Application Note

Cancer Research

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Image-Based Cell Cytotoxicity Screening Using Agilent 384-Well Microplates

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Introduction

As many as a third of all putative drugs fail from toxicity issues, which contribute to the high cost of pharmaceutical R&D, particularly if toxicity is proven in clinical trials. Thus, toxicity testing of small molecules is typically performed preclinically using cell-based, in-vitro assays in microplates, as a first step to establishing any toxicity concerns. For statistically significant determinations, it is important to capture enough cellular events within a well along with sufficient replicate experimental wells.

This application note demonstrates that the Agilent 384-well clear-bottom, black-sided cell culture and imaging plate (Figure 1) is a versatile microplate to effectively conduct cytotoxicity screening assays, and a wide range of other applications. This plate provides a four-fold increase in the number of wells in the same footprint as a standard 96-well microplate, while adhering to ANSI/SLAS standards for outside dimensions and well-to-well internal spacing, allowing for reagent additions and wash cycles to be automated.

Figure 1. Agilent 384-well black-sided, clear-bottom microplate.

In addition, the coverslip-thin 190 µm imaging bottom enables the capture of high-quality images using widefield and confocal microscopy (Figure 2). Using a library of previously identified cytotoxic compounds, this study describes the use of Agilent 384-well tissue culture plates to perform assay optimization and screening applications to ascertain viability of compound-treated cells.

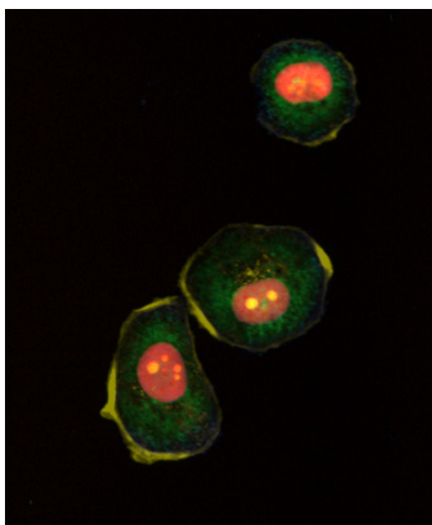

Figure 2. High-resolution imaging of T47D-Red cells. Cells were fixed, stained, and imaged with an Agilent BioTek Cytation C10 confocal imaging reader using a 40x objective to demonstrate the microplate's compatibility with confocal imaging. Images reveal detailed cellular features that can aid in the evaluation of treatment responses.

Materials and methods

RPMI-1640, fetal bovine serum (FBS), and antibiotics were purchased from Life Technologies (Carlsberg, CA). T47D cells (part number HTB-133) were originally purchased from ATCC (Manassas, VA) and modified to express mKat2 fluorescent protein in their nucleus using Agilent eLenti-Red particles (part number 8711011) as described in the reagent instructions. Tamoxifen and mechlorethamine were from Tocris BioSciences (Minneapolis, MN). The Selleck-Pfizer licensed library (part number L2400) was purchased from Selleck Chemicals LLC (Houston, TX). Agilent eTox Green (part number 8711008) and black-sided, clear-bottom 384-well microplates (part number 204628-100) were used in this study.

T47D-Red cells were cultured in RPMI-1640 media supplemented with 0.2 U/mL bovine insulin, 10% fetal bovine serum, and penicillin-streptomycin at 37 °C in 5% CO_2. Cells were plated into Agilent 384-well microplates such that there were 2,000 total cells per well in 50 µL media. After 24 hours, to allow for attachment, dose-titrations of various compounds were added to the cultures in 25 µL at three times the final drug concentration. After 23 hours exposure, 25 µL media with eTox Green was added to a final concentration of 250 nM, and after 60 minutes of incubation cells were fixed with 4% paraformaldehyde.

For screening purposes, all wells were imaged in widefield using an Agilent BioTek Cytation 5 cell imaging multimode reader in the TRITC and GFP channels with a 4x objective. Camera exposure values were set with the Agilent BioTek Gen5 microplate reader and imager software auto-exposure routine for both channels on control wells before imaging the plate. Separate cell count analysis routines for nuclei in the GFP and TRITC channels were performed, and the data output expressed as the ratio of the GFP (dead) to the TRITC (live) counts. Using a dead/live ratio rather than the total number of dead cells served to correct for the physical loss of cells due to cytotoxicity.

Results and discussion

Increases in cytotoxicity caused by the presence of drug compounds can be monitored by an increase in green positive cells as determined by image analysis. eTox Green can be added at the beginning of the assay and be present during the exposure or for a short time before cell fixation. The late addition of the eTox Green dye can also be used in conjunction with other cellular dyes to identify nuclei or mitochondria. These dyes can offer further phenotypic information regarding cellular health.

