# Automated Technique to Examine the Cell Viability of 3D Cancer Spheroids using the OT-2

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## OVERVIEW

- We implemented a technique for assessing the cellular viability of three-dimensional cancer spheroids after therapeutic intervention involving the following:
  - A **resazurin-based** reagent known as **PrestoBlue HS** for reading fluorescence at excitation/emission maxima at 560 nm/590 nm
  - Automation of steps using **Opentrons OT-2 liquid handling platform** including seeding of cells for generation of spheroids, and administration of drugs to the spheroids
  - Utilization of three cell lines for generating 3D spheroids, lung carcinoma cell line A549, hepatocellular carcinoma cell line HepG2, and pancreatic cancer cell line PANC1.
  - **Cultivation of spheroids on Nunclon Sphera plates** with low attachment properties to promote the development of well-formed and compact spheroids.
  - **Dose-response curves** using Gambogic Acid for A549 cells, and Doxorubicin for HepG2 and PANC1, respectively.
  - A **non-linear regression analysis** to determine the variable slope of the logarithm of the inhibitor in relation to the response.
  - Study was performed using GraphPad Prism 10 software to compute the EC50.
- We carried out the **automation of 3D cell culture technique** with precision, reliability and reproducibility with consistency of spheroid size across replicates

## INTRODUCTION

3D cultures have emerged as the preferred model for preclinical research in the field of cancer biology. The process of generating 3D models of cancer entails several steps.

- First, it is necessary to select an appropriate cell type that accurately represents cancer.
- Next, growth and maintenance conditions for the 3D cultures must be optimized.
- Once the models are established, they need to be characterized to ensure their accuracy and reliability.
- Finally, the drug responses of these models are assessed to gain insights into the physiological effects of the drugs.

The development of a 3D spheroid model involves a series of five steps: culture, generation, optimization, characterization, and measurement of the response. The cell lines utilized in this study include A549, PANC1, and HepG2.

The PrestoBlue HS is capable of eliminating false positives and has a broad dynamic range when compared to environmental signals. The Presto Blue HS assay has the smallest detection threshold when compared to the assays that utilize resazurin fluorescence. By employing an enhanced reduction potential assay, one can thoroughly examine the dose-response curves of different drugs and their corresponding cell lines.

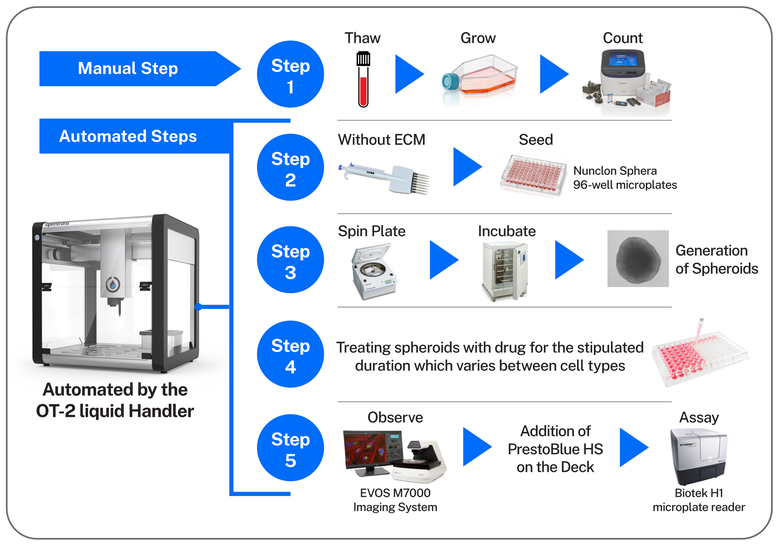
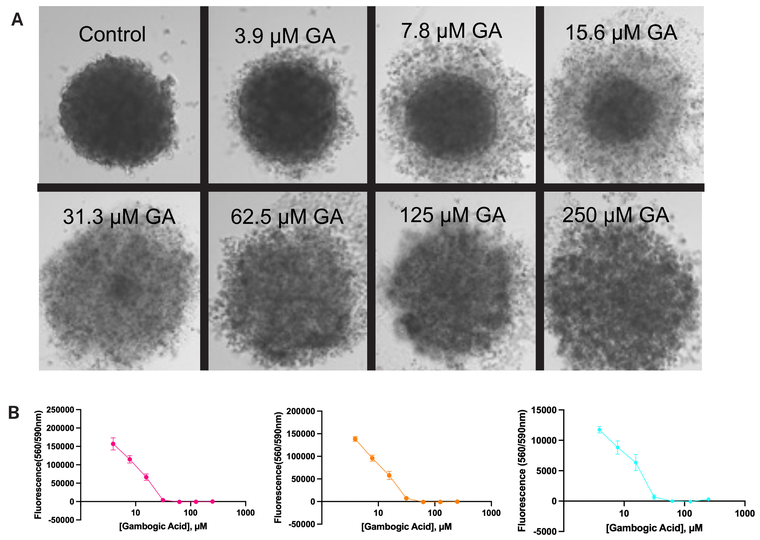



**Figure 1. Schematic representation of the process of generation of spheroids on Nunclon Sphera plates.** Adapted from Application note "Analysis of cancer spheroids through high-throughput screening assays", ThermoFisher Scientific.

