Multiplex assays for robust cell health analyses

CyQUANT Direct and PrestoBlue viability assays work together.

Plate-based viability assays are a fundamental tool in drug discovery for evaluating the potency of compounds and the sensitivity of cell lines to specific agents. These homogeneous assays typically measure a single parameter—such as ATP concentration, cell membrane permeability, or reductive capacity (redox potential)—to determine viability. Viability, however, is not easily defined in terms of a single physiological or morphological parameter, and relying on a single measure can generate bias in the experiment.

The luciferin/luciferase bioluminescence assay provides an extremely sensitive measure of ATP and is commonly used to determine viable cell numbers, cell proliferation, and cytotoxicity in both bacterial and mammalian cells. Recent reports caution, however, that results from firefly luciferase (FLuc)–based ATP assays can be misinterpreted due to errors introduced both by a drug's mechanism of action and by cell line–specific responses [1–3]. One study found that as many as 60% of the active compounds originally identified for their antiproliferative activity (using FLuc reporter assays) are actually compounds that inhibit FLuc activity itself [4]. Moreover, at least 12% of the compounds in the NIH Molecular Libraries Small Molecule Repository reportedly exhibit FLuc-inhibiting activity [5].

Multiplex or orthogonal assay approaches, which use a combination of different measures of cell health, provide more stringent viability determinations than possible with any single-parameter assay. Here we describe a robust and convenient multiplex viability assay that employs both the Invitrogen™ CyQUANT™ Direct Cell Proliferation Assay, which measures cellular DNA content and membrane integrity, and the Invitrogen™ PrestoBlue™ Cell Viability Reagent, which detects cell metabolism. The combination of a DNA content–based assay, which has been shown to be among the most sensitive indicators of cell health, and a metabolism-based assay produces an effective orthogonal assay that provides a more complete measure of cell viability. Additionally, because neither of these assays requires cell lysis, they can be further multiplexed with other fluorescence-based cell function probes.

Determining cell proliferation based on DNA content

The CyQUANT Direct Cell Proliferation Assay provides a fluorescence-based method for determining the number of viable cells in a population across a range of growth conditions and cell types. This assay uses two reagents: a green-fluorescent nucleic acid stain and a background suppression dye. The nucleic acid stain is a cell-permeant dye that binds to DNA and concentrates in nuclei of mammalian cells; binding of this dye to DNA is proportional to the amount of DNA present in the cell and independent of the cell's metabolic state. The background suppression dye is impermeant to live cells; however, the outer membranes of dead and dying cells are typically compromised, allowing the suppression dye to enter the cells and mask the fluorescent signal from the DNA-binding dye.

The combination of these two components produces an assay that measures both DNA content and membrane integrity, both important for accurate determination of viability. Because cellular DNA content is highly regulated, the CyQUANT Direct assay can be used at multiple time points to calculate the average proliferation rate of a cell population. In addition, cell number determined with the CyQUANT Direct assay can be used as a highly sensitive indicator of cytotoxicity (Figure 1).

Determining cell proliferation based on metabolism

The PrestoBlue Cell Viability Reagent is both a fluorescence- and absorption-based reagent that is used to measure the reductive capacity of cells. This proven cell viability indicator uses the →

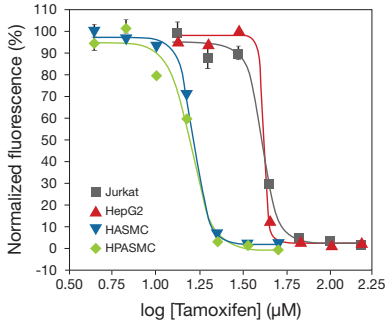

Figure 1. Measurements of cytotoxicity differences across different cell types using the CyQUANT Direct assay. Jurkat cells, HEPG2 cells, human aortic smooth muscle cells (HASMCs), and human pulmonary aortic smooth muscle cells (HPASMCs) were seeded in 384-well plates at a density of 5,000 cells per well with 30 µL medium containing 10% FBS. Following incubation at 37°C for 48 hr with increasing concentrations of tamoxifen, 30 µL of CyQUANT™ Direct reagent (a component of the CyQUANT Direct Cell Proliferation Assay Kit, Cat. No. C35011) was added to each well. Fluorescence was measured after 60 min. Fluorescence intensities were normalized to DMSO-alone treatment, and dose-response curves were generated. Each point graphed represents the average of the fluorescence of eight wells. As shown, the two primary cell types (HASMCs and HPASMCs) were significantly more sensitive to tamoxifen than the two transformed cell lines (adherent HepG2 and suspension Jurkat cells).