As an assay optimization step and proof-of-concept demonstration, exposure of T47D-Red cells to 25 µM tamoxifen results in measurable cell toxicity after 12 hours of exposure. With further exposure, greater numbers of cells registered positive for green fluorescence. Confirmatory experiments were then performed using a single time point and a range of concentrations of a second known toxicant, mechlorethamine (Figure 3). T47D-Red cells exposed to increasing concentrations of mechlorethamine for 24 hours demonstrate substantially more green fluorescence than untreated cells. When exposed to 50 µM mechlorethamine, all cells observed emit green fluorescence. Additionally, there is a near absence of nuclear TRITC fluorescence.

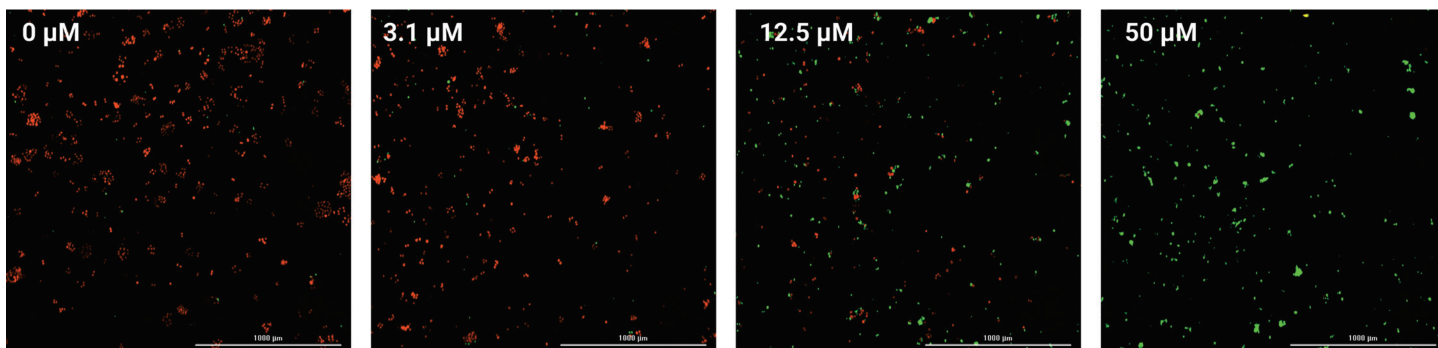

Figure 3. T47D-Red cells were treated with mechlorethamine or left untreated for 24 hours. After treatment, cells were stained with Agilent eTox Green for 30 minutes. Cells were fixed with 4% paraformaldehyde and imaged with an Agilent BioTek Cytation 5 cell imaging multimode reader using a 10x objective. Images represent a 2 x 2 montage that was preprocessed for background subtraction and stitched into a single file.

Finally, the assay was run using a compound library consisting of a total of 89 compounds, with the remaining seven sample wells serving as untreated controls (Figure 4). The mean dead/live value for the untreated controls was determined to be 0.22. Using three standard deviations, a cutoff value for potentially toxic compounds was calculated to be 0.5. A total of 17 compounds were identified as possibly being toxic. Of these, seven compounds were deemed highly toxic, using a criterion of a dead-to-live ratio of 1 as a cutoff. One of these compounds was oridonin, which was added to the compound library as a known positive control. Except for ziprasidone HCl, which is used as an atypical antipsychotic, the remaining compounds; bosutinib, bazedoxifene, sunitinib, crizotinib, and dacomitinib are all compounds used for the treatment of cancers.

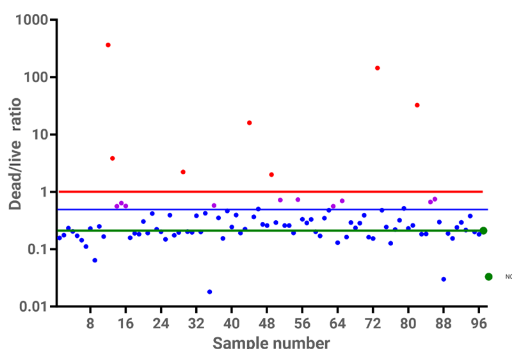

Figure 4. Compound library screen. T47D-Red cells plated in Agilent 384-well microplates were exposed to a small compound library with each compound present at a final concentration of 25 µM. Agilent eTox Green was then added for the final 60 minutes and the cells were fixed with 4% paraformaldehyde and imaged using an Agilent BioTek Cytation 5 cell imaging multimode reader with 4x WFOV camera in the TRITC and GFP channels. Cellular analysis was used to count GFP (dead) and TRITC positive (live) objects for each well. Data are expressed as the ratio of dead-to-live counts over time. Data represent the mean and standard deviation of four replicates.

Conclusion

These data indicate that Agilent 384-well microplates are well suited for a range of cell-based applications, including cytotoxicity screening. The plates demonstrate low background fluorescence allowing the use of commonly used cytotoxicity reagents, while the tissue culture-treated substrate provides an excellent surface for cell growth and supports long-term, cell-based assays. The well density supports rigorous quantitative analysis and provides an ideal platform for high-throughput image-based compound screening with fewer plates and reagents than it would be required with 96-well microplates. Additionally, the coverslip-thin bottom enables high-resolution imaging for detailed analysis of cellular features.

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