In Vitro Application

APPLICATION NOTE:

LifeGel PANC-1 IC_{50} Determination Using CellTiter-Blue® Assay and Hoechst.

3D spheroid cultures are typically less sensitive to cytotoxic compounds than standard 2D cell cultures. Recognizing these differences is key to improving the clinical relevance of in vitro drug testing.

This application note outlines how to measure drug sensitivity (IC_{50}) using 96-well LifeGel plates with a resazurin-based viability assay (CellTiter-Blue). While resazurin assays are a convenient, indirect measure of cell health, they may not always accurately reflect cell number. DNA quantification (e.g., using Hoechst 33342) is more labor-intensive—especially in hydrogels—but often provides a better estimate of cell count.

Both viability and DNA-based assays were tested for use with LifeGel plates. For accurate comparison between 2D and 3D cultures, experiments should ensure similar cell proliferation rates, which were confirmed under our test conditions. The DNA assay can also be performed on the same plates after the CellTiter-Blue readout.

Materials required:

- PANC-1 human pancreatic cancer cells
- Complete culture medium (e.g., DMEM + 10% FBS + antibiotics)
- 96- or 384-well LifeGel plate
- Tested compoung/drug (doxorubicin, SN38, 5-FU, oxaliplatin)
- CellTiter-Blue® Cell Viability Assay (Promega)
- Hoechst
- Pipette/multichannel pipettor
- Plate reader (fluorescence, ex/em: 560/590 nm)
- GraphPad Prism or equivalent software for IC_{50} calculation

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3D Protocol Overview

Seed 600–1200 cells per well in 150 µL medium (and include some no cell controls). Allow 3D spheroids growth over 7–14 days.

DAY 1 - Prepare test compound dilutions in culture medium – 150 µL of solution for each assay well.

Aspirate medium from spheroid containing assay plate and replace with 135 µL of the appropriate medium + /- test compound dilution. Incubate assay plate in a humidified CO2 incubator for 72 hours.

DAY 4 - Add 25 µL of CellTiter-Blue reagent to all wells and incubate plate at 37 °C for 1–4 hours, then read fluorescence (572 ex / 615 em)

2D Protocol Overview

Seed 2400 PANC-1 cells per well in 135 µL medium (and include some no cell controls).

DAY 2 - Prepare test compound dilutions in culture medium – 150 µL of solution for each assay well.

Quickly aspirate medium from seeded assay plate and replace with 135 µL of the appropriate medium + /- test compound dilution. Incubate assay plate in a humidified CO2 incubator for 72 hours.

DAY 5 - Add 25 µL of CellTiter-Blue reagent to all wells and incubate plate at 37 °C for 1 hour, then read fluorescence (572 ex / 615 em). If DNA quantitation is also required, aspirate well contents and replace with 150 µL of 70% ethanol, storing sealed plates at 4°C until required

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PROTOCOL:

1. Plate setup

- An actively dividing 2D monolayer of PANC-1 cells was detached using trypsin, following standard procedures. Repeated pipetting and microscopic examination were used to eliminate cell clumps.
- A final cell suspension was prepared in DMEM supplemented with 10% FBS, such that each 150 µL aliquot contained 600–1200 cells.
- Each 96-well LifeGel plate was used to assay 2–4 compounds. For each compound, a **9- or 10-point 3-fold dilution series** was prepared and added to wells in **duplicate or triplicate**. At least **4 wells without compound** and **4 wells without cells** were included as controls.
- Before adding cells, the medium above the LifeGel surface was carefully aspirated. Then, **150 µL of the cell suspension** was added to the wells requiring cells, and **150 µL of medium alone** was added to the 'no-cell' control wells.
- Plates were incubated in a humidified incubator at **37 °C with 5% CO_2** for **7–14 days** to allow spheroid formation. Culture medium was replaced every **4–6 days**.

2. Compound Preparation

- Pre-prepared dilutions of **doxorubicin**, **SN38**, and **5-FU** were used, as they remained stable when frozen at −20 °C. However, **oxaliplatin** was prepared fresh due to instability after dilution.
- Since each well contained approximately **135 µL of LifeGel**, the addition of an equal volume (135 µL) of drug-containing medium resulted in the **effective halving** of the final drug concentration after diffusion. Stock concentrations were therefore adjusted accordingly (If your desired final concentration in the well is *X*, you must prepare your dosing solution at **2X).**

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3. Test Compound Addition

- Once spheroids had formed, the medium above the LifeGel was carefully aspirated. Then, using a multichannel pipette, **135 µL of each compound dilution or control** was transferred to the corresponding wells.
- Plates were incubated at **37 °C with 5% CO_2** for **72 hours**.

4. CellTiter-Blue® Assay

- After 72 hours of treatment, **CellTiter-Blue reagent** was thawed at room temperature, protected from light. The reagent was placed in a reservoir, and **25 µL was added** to each well.
- The assay plates were returned to the incubator. Fluorescence was measured at **1, 2, 3, and 4 hours** post-reagent addition using a plate reader (e.g., **572 nm excitation, 615 nm emission**). A single 1-hour reading was usually sufficient, provided that the 'cells + no compound' wells had at least twice the signal of the 'no cell' controls.

*Longer incubation times improved results if cell growth was slow, but excessively long incubation or too many cells could lead to substrate depletion and nonlinear fluorescence.

