SOUTHERN RESEARCH

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Evaluation of Viral ToxGlo™ as an Endpoint Reagent for Cell-based, High-throughput Anti-viral Discovery.

Abstract No. TP 138

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Abstract

Influenza continues to cause significant global morbidity and mortality in spite of the development of vaccines and therapeutics. Flu vaccines range from very effective to ineffective depending on how well the vaccine serotypes match the circulating strains. A number of effective anti-influenza therapeutics have been developed but the emergence of drug-resistant strains of virus limits their long term usefulness, so there is still a need to develop new therapeutics. Southern Research has a large anti-influenza drug discovery program that screens diverse small molecules against many virus strains using assays that are phenotypic and based on monitoring the viral-induced cytopathic effect (CPE) on the host cell. To validate the performance of a new, commercially-available reagent (Viral ToxGlo™ *, Promega), multiple viruses and cell types were assayed in the cell-viability assay format. These were Venezuelan equine encephalitis virus (strain TC83) in VeroE6 cells, respiratory syncytial virus (strain A2) in A549 cells, and Dengue virus (serotype 2) in BHK-21 cells. Viability data was compared with the tetrazolium dye MTT, which is a standard endpoint reagent for antiviral assays. Signal-stability and linearity were established in uninfected cells, and signal-to-background ratio, noise ratio, assay sensitivity limits, and Z-values were defined using infected and uninfected cells. As part of the validation, ~30,000 compounds from the Enamine chemical diversity library was screened in parallel against H3N2, H1N1 and H5N1 influenza virus strains. 320 active compounds were identified in these HTS screens, and these were further evaluated using Viral ToxGlo™ by dose response to determine comparative potency and broad spectrum activity against influenza.

Methods

Reagent validation and HTS:

Experimental parameters evaluated in triplicate:

- Signal to background ratio (S/B)
- Signal linearity
- Assay sensitivity limits
- Comparison with the MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay endpoint format.
- Read stability in uninfected cells.

CPE assays using three different RNA viruses:

- Venezuelan equine encephalitis virus (VEEV), strain TC83 in VeroE6 cells, which has a rapid CPE phenotype (72 h).
- Dengue virus (DENV2), serotype 2 in BHK-21 cells, with a moderate CPE (96 h).
- Respiratory syncytial virus (RSV), strain A2 in A549 cells, with syncytial formation and slower CPE (144 h).

HTS of three Influenza A strains with ~30K compounds in single dose format:

- Compounds were pre-plated in assay plates.
- Virus stock was mixed with MDCK cells at a stock dilution of 1:5000, and added to assay plates. Uninfected cell controls were treated in the same manner, but contained no virus.
- Influenza strains A/California/7/2009, A/Udorn/1972, and A/Vietnam/1203/2004 were used.
- The assay was endpointed 72 h post cell-plating using Viral ToxGlo™ was used as the endpoint reagent.

Data acquisition and analysis:

Statistical calculations were made as detailed in (1) Compounds showing greater than 50% inhibition of viral-induced CPE were considered "hits".

Results

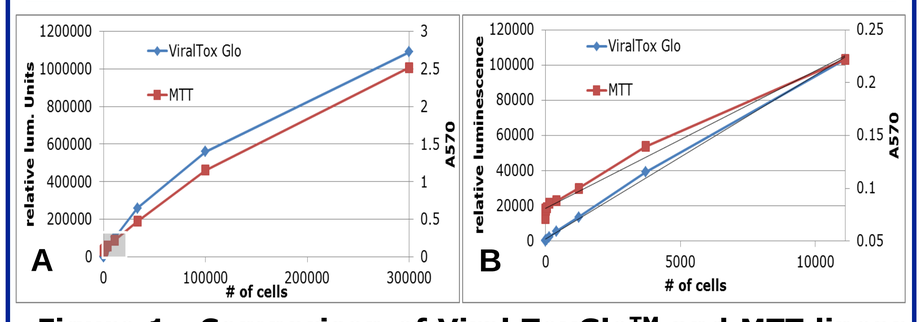


Figure 1. Comparison of Viral ToxGlo™ and MTT linear range of detection. Vero E6 cells were plated and both Viral ToxGlo™ and MTT were used to measure cell viability in 96-well microplates. Panel A-10,000-300,000 cells/well. Panel B 0-10,000 cells/well (gray inset from panel A). Viral ToxGlo™ maintains a generally linear range between 20-300,000 cells/well, where MTT linearity deteriorates below 2000 cells/well.

