

A High-Throughput System for Screening Potential Antiviral Compounds Using the CellTiter 96® AQ_{ueous} One Solution Assay

By Thomas Fletcher III, Ph.D., Roger Ptak, B.S., Stacy Bartram, B.S., Susan Halliday, M.S. and Robert Buckheit, Jr., Ph.D.
Infectious Disease Research Department, Serquest, Southern Research Institute, Frederick, MD; and
Rich Moravec, B.S., and Terry Riss, Ph.D., Promega Corporation

Promega's CellTiter 96® AQ_{ueous} One Solution Assay^(a) features the MTS tetrazolium compound, which is converted by viable cells into an intensely colored formazan product that is directly soluble in tissue culture media. The reagents in the CellTiter 96® AQ_{ueous} One Solution System enable design of homogeneous high-throughput screening assays to monitor cell viability as an endpoint. In this article, a virus-induced cytopathic effect (CPE)-inhibition assay using Promega's CellTiter 96® AQ_{ueous} One Solution Assay is used to evaluate compounds for antiviral activity against a variety of viruses.

INTRODUCTION

During replication, many viruses destroy not only the host cells that they infect but also neighboring uninfected cells by cytopathic effects (CPE). The antiviral activity of potential therapeutic agents against many of these viruses can be determined by evaluating the inhibition of this virus-induced cell death. Using a cell-based CPE assay, we have developed an automated, high-throughput method for identifying inhibitors of viruses including Human Immunodeficiency Virus Type 1 (HIV-1), Bovine Viral Diarrhea Virus (BVDV), human herpesviruses (HSV-1 and HSV-2) and respiratory viruses (Table 1). Using this assay methodology, we can screen up to 15,000 compounds per week against an individual virus. This high-throughput screening method can also be used to determine the activity of potential antiviral agents against a panel of diverse viruses. This assay system allows rapid screening of both natural compounds and combinatorial chemistry libraries for antiviral compounds, thus reducing the time interval between drug discovery and lead optimization.

Table 1. Viruses and Cell Lines Analyzed Using CPE-Inhibition Assay and the CellTiter 96® AQ_{ueous} One Solution Reagent.

Virus	Cell Line	Length of Assay
HIV-1	CEM-SS	6 days
HSV-1	Vero	5 days
HSV-2	Vero	5 days
BVDV	MDBK	6 days
CMV	MRC-5	7 days
Coxsackie	MRC-5	5 days
Echovirus	MRC-5	4 days
Enterovirus	MRC-5	4 days
Rhinovirus	MRC-5	5 days
Adenovirus	HeLa	3–4 days
Influenza	MDCK	2–3 days
Parainfluenza	Hep2	3 days
RSV	Hep2	4 days

HIGH-THROUGHPUT ASSAY FORMAT

Cells are cultured in 96 well plates using the appropriate culture medium. Automated antiviral assays are performed using a Beckman Coulter SAGIAN™ Core System (Figure 1). The assays are designed to test twenty-four compounds per plate at a single dose against the challenge virus (Figure 2). A 100µl aliquot of the test drug compound and 100µl of the virus suspension are added to each sample well. Controls are included on each plate (Table 2). The plates are incubated at 37°C in a humidified atmosphere containing 5% CO₂. Incubation is continued until maximum CPE is observed through the performance of control assays (see Table 1).

Table 2. Antiviral Assay Controls.

Control	Well Contents			
	Virus	Drug	Cells	Media
Cell			✓	✓
Virus	✓		✓	✓
Toxicity		✓	✓	✓
Colorimetric		✓		✓
Media				✓



Figure 1. The SAGIAN™ Core System automated robotic workstation (Beckman Coulter).

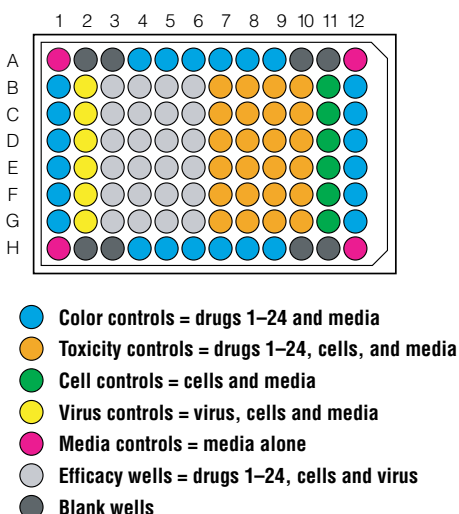


Figure 2. Plate layout for single-dose testing of antiviral compounds. Color controls determine background absorbance due to drug color and this value is subtracted from corresponding efficacy and toxicity values. Toxicity controls differentiate between antiviral activity and toxicity. Cell controls determine the absorbance of untreated cells and give values equal to 100% viability. Virus controls determine the absorbance of cells killed by virus treatment and equal 0% viability. Media controls determine the background absorbance of the tissue culture media. Efficacy wells determine the effect of the drug compound in terms of percent viability with respect to the control wells.

