SARTORIUS

Protocol

Incucyte® Single Spheroid Assay

For the Quantification of Cell Viability and Spheroid Size

This protocol describes a solution for creating single spheroids using a 96- or 384-well round-bottom, ultra-low attachment plate. This method utilizes the Incucyte® Live-Cell Analysis System and the Incucyte® Spheroid Analysis Software Module for image-based Brightfield and fluorescence within the Brightfield boundary of spheroid area measurements.

Cell health reagents are used to report cell viability (Incucyte® Cytotox Dye) and apoptosis (Incucyte® Annexin V Dye or Incucyte® Caspase 3/7 Dyes) in parental (non-transduced) cells. Alternatively, cell lines expressing fluorescent protein can be used to monitor spheroid health.

Single Spheroid Assay—Cell Health Reagent

Materials

- Incucyte® Spheroid Analysis Software Module (Sartorius Cat. No. 9600-0019), required
- Incucyte® Cell Health Reagent
  - Incucyte® Caspase 3/7 Dye
  - Incucyte® Annexin V Dye
  - Incucyte® Cytotox Dye
  - Incucyte® Nuclight Lentivirus
- Matrigel® (Corning® Cat. No. 356234), optional
- 96-well round-bottom, ultra-low attachment plate (e.g., Corning® Cat. No. 7007, S-BIO Cat. No. MS-9096UZ, BRANDplates Cat. No. 7816 60, 7819 00, 7819 60)
- 384-well round-bottom, ultra-low attachment plate (e.g., S-BIO Cat. No. MS-9384UZ)

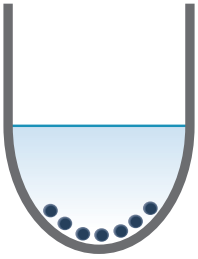
  Note: Combination of cell health reagents (Cytotox, Annexin V, Caspase 3/7, etc.) with Nuclight labeled cells is **not recommended** for use in the Incucyte® S-Series, but may be used in the Incucyte® CX3.

General Guidelines

- 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.

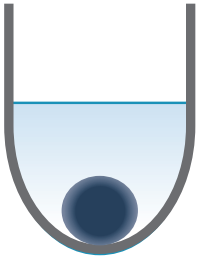
Protocol

1. Cell seeding
(Day 0)



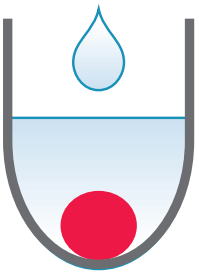
Seed cells into a 96-well or a 384-well ultra-low attachment plate. Centrifuge.

2. Spheroid formation
(Day 0–3)



Place plate inside the Incucyte® Live-Cell Analysis System and scan every six hours.

3. Add treatments and cell health reagent
(Day 3)



Add treatments to plate.
Monitor spheroid growth and shrinkage.

Day 0: Seed Cells

1. Seed cells of interest (100 µL per well for 96-well, 50 µL for 384-well) at an appropriate density into a 96- or 384-well ultra-low attachment (ULA) plate such that by day 3, spheroids have formed with the desired size (e.g., 200–500 µm after 3 days). Seeding density will need to be optimized for each cell line used, however, we recommend a range of 1,000–5,000 cells per well (10,000–50,000 cells per mL seeding stock).
   Note: Some cell lines may require the addition of a basement membrane extract, typically 2.5% v/v Matrigel®, to promote tight spheroid formation.
2. Centrifuge the ULA plate (125 g, 10 minutes) at room temperature (20–25 °C).
3. Place plate in a 37 °C incubator for 30 minutes prior to scanning.