5. DNA Quantification with Hoechst H33342 (Optional)

- After the CellTiter-Blue assay, all media and compound solutions were aspirated. **165 µL of 70% ethanol** was added to each well to fix the cells. Plates were sealed and stored at **4 °C overnight** (or up to several days) or frozen at **−20 °C** for longer-term storage.
- A staining solution containing **20 µM Hoechst H33342** in buffer (20 mM Tris-HCl, 150 mM NaCl) was prepared. After ethanol was aspirated from each well, **150 µL of stain** was added and incubated at **room temperature for 60 minutes**.
- The stain was removed using a multichannel pipette or aspiration head adapted to LifeGel depth. Wells were washed **twice for 30 minutes each** with **pH 5 wash buffer** (2 mM $CaSO_4$, 2 M NaCl, 0.2% Tween 20, 20 mM citrate buffer, pH 5.0), followed by **four 30-minute washes** with **pH 7 wash buffer** (150 mM NaCl, 20 mM phosphate buffer, pH 7.0).
- Fluorescence was measured immediately after the final wash.

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Results were improved by reading the plate in 1536-well format since this increased resolution and well area coverage detects any fluorescence localised away from the centre of the wells e.g. in spheroids growing towards the edges of wells

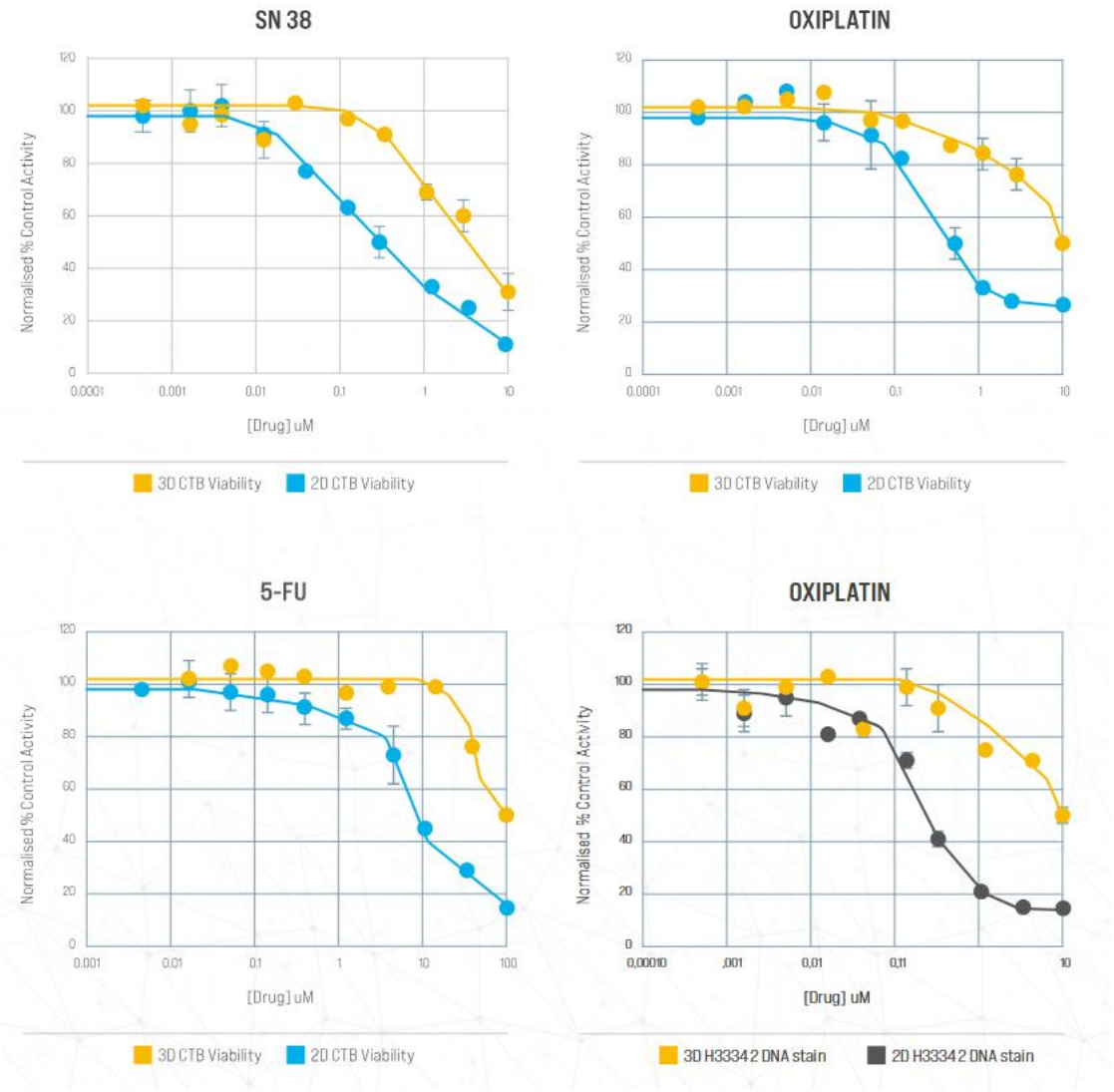

Fig.1. Initial 2D versus 3D IC50 data for Panc-1 CellTiter-Blue Viability Assay Results

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Tips

Normalize Fluorescence Readings
After you run the CellTiter-Blue® assay:

- Subtract background:

$$F_{\text{net}} = F_{\text{sample}} - F_{\text{no cells control}}$$

- Normalize to no-drug control (assumed 100% viability):

$$\text{Relative Viability (\%)} = \left(\frac{F_{\text{net}}}{F_{\text{no drug control}}} \right) \times 100$$

Plot and Determine IC_{50}

Plot **$\log_{10}$(actual final concentration)** (after diffusion correction!) vs **% viability**.
Fit a sigmoidal dose-response curve (e.g., using GraphPad Prism or similar).
Extract **IC_{50}** from the fitted curve.