

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) 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® Cytotox Green or Red Dye (Sartorius Cat. No. 4633 or 4632)
- Incucyte® Annexin V Green or Red Dye (Sartorius Cat. No. 4642 or 4641)
- 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)

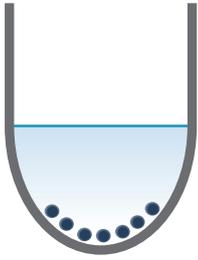
Note: Combination of cell health reagents (Cytotox, Annexin V, etc.) with Nuclight or Cytolight labeled cells is not recommended.

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.

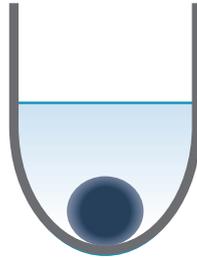
Protocol

1. Cell seeding (Day 1)



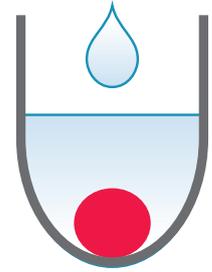
Seed cells into a 96-well or a 384-well ultra-low attachment plate. Centrifuge.

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 μ L per well for 96-well, 50 μ L 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 μ m 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).

Day 0–3: Spheroid Formation

1. Place the cell plate into the Incucyte® Live-Cell Analysis System and schedule 24 hour repeat scanning:
 - a. Objective: 4X or 10X (96-well ULA) or 10X (384-well ULA), 1 image per well
 - b. Channel selection: Phase Contrast | Brightfield + Fluorescence if using fluorescent label OR if a cell health reagent will be added post spheroid formation.
 - c. Scan type: Spheroid
 - d. Scan interval: Every 6 hours

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

1. Once spheroids have reached desired size (e.g., 200–500 μ m), remove the ULA plate from the incubator and carefully add culture media supplemented with cell health reagent (100 μ L per well for 96-well, 25 μ L per well for 384-well) containing test material (e.g. small molecules, antibodies; prepared at 2X final assay concentration for 96-well, 3X final assay concentration for 384-well).
2. Cell Health Reagents suggested concentration (optimize for specific conditions)
 - Incucyte® Cytotox Red Dye (Sartorius Cat. No.4632)—250 nM
 - Incucyte® Cytotox Green Dye (Sartorius Cat. No.4633)—25 nM
 - Incucyte® Annexin V Red Dye (Sartorius Cat. No.4641)—1:100 dilution
 - Incucyte® Annexin V Green Dye (Sartorius Cat. No.4642)—1:100 dilution
3. Continue to monitor spheroid growth (e.g., every 6 hours for 10 days).

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 μ L of media per well and replace with 100 μ L of culture media supplemented with cell reagent and 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 hours).

Single Spheroid Assay—Fluorescent Label

Materials

- Incucyte® Spheroid Analysis Software Module (Sartorius Cat. No. 9600-0019), required
- Incucyte® Nuclight Red, Green, Orange or NIR Lentivirus (EF-1 α , Puro) for nuclear labeling (Sartorius Cat. No. 4624, 4625, 4771 or 4805)
- Incucyte® Cytolight Red or Green Lentivirus (EF-1 α , Puro) for cytoplasmic labeling (Sartorius Cat. No. 4481 or 4482)
- 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)

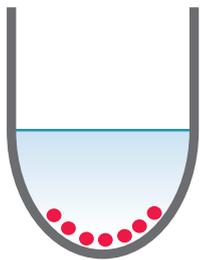
Note: Combination of cells expressing fluorescent proteins with cell health reagents (Cytotox, Annexin V) is not recommended

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.

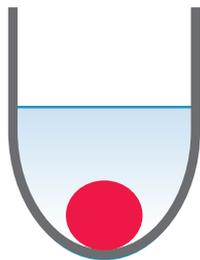
Protocol

1. Cell seeding (Day 1)



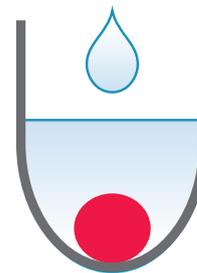
Seed cells into a 96-well or a 384-well ultra-low attachment plate. Centrifuge.

2. Spheroid formation (Day 0-3)



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

3. Add treatments (Day 3)



Add treatments to plate. Monitor spheroid growth and shrinkage.

Day 0: Seed Cells

1. Seed cells of interest (100 μ L per well for 96-well, 50 μ L 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 μ m 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).

Day 0-3: Spheroid Formation

1. Place the cell plate into the Incucyte® Live-Cell Analysis System and schedule 24 hour repeat scanning:
 - a. Objective: 4X or 10X (96-well ULA) or 10X (384-well ULA), 1 image per well
 - b. Channel selection: Phase Contrast | Brightfield + Fluorescence.
 - c. Scan type: Spheroid
 - d. Scan interval: Every 6 hours

Day 0–3: Add Treatments

1. Once spheroids have reached desired size (e.g., 200–500 μm), remove the ULA plate from the incubator and carefully add culture media supplemented with cell health reagent (100 μL per well for 96-well, 25 μL per well for 384-well) containing test material (e.g., small molecules, antibodies; prepared at 2X final assay concentration for 96-well, 3X final assay concentration for 384-well).
2. Continue to monitor spheroid growth (e.g., every 6 h for 10 days).
Note: It is not recommended to change media in this assay as it will disrupt spheroids containing necrosing or apoptotic cells.

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 (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 (e.g., Apoptosis: Annexin V, or Cytotox Dyes)
 - Suggested metric: Mean Intensity
3. For cells expressing fluorescent protein—Fluorescent and Brightfield Boundary Measurements
 - Result: Size and viability measurements
 - Suggested Metric: Integrated Intensity
 - Secondary metric: Mean Intensity

Find more information at www.sartorius.com/incucyte

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For further contacts, visit
www.sartorius.com

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