Application Note

LIFEGEL PANC-1 IC50 DETERMINATIONS USING CELLTITER-BLUE AND HOECHST

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DESCRIPTION

3D spheroid structures are generally reported to be less sensitive to many cytotoxic tool compounds than conventional 2D cell cultures. Identifying these potential differences in drug sensitivity can be an important step in understanding the physiological relevance of laboratory results to those in the clinic. This application note primarily describes how to perform drug sensitivity testing (IC50) using 96-well LifeGel plates and a resazurin-based cell viability readout (CellTiter-Blue).

There are many biochemical assays available for the determination of cell viability – we have concentrated on the resazurin-based Promega CellTiter Blue reagent, but the assay techniques are equally applicable to other reagents. Viability assays are a good indirect way to easily determine general cell health and can also reflect with reasonable accuracy the number of healthy cells. However, there will be scenarios where cell health and cell number are not fairly reflected. Quantitation of cellular DNA on the other hand is generally more involved, particularly in hydrogels; however, it should also be a more accurate indirect measure of cell number compared with viability assays. Since both cell health and cell number are helpful parameters with which to assess the effect of potential chemotherapeutic drugs, both assay end-points have been evaluated for suitability in LifeGel plates.

A valid comparison between 2D and 3D drug sensitivities should include a check on whether a similar number of control cell divisions has occurred during the time frame of both experiments. We have previously shown that very similar replication rates occur in 2D and 3D assays under our seeding and testing conditions. Note that with the assay protocols described here, the H33342 assay for DNA can be carried out on the same IC50 plates after first measuring cell viability using CellTiter-Blue reagent.

EQUIPMENT

1. laminar chamber
2. incubator
3. centrifuge
4. fluorescent reader
5. multichannel pipette
6. preferably a vacuum line for aspiration

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MATERIALS AND REAGENTS

1. LifeGel plates
2. DMEM medium
3. FBS
4. eppendorfs
5. falcons
6. pipette tips
7. CellTiter-Blue
8. doxorubicin
9. SN38
10. 5-FU
11. oxiplatin
12. trypsin
13. ethanol
14. Hoechst

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PRIOR TO ASSAY

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 CO_2 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 CO_2 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.

PROCEDURE

Cell seeding

An actively dividing 2D monolayer culture of PANC-1 cells is removed from a culture flask with trypsin treatment according to standard laboratory procedures. Repeated pipetting action and microscopic examination are used to ensure that cell clumps are eliminated as much as possible.

A final cell suspension in DMEM + 10% FBS is made such that every 150 µL of medium needed per well would contain between 600 - 1200 cells. Normally, 9- or 10-point 3-fold dilution series of compound would be assayed in duplicate or triplicate wells, plus at least 4 'no compound' control wells and 4 'no cells' control wells. In this way, one 96-well plate yields IC50 data for between 2 - 4 compounds.

Medium above the LifeGel surface in wells is first carefully aspirated. 150 µL of cell suspension are then added to all wells requiring cells, and 150 µL of just medium added to 'no cell' control wells.

Cells are allowed to attach and form 3D spheroid structures in a humidified incubator (5% CO_2) at 37 °C over a period of 7 – 14 days. Culture medium is replaced in all wells every 4–6 days during the spheroid growth stage.

Preparation of test compound dilutions

Pre-prepared dilutions of doxorubicin, SN38 and 5-FU have been shown to be relatively stable when frozen at -20 C. However, oxiplatin is particularly unstable after dilution. It is therefore recommended to prepare test compound dilutions fresh unless stability is known in advance. Remembering that in 96-well LifeGel plates the volume of LifeGel is ~ 135 µL, when another 135 µL of medium + / - test compound is added to the LifeGel surface, the final compound concentration is effectively halved (after a few hours of diffusion at 37°C). Appropriate highest concentrations of test compounds need to be determined from the literature and / or experimentation and doubled to take into account dilution in the LifeGel matrix.

Test compound addition and incubation

Culture medium is carefully aspirated from above the LifeGel surface of the seeded 96-well assay plate. Using a (multichannel) pipette, 135 µL of each control/compound dilution is transferred from a drug dilution plate to the corresponding wells of the assay plate.

The lidded assay plates are then incubated for the desired growth period in a humidified incubator; our protocols have been validated for 72 hours compound treatment time.

CellTiter Blue Assay

After completion of 72 hours growth, sufficient CellTiter-Blue reagent was defrosted at room temperature (25 µL per well), protecting the reagent from bright light if possible. Defrosted reagent was placed in a reservoir, and 25 µL gently added to the side wall of each well.

Assay plates are returned to the incubator, and fluorescence measurements taken at 1, 2, 3 and 4 hours, if possible. A single reading after 1 hour is usually acceptable if the assay is performing as it should – 'cells plus no compound' control values should be at least double the 'no cells' control values if possible – the higher the signal to background ratio the better. Incubation times longer than 1 hour can help if fewer cells were seeded or growth has been relatively slow. However, be aware that overly long incubation periods or too many seeded cells can lead to the CellTiter-Blue substrate being depleted during the assay and the fluorescence readout no longer being directly proportional to cell viability or cell number (monitoring several time points can help in deciding the most relevant time point to use). Fluorescent resorufin product is quantitated using a plate reader and the appropriate filters (e.g. 572 nm excitation, 615 nm emission).

