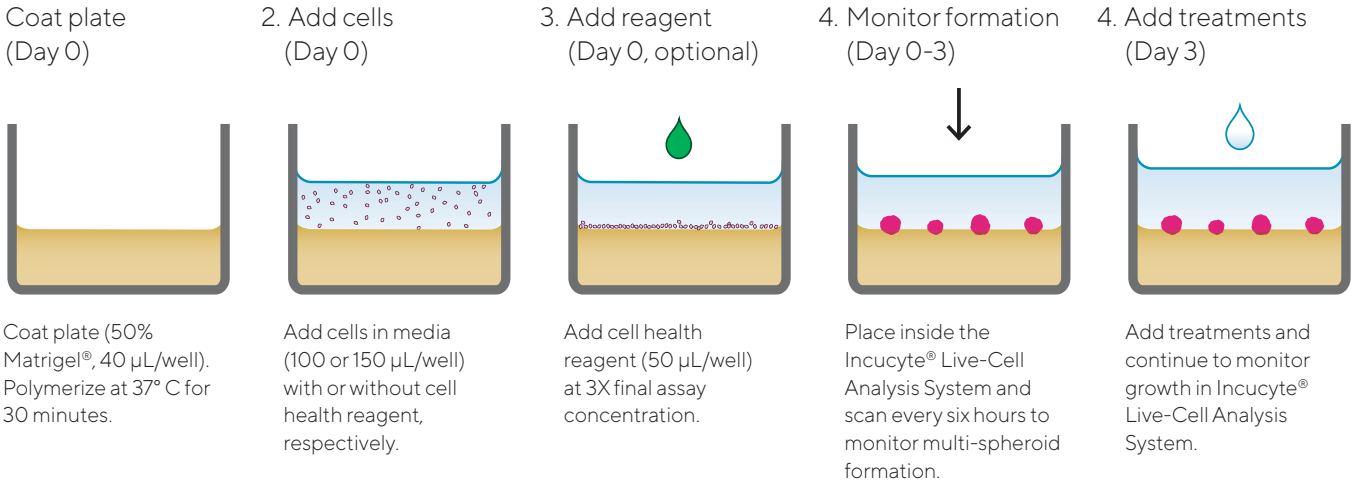
  Note: Transfect cells with Nuclight or Cytolight Lentivirus prior to performing multi-spheroid experiments by following the protocols supplied with the reagents.

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 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.

Protocol

Quick Guide



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 4 hours:
   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

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®. As a guideline, a total volume of 5 mL diluted Matrigel® will adequately coat a single 96-well plate.

   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®.

Seed Cells

2.1 Seed cells of interest (100 µL per well if using cell health reagent, 150 µL 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 µm 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.

Add Cell Health Reagent

Note: Annexin V Dye requires solubilization in assay media before use. Centrifuge briefly to collect solid in bottom of vial and add 100 µL 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 Dyes at 250 nM final assay concentration, Annexin V Dyes at 1% final assay concentration, and Caspase 3/7 Dye (green only) at 2.5 µM final assay concentration.
   a. Incucyte® Cytotox Dye: Dilute the stock solution 1:1333 in complete medium to make a 750 nM (3X final assay concentration) working solution.
   b. Incucyte® Annexin V Dye: Dilute the stock solution 1:33 in complete medium to make a 3% (3X final assay concentration) working solution.
   c. Incucyte® Caspase 3/7 Green Dye: Dilute the stock solution 1:666.7 in complete medium to make a 7.5 µM (3X final assay concentration) working solution.

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

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

Day 0—3

Monitor Multi-Spheroid Formation

4.1. 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 Fluorescence depending on reagent used
   c. Scan type: Spheroid, Spheroid Type: Multi
   d. Scan interval: Every 6 hours

Day 3

Add Treatments

5.1. Once multi-spheroids have reached desired size, remove the plate from the Incucyte® Live-Cell Analysis System and carefully add appropriate treatments at:
   a. No cell health reagent utilized: 4X final assay concentration (50 uL per well)
   b. With cell health reagent utilized: 11X final assay concentration (15 µL per well).

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

Re-feed cultures (optional and not recommended when using Annexin, Cytotox, or Caspase 3/7 Dyes).

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

Find more information at www.sartorius.com/incucyte

Sales and Service Contacts

For further contacts, visit www.sartorius.com

Essen BioScience, A Sartorius Company
www.sartorius.com/incucyte
E-Mail: AskAScientist@sartorius.com

North America
Essen BioScience Inc.
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA
Telephone +1 734 769 1600
E-Mail: orders.US07@sartorius.com

Europe
Essen BioScience Ltd.
Units 2 & 3 The Quadrant
Newark Close
Royston Hertfordshire
SG8 5HL
United Kingdom
Telephone +44 1763 227400
E-Mail:
euorders.UK03@sartorius.com

APAC
Essen BioScience K.K.
4th Floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001
Japan
Telephone: +81 3 6478 5202
E-Mail: orders.US07@sartorius.com

Publication No.: 8000-0592-C00
Status: 09 | 2020