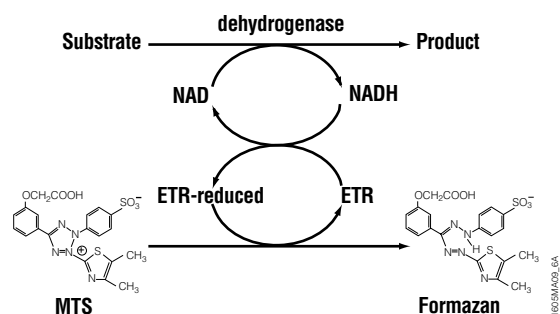


Figure 3. Schematic showing cellular metabolic conversion of MTS to formazan. The primary means of reducing MTS to its formazan product by metabolically active cells is thought to occur as a result of the action of dehydrogenase enzymes generating reducing equivalents such as NADH or NADPH. NADH can transfer its electrons to an electron transfer reagent (ETR) such as phenazine methosulfate (PMS) or phenazine ethosulfate (PES), resulting in reduction of these compounds. The reduced ETRs, in turn, can directly interact with and reduce the MTS tetrazolium compound, producing the deeply colored formazan product.

CPE inhibition is determined by a cell viability assay using Promega's CellTiter 96® AQ_{ueous} One Solution Assay (Cat.# G3580, and G3581). This assay measures cell viability and is based on the bioreduction of MTS tetrazolium^(a) into formazan by NADH and NADPH produced by dehydrogenase enzymes in viable host cells (1; Figure 3). The CellTiter 96® AQ_{ueous} One Solution reagent (10% total well volume) is added to each well. Plates are incubated at 37°C until sufficient color development has occurred (usually 2–6 hours, depending on cell line). Purple formazan product is then measured spectrophotometrically at 490nm with a reference wavelength of 650nm. The optical density (O.D.) value of each culture is a function of the amount of formazan produced and is proportional to the number of viable cells (2).

Compounds that exhibit activity in single-dose testing are further evaluated to generate dose response curves, which are used to determine IC₅₀ (concentration at which the compound inhibits virus-induced cell killing by 50%) and TC₅₀ (concentration at which the compound alone kills 50% of uninfected cells) values. The plate layout for the dose response testing is shown in Figure 4. Typical results for control compounds against HIV-1, HSV-2 and CMV are shown (Figure 5, Panels A–C).

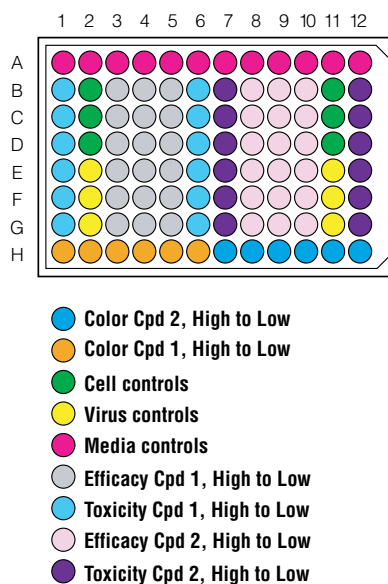
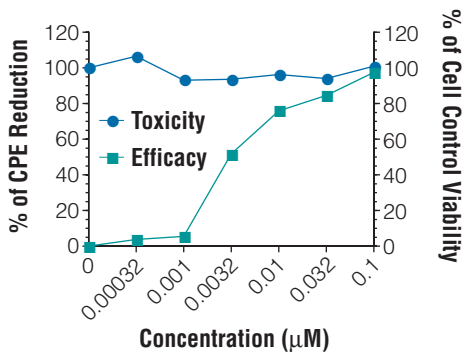
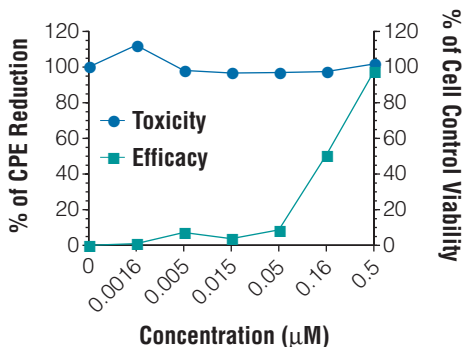


Figure 4. Plate layout for dose response testing of antiviral compounds. Controls are the same as described in Figure 2. Compounds are plated from high to low concentrations in the indicated wells.