If H33342 staining for DNA quantitation is to be performed, all of the culture medium and test compound solutions should be aspirated carefully from above the LifeGel surface in wells. Cells are then fixed by addition of 165 µL of 70% ethanol to all wells, a sealing film applied to the plate, and the plate then left overnight at 4°C ready for DNA staining. Plates may be left at 4°C for several days before staining, or frozen at -20°C for longer periods of time before staining.

Hoechst H33342 DNA staining

A 20 µM stock of H33342 in {20 mM Tris HCl, 150 mM NaCl} sufficient for 150 µL per assay well is prepared and protected from light. After aspiration of the 70% ethanol from wells, 150 µL of 20 µM H33342 stain are gently added to all wells and left at room temperature for 60 minutes.

The staining solution is removed using multichannel pipette tips (or an 8-channel aspiration head adapted for the LifeGel depth), replaced with 165 µL of pH 5 wash buffer {2 mM CaSO4, 2M NaCl, 0.2% (v/v) Tween 20, 20 mM citrate buffer pH 5.0} and left for 30 minutes. A second 30 minute pH 5 washing step is performed in the same way, followed by four 30 minute washes with pH 7 wash buffer {150 mM NaCl, 20 mM phosphate buffer pH 7.0}. Immediately after addition of the fourth pH 7 wash buffer the assay plate could be read for DNA-bound H33342 fluorescence. Results are 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.

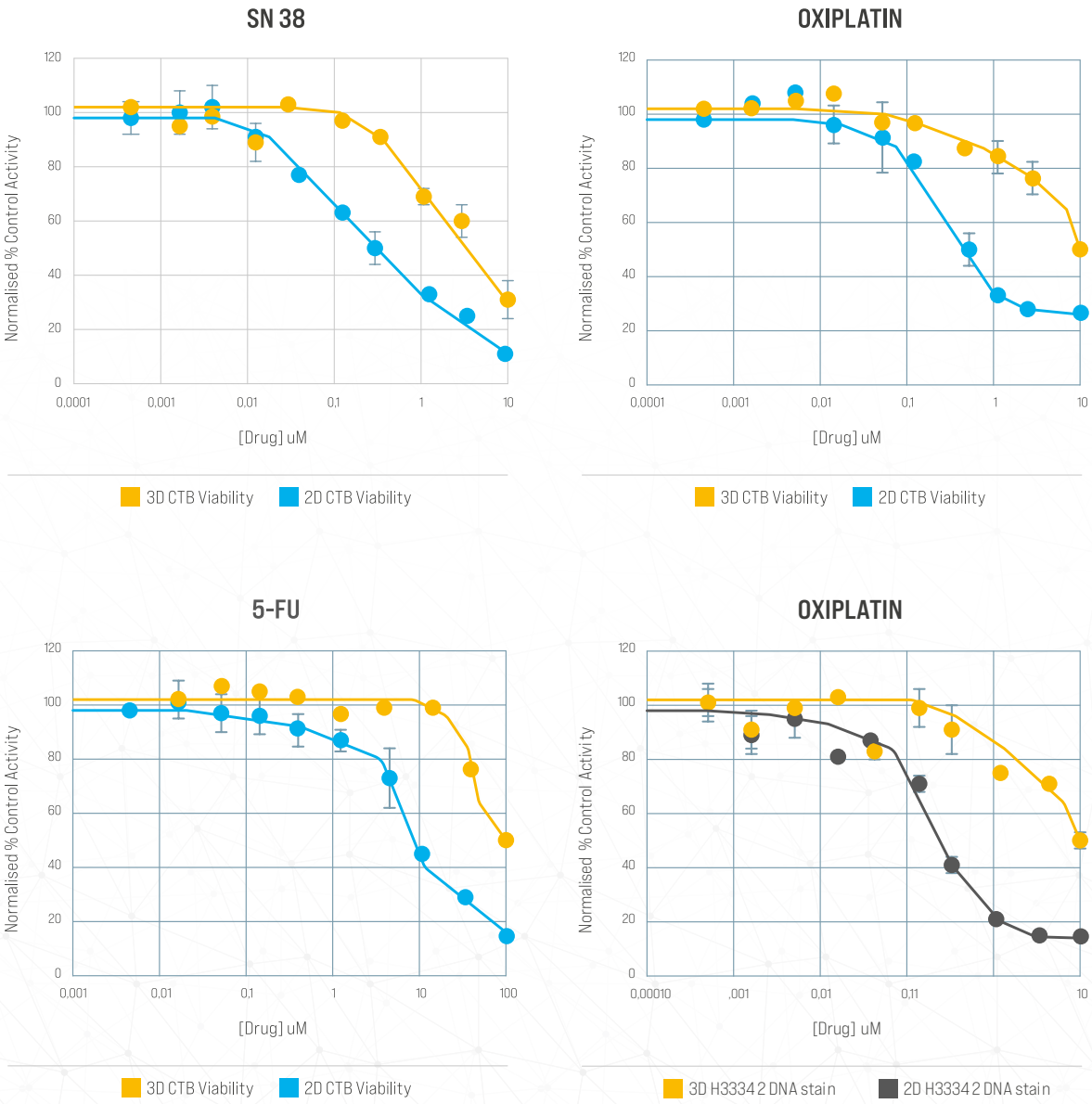

Fig.1. Initial 2D versus 3D IC50 data for Panc-1 CellTiter-Blue Viability and Hoechst H33342 DNA Quantitation Assay Results.

Find out more on LifeGel and another application notes at
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