## MATERIALS AND METHODS

- OT-2, P300 Single GEN2 pipette, P20 Single GEN2 pipette
- Nunclon Sphera 3D culture system, (ThermoFisher Scientific)
- Phenol red free medium- Ham's F12K medium (Crystalgen, # 226-062), DMEM high glucose (Gibco, # 31053028), MEM (Gibco, # 51200038)
- Presto Blue HS reagent (ThermoFisher Scientific, # P50201)
- A549 cells (ATCC, Manassas, VA, USA) (CCL-185) , HepG2 cells (ATCC, Manassas, VA, USA) (HB-8065), PANC1 (ATCC, Manassas, VA, USA) (CRL-1469)
- Doxorubicin (Selleckchem, S1208)
- Gambogic Acid (Selleckchem, S2448)
- Fetal Bovine Serum (FBS) not Heat Inactivated (ATCC, Cat No. 30-2020)
- Labware a) Opentrons 96 Filter Tip rack 200 µL b) Opentrons 96 Filter Tip rack 20 µL c) Opentrons 10 Tube Rack with Falcon 10X15 mL Conical d) Opentrons 24 Tube Rack with Eppendorf 1.5 mL Safe-Lock Snap cap (Two)

## RESULTS




**C A549 Cells**

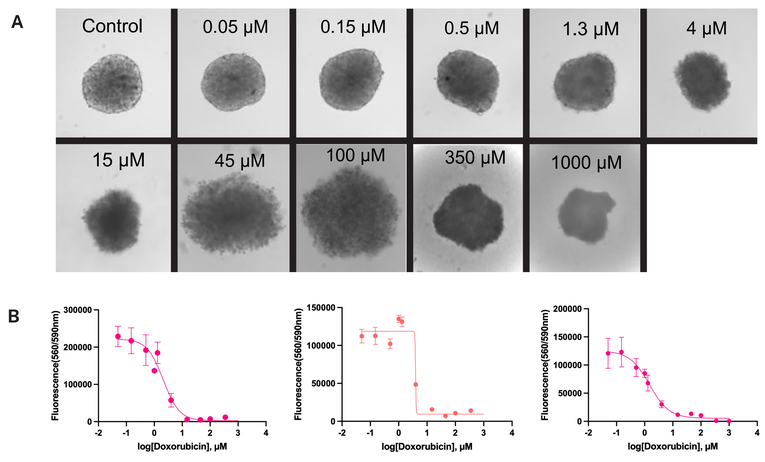
**Day 0** — 1 X $10^4$ cells/ well of A549 after taking a cell count and seed on Nunclon Sphera plates

**Day 2** — Treating spheroids after 48 hours of seeding with Gambogic Acid ( GA)

**Day 6** — Presto Blue HS addition to the spheroids after 96 hours that have been treated with GA, fluorescence recorded after 3 hours of addition

- Images captured using the EVOS M7000 (10X magnification) before and after the duration of the treatment
- Concentration of Gambogic acid is in µM concentrations
- The spheroids with higher concentration of drug show a reduction in spheroid size
- 3.9 – 250 µM Gambogic Acid concentrations used for the study
- At 48 hours after seeding, prior to drug treatment, the spheroids are loose and not compact
- Tight spheroids seen after 72 hours in case of untreated cells
- As concentration of GA acid increases, the size of spheroid diminishes. In case of 125 and 250 µM GA, the spheroids disintegrate and the tight structure is lost

**Figure 2. Measuring cellular health on A549 3D spheroids cellular model.** (A) Effects of Gambogic Acid on A549 spheroids in 3D cultures grown on Nunclon Sphera plates. (B) Dose response curves for gambogic acid-treated A549 spheroids in 3D cultures; EC50 value is 7.84. (C) Diagram illustrating the A549 spheroid assay and its essential components.