A. AZT vs. HIV-1 RF in CEM-SS Cells



B. Acyclovir vs. HSV-2 MS in VERO Cells



C. Ganciclovir vs. CMV AD169 in MRC-5 Cells

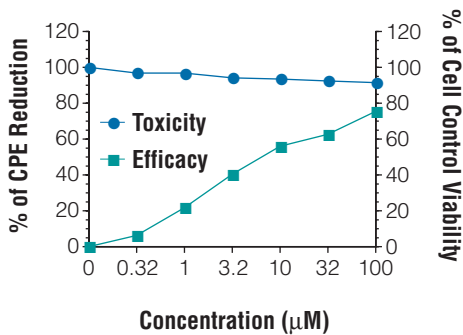


Figure 5. Dose response curves for three known antiviral compounds. CPE-inhibition assays were performed using the CellTiter 96[®] AQ_{ueous} One Solution Assay as described. The results show compound toxicity as percent viability compared to the cell controls and efficacy as a percent of CPE reduction. The three compounds decrease the virus-induced cytopathic effect in a concentration-dependent manner. Additionally, the compounds show minimal toxicity (i.e., 100% viability) in the concentration range tested. **Panel A:** Zidovudin (AZT) against Human Immunodeficiency Virus Type 1, Strain RF (HIV-1 RF). **Panel B:** Acyclovir against Human Herpesvirus, Type 2 (HSV-2). **Panel C:** Ganciclovir against Cytomegalovirus, Strain AD169 (CMV AD169).

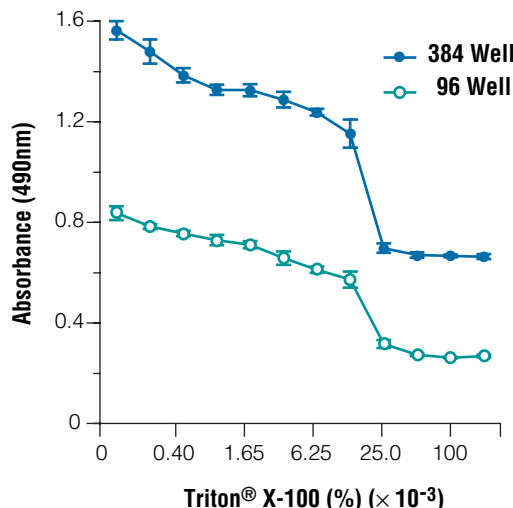


Figure 6. Comparison of 384 and 96 well format cytotoxicity assays. Mouse hybridoma cells in log phase were harvested by centrifugation and suspended in F12/DME medium supplemented with 10% calf serum. The cells were dispensed into a Corning[®] 96 well tissue culture plate in a final volume of 90µl (45,000 cells/well) and into a Corning[®] 384 well plate in a final volume of 35µl (17,500 cells/well). The culture wells were treated with various dilutions of Triton[®] X-100 (to kill cells) prepared in culture media and added at 4µl/well to the 384 well plate or 10µl/well to the 96 well plate. CellTiter 96[®] AQ_{ueous} One Solution Reagent was added (8µl/well to the 384 well plate and 20µl/well to the 96 well plate) and the cultures were incubated at 37°C in a 5% CO₂ incubator for 4 hours. Absorbance was recorded using a Wallac[™] 1420 VICTOR^{2™} plate reader. Absorbance values are the mean ± standard deviation of 4 replicates for each treatment.

DISCUSSION

The CellTiter 96[®] AQ_{ueous} One Solution Assay enables us at Serquest to provide faster and better high-throughput screening. The easy “add and read” format of the aqueous soluble formazan assay eliminates the need to remove culture media or perform other sample manipulations. This results in increased efficiency and throughput as well as decreased well-to-well variation. The CellTiter 96[®] AQ_{ueous} One Solution Assay can also be used for 384 well plate applications while still retaining performance properties similar to those of the 96 well format (2; Figure 6). The process presented here demonstrates that the CellTiter 96[®] AQ_{ueous} One Solution Assay is a useful tool for determining antiviral efficacy and cytotoxicity of test compounds in a high-throughput setting.

REFERENCES

1. Barltrop, J.A. *et al.* (1991) *Bioorg. & Med. Chem. Lett.* **1**, 611.
2. Riss, T. and Moravec, R. (1998) *Promega Notes* **69**, 15.



THOMAS FLETCHER III



ROGER PTAK



STACY BARTRAM



SUSAN HALLIDAY



ROBERT BUCKHEIT



RICH MORAVEC



TERRY RISS

Ordering Information

Product	Size	Cat.#
CellTiter 96 [®] AQ _{ueous} One Solution Cell Proliferation Assay	1,000 assays	G3580
	5,000 assays	G3581
	200 assays	G3582

Related Products

Product	Size	Cat.#
CytoTox 96 [®] Non-Radioactive Cytotoxicity Assay	1,000 assays	G1780
CellTiter 96 [®] AQ _{ueous} MTS Powder ^(a)	1g	G1111
	250mg	G1112
CellTiter 96 [®] AQ _{ueous} Non-Radioactive Cell Proliferation Assay	1,000 assays	G5421
	5,000 assays	G5430
	50,000 assays	G5440

CellTiter 96 and CytoTox 96 are trademarks of Promega Corporation and are registered with the U.S. Patent and Trademark Office.

Corning is a registered trademark of Corning, Inc. SAGIAN is a trademark of Beckman Coulter, Inc. Triton is a registered trademark of Union Carbide Chemicals and Plastics Co., Inc. VICTOR² and Wallac are trademarks of Perkin Elmer Life Sciences.

^(a)The MTS tetrazolium compound is the subject of U.S. Pat. No. 5,185,450 assigned to the University of South Florida and is licensed exclusively to Promega Corporation.