Day 0–3: Spheroid Formation

1. Place the cell plate into the Incucyte® Live-Cell Analysis System and schedule 24 hour repeat scanning:
   For S-series: Set up a 24-hour repeat scanning schedule with the following parameters:
   a. Scan type: Spheroid
   b. Spheroid type: Single Spheroid
   c. Image Channels; Phase Contrast + Brightfield and Fluorescence depending on reagent used
   d. Objective: 4X or 10X
   e. Scan interval: Every 6-8 hours
   For CX3: Confocal Multi-plane scanning is not recommended until day 3 post spheroid formation.
   For Brightfield and wide-field single-plane fluorescence acquisition: Configure the Incucyte® Live-Cell Analysis System with the following settings for a 24-hour scanning schedule:
   a. Scan type: Spheroid
   b. Spheroid type: Single Spheroid
   c. Imaging mode: Wide-Field, Single-plane
   d. Image Channels: Phase Contrast + Brightfield and Fluorescence depending on reagent used
   e. Objective: 4X or 10X
   f. Scan interval: Every 6-8 hours

Day 0–3: Add Treatments and Cell Health Reagent (optional)

1. Once single spheroids have reached desired size (e.g., 200–500 µm), prepare Cell Health Reagent at 4X final assay concentration 50 µL addition per well for a 96-well plate. For a 384 well plate, prepare reagents at a 6X final assay concentration, and add 12.5 µL per well.
2. Cell Health Reagents suggested final concentrations (optimize for specific conditions)
   - Incucyte® Cytotox Green Dye (Cat. No.4633)—25 nM
   - Incucyte® Cytotox Red Dye (Cat. No.4632)—250 nM
   - Incucyte® Cytotox NIR Dye (Cat. No.4846)—0.6 µM
   - Incucyte® Annexin V Green Dye (Cat. No.4642) —1:200 dilution
   - Incucyte® Annexin V Red Dye (Cat. No.4641) —1:100 dilution
   - Incucyte® Annexin V Orange Dye (Cat. No.4759) —1:200 dilution
   - Incucyte® Annexin V NIR Dye (Cat. No.4768) —1:200 dilution
   - Incucyte® Caspase 3/7 Green Dye (Cat. No.4440) —1:1000
   - Incucyte® Caspase 3/7 Red Dye (Cat. No.4704)—1:200
3. **Add Treatments**
   a. If no Cell Health Reagent is applied: Use a 2X final assay concentration and add 100 µL per well for a 96-well plate. For 384 well plates, use a 3X final assay concentration and add 25 µL to each well.
   b. If a Cell Health Reagent is applied: Use a 4X final assay concentration and add 50 µL per well for 96-well plates. For 384 well plates, use a 6X final assay concentration and add 12.5 µL per well.

4. 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.
5. Place plate in a 37 °C incubator for 30 minutes prior to scanning.
   For S-Series: Continue to monitor spheroid growth as per the previous specifications (e.g., every 6-8 hours for 7 days).
   For CX3:
   a. If imaging in confocal mode with multi-plane acquisition is preferred, a new vessel must be created and scheduled to scan every 8 hours for a duration of up to 7 days.
   b. For wide-field mode with single-plane acquisition, continue imaging the test vessel every 6-8 hours for up to 7 days, using the settings previously specified.

Day 7 Onwards (optional, for 96-well plates)

1. Re-feed spheroids every 96 hours. Remove ULA plate from the Incucyte® Live-Cell Analysis System. Carefully remove 100 µL of media per well and replace with 50 µL of culture media supplemented with cell reagent and 50 µL containing test material at 1X final assay concentration.
2. Return plate to the Incucyte® Live-Cell Analysis System and continue imaging (e.g., every 6-8 hours).

Analysis Guidelines

Note: Utilize the Incucyte® Spheroid Analysis Software Module in the Brightfield channel to identify spheroid boundaries and analyze fluorescent label(s) as needed. See "Guidelines for Analysis," which can be accessed from the Incucyte® Technical Notes folder as part of the GUI installer.

1. For parental (non-transduced) cells—Brightfield Boundary Measurements
   - Result: Size of spheroid measurement
   - Suggested Metric: Largest Brightfield Object Area (avoid segmentation of small fragments)
2. For parental cells with cell health reagent(s)—Fluorescent and Brightfield Boundary Measurements
   - Result: Size, viability and mechanism of action based on the cell health reagent used
   - Suggested metric: Mean Intensity
3. For cells expressing fluorescent protein—Fluorescent and Brightfield Boundary Measurements
   - Result: Size and viability measurements
   - Suggested metric: Largest Brightfield Object Integrated Intensity
   - Secondary metric: Largest Brightfield Object Mean Intensity

Find more information at www.sartorius.com/incucyte

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