**C HepG2 Cells**

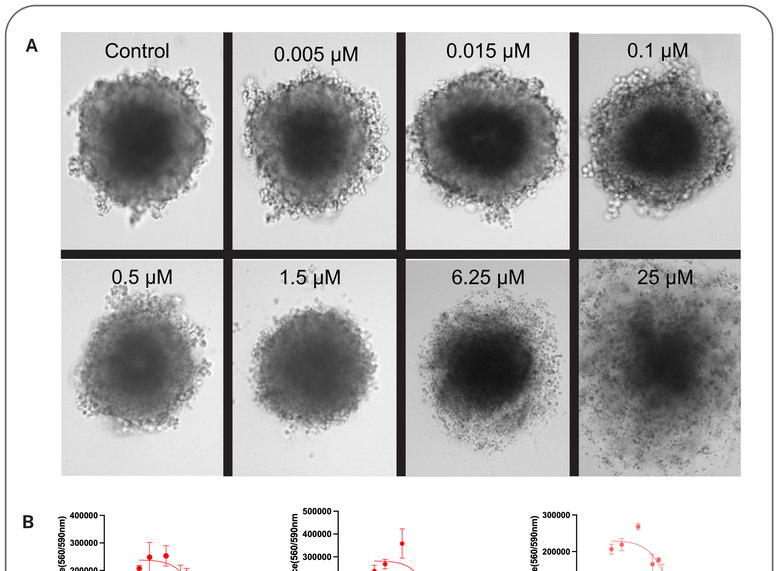
**Day 0** — 2500 cells/ well of HepG2 after taking a cell count and seed on Nunclon Sphera plates

**Day 4** — Treating spheroids after 96 hours of seeding with Doxorubicin

**Day 7** — Presto Blue HS addition to the spheroids 72 hours post treatment with Dox

- Images captured using the EVOS M7000 (10X magnification) before and after the duration of the treatment
- Concentration of Doxorubicin is in µM concentrations
- The spheroids with higher concentration of drug show a reduction in spheroid size
- 0.05 – 1000 µM Doxorubicin concentrations
- At 96 hours after seeding, prior to drug treatment, the spheroids have an irregular shape
- Tight spheroids are seen after 72 hours of treatment
- As concentration of DR increases, the spheroids become fuzzy in appearance . The tight structure is lost and at concentrations 4 and above the spheroids become loose and fluffy like cotton balls

**Figure 3. Morphology and effectiveness of doxorubicin treatment on HepG2 spheroids in 3D cultures.** (A) Morphology of control and doxorubicin-treated HepG2 spheroids after 72 hours. Scale bar: 350 µm. (B) a, b, c Dose response curves for doxorubicin-treated HepG2 spheroids in 3D cultures; EC50 value is 2.3. (C) Key features of the HepG2 spheroid assay




**C PANC1 Cells**

**Day 0** — 2500 cells / well of PANC1 after taking a cell count and seed on Nunclon Sphera plates

**Day 7** — Treating spheroids after 7 days of seeding with Doxorubicin

**Day 10** — Presto Blue HS addition to the spheroids after 72 hours that have been treated with DR

- Images captured using the EVOS M7000 (10X magnification) before and after the duration of the treatment
- Concentration of Doxorubicin is in µM concentrations
- The spheroids with higher concentration of drug show a reduction in spheroid size
- 0.005– 500 µM Doxorubicin concentrations
- At 7 days after seeding, prior to drug treatment, the spheroids are loose and not compact
- Tight spheroids seen after 72 hours in case of untreated cells
- At concentration of 6.25 µM, the size of spheroid diminishes. In case of 25 and 500µM DR, the spheroids become loose and disintegrate, the tight structure is lost

**Figure 4. Morphology and efficacy of doxorubicin on 3D PANC1 spheroids.** (A) Structure of control and doxorubicin-treated PANC1 spheroids Scale bar: 350 µm. (B) Dose response profiles for PANC1 spheroids treated with doxorubicin in three-dimensional cultures; EC50 value is 2.39. (C) Schematic showing the timeline and key features of PANC1 spheroid assay

## CONCLUSION

- OT-2 liquid handler can perform the automation of an assay to determine the cell viability of 3D spheroids after drug treatment
- OT-2 can process a moderate to high throughput number of samples in a 96 well format, in this case Nunclon Sphera plates with low attachment.
- OT-2 can mimic the manual protocol without loss of time and highly reproducible

## DISCUSSION

Though cancer spheroids are difficult to analyze , various cell-culture based assays can be optimized to test drug responses in cancer cells grow in 3D. A microplate readout using PrestoBlue HS for measuring cell viability is used for analysis of 3D spheroids after drug treatment. The spheroids and the efficacy of the assay can be increased by increasing the incubation time with the drug and also increasing the incubation time of the Presto Blue HS reagent with the media containing spheroids.

## REFERENCES

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2. Analysis of cancer spheroids through high-throughput screening assays, Application Note, ThermoFisher Scientific, 2020.
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