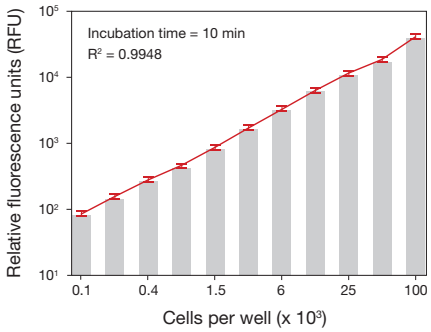

Figure 2. Linear detection range for the PrestoBlue viability assay. PrestoBlue™ Cell Viability Reagent (Cat. No. A13261) exhibits a linear response over three orders of magnitude, with a one-step protocol and a 10 min incubation in culture medium.

natural reducing power of live cells to convert resazurin to the fluorescent molecule resorufin. The active ingredient in the PrestoBlue reagent (resazurin) is a nontoxic, cell-permeant compound that is blue in color and virtually nonfluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity. Damaged (which are likely to be nonproliferating cells) and nonviable cells have decreased reductive capacity and thus generate proportionally lower signals.

The PrestoBlue reagent provides data in as little as 10 minutes, whereas most other commercially available resazurin-based cell viability reagents require a 1- to 4-hour incubation. Moreover, the PrestoBlue reagent provides a measure of relative viable cell number that can be used to assess the average proliferation rate of the populations when performed at multiple time points. The PrestoBlue assay exhibits linear responses over three orders of magnitude in culture medium (Figure 2) and is compatible with downstream functional analyses because it does not require cell lysis.

Multiplexing PrestoBlue and CyQUANT Direct viability assays

We have found that the PrestoBlue and CyQUANT Direct assays can be applied to cells in tandem (first the PrestoBlue assay and then the CyQUANT Direct assay), producing a viability assay that detects changes in cell metabolism, DNA content, and membrane integrity, all in the same sample. This multiplex assay, with its simple, high-throughput mix-and-read protocol and fluorescence readout, is compatible with standard fluorescence-based microplate readers; no special equipment is required. The tandem PrestoBlue and CyQUANT Direct protocol requires no wash steps and no cell lysis. Simply add the PrestoBlue reagent to drug-treated cells, incubate for 10 minutes, and read the fluorescence. Following the PrestoBlue readout, add the CyQUANT Direct reagent, incubate an additional 60 minutes, and re-read the fluorescence (Figure 3). This combined assay can be used on the same cell sample to generate two fluorescent signals (a PrestoBlue signal and a CyQUANT Direct signal), both of which produce measurements of pharmacological parameters very similar to those obtained with the Promega CellTiter-Glo™ Luminescent Cell Viability Assay, a homogeneous luciferase-based assay for measuring ATP levels (Figure 4).

Assessing cytotoxicity with orthogonal assays

An ongoing collaboration between scientists at Thermo Fisher Scientific and the NIH/NCATS High Content Imaging & Discovery Lab aims to explore the potential advantages of using orthogonal viability assays for toxicity profiling. A collection of 1,408 small-molecule compounds with known toxic mechanisms of action were tested on U2OS cells. Three high-throughput screening assays for cytotoxicity were then performed on the cells using the multiplexed PrestoBlue and CyQUANT Direct viability assays, as well as the CellTiter-Glo Luminescent Cell Viability

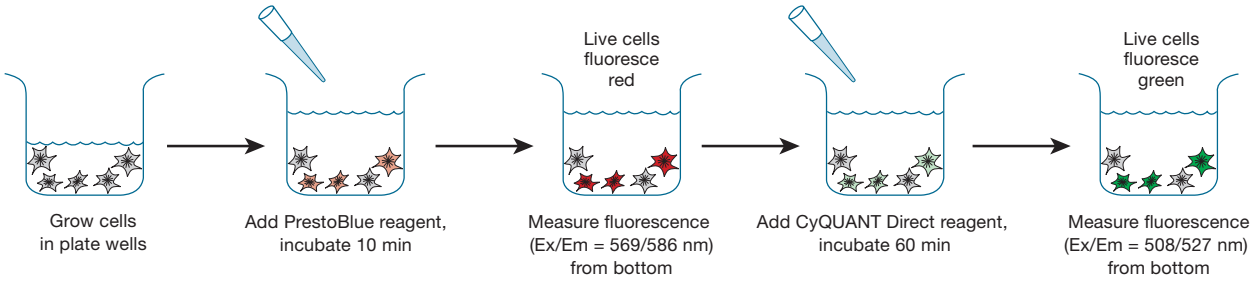

Figure 3. Multiplexing the PrestoBlue Cell Viability Assay and the CyQUANT Direct Cell Proliferation Assay. To perform the multiplex viability assay, add PrestoBlue™ reagent to drug-treated cells, incubate 10 min, and read fluorescence. Following the PrestoBlue readout, add CyQUANT™ Direct reagent to cells, incubate an additional 60 min, and re-read fluorescence. The use of the PrestoBlue and CyQUANT Direct assays together produces a viability assay that is sensitive to cell metabolism, DNA content, and changes in membrane permeability.

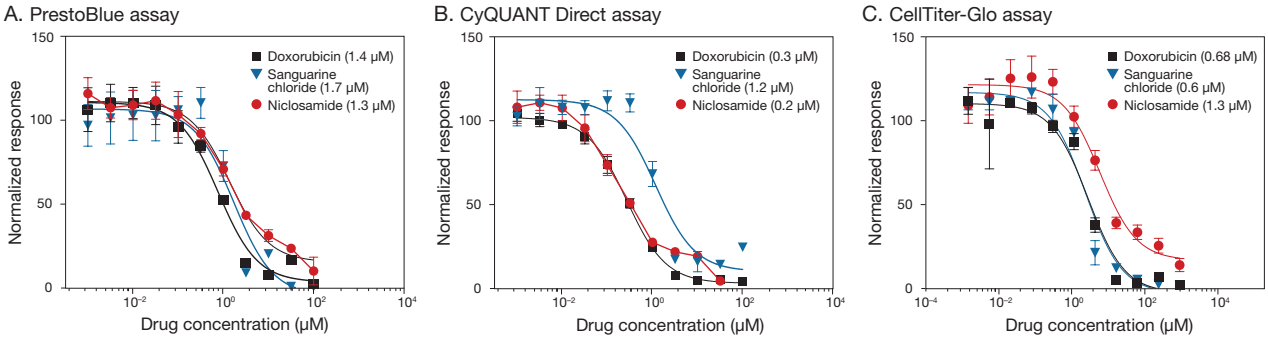

Figure 4. Pharmacological testing with three different cell viability assays. HeLa cells were exposed to three different drugs at the indicated concentrations for 24 hr. The cells were then assayed using either the combined **(A)** PrestoBlue™ cell viability assay and **(B)** CyQUANT™ Direct cell proliferation assay (see Figure 3), or **(C)** the CellTiter-Glo™ cell viability assay. The combined assay can generate two fluorescent signals (a PrestoBlue signal and then a CyQUANT Direct signal), both of which are very similar to the results produced by the CellTiter-Glo assay. IC_{50} values for each drug are shown in parentheses in the key for each assay.

Assay. Of the 1,408 compounds tested, 590 displayed no cytotoxic effect in any of the assays. Of the remaining 818 active compounds, only 395 (48%) were identified by all three tests (Figure 5). Work is in progress to further characterize the toxic compounds identified as active in only one or two of the cytotoxicity assays. The goal of these additional analyses is to understand their mechanisms of action and also to explain false hits (both positive and negative) and singleton compounds (i.e., those identified as active in only a single assay).

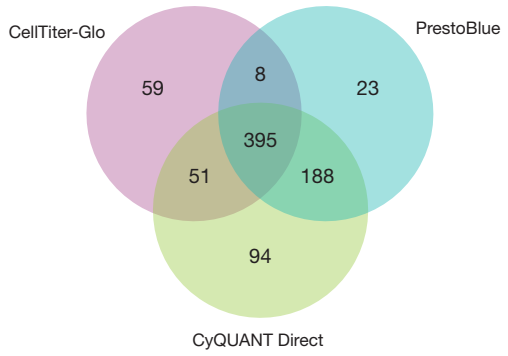

Figure 5. Venn diagram representing the dose-response screens of over 1,000 compounds from a focused library of drugs relevant for clinical use in cancer. U2OS cells were seeded at a density of 1,000 cells/well in 5 µL of DMEM containing 10% fetal calf serum, grown at 37°C for 8 hr, treated with a compound, and incubated for an additional 2 days prior to being assessed for cytotoxicity. A collection of 1,408 small-molecule compounds with known toxic mechanisms of action were tested in a 11-point dose response at final concentrations ranging from 780 pM to 46 µM. Controls were used to normalize for 100% toxicity (50 µM camptothecin) and 0% toxicity (DMSO only). High-throughput screening assays were then performed using the combined PrestoBlue™ cell viability assay and CyQUANT™ Direct cell proliferation assay (see Figure 3), or using the Promega CellTiter-Glo™ Luminescent Cell Viability Assay. For each assay, whole-well fluorescence intensity values were analyzed, dose-response curves were fit using in-house software [6], the curve class was assigned and characterized [2,7], and the three-way Venn diagram was computed. Data used with permission from Steve Titus and Rajarshi Guha, NIH/NCATS High Content Imaging & Discovery Lab, Rockville, Maryland.

Cell viability assays for every application

Multiplex viability assays can provide measures of different aspects of cell viability, producing a more informative picture of the state of cells. Find out more about our wide selection of cell viability assays, available for microplate, imaging, and flow cytometry platforms, at thermofisher.com/cellviabilitybp73. ■

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Product	Quantity	Cat. No.
CyQUANT™ Direct Cell Proliferation Assay	10 microplates 100 microplates	C35011 C35012
PrestoBlue™ Cell Viability Reagent	25 mL 100 mL	A13261 A13262