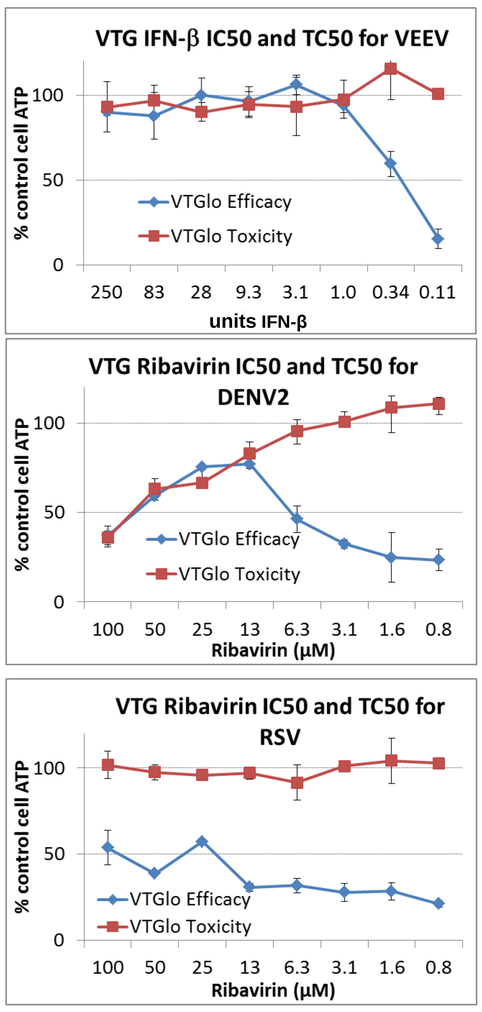


Figure 2. Validation of Viral ToxGlo™ (VTG) with three different viruses. Comparison of compound toxicity (red) and antiviral efficacy (inhibition of CPE-blue) are shown for three different viruses using standard antiviral controls. Standard deviations are given as +/- values. Z-values ranged from 0.6-0.73 (MTT = 0.44) S/B ranged form 5-10 (MTT <5), and read stability was > 5 h. The ability of each control compound to inhibit viral-induced CPE was determined and compared to previously published values. The IC50 were: Interferon-β (IFN-β) against VEEV = 0.20 U; RBV against DENV2 = 7.5 μM; RBV against RSV = 18 μM. These values are comparable to published values and our historical values.

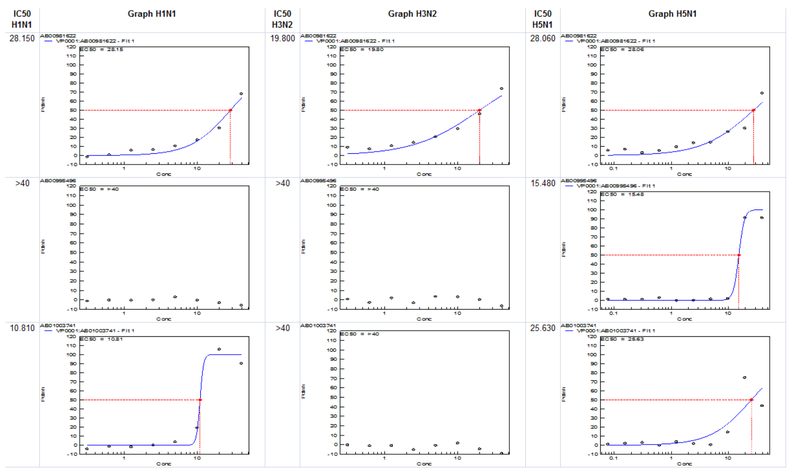
Table 1. Summary of the Influenza HTS Primary Assays Z values, S/B, and primary screen hit rates are shown for screens against three influenza A strains. Standard deviations are given as +/- values. Typically, a S/B value greater than 5 is an adequate detection window and a Z-factor above 0.5 indicates an assay that is considered robust enough for HTS (2). These assay values (given in Table 1 below) indicate that use of Viral ToxGlo™ results in an assay that is sufficiently robust and reproducible for use in HTS.

Screen	H1N1	H3N2	H5N1
# compounds	29920	29920	29920
Z	0.73 ± 0.11	0.80 ± 0.06	0.73 ± 0.07
S/B	8.6 ± 3.4	46 ± 31	79 ± 57
primary screen hit rate at 50%	0.70%	0.72%	0.05%

Table 2. Confirmatory assay results. 320 compounds that showed at least 40% inhibition of viral-induced CPE for at least one influenza strain were selected for confirmatory assays. IC50 values were returned for 21 of the compounds tested. Some were active against more then one strain of influenza.

Dose Response	H1N1	H3N2	H5N1
# compounds with a calculated IC50	12	8	12
IC50 Range (μg/mL)	4.33-34.73	2.36-37.94	3.63-35.6

Figure 3. Representative Dose Response Curves. DR curves for compounds showing activity in one or more of the Influenza A strains tested.



Discussion

A high-throughput, cell-based assay was validated using a new, luminescence-based reagent (Viral ToxGlo™). The results of this reagent were superior to MTT, the standard for virology. In addition, the add and read protocol and long signal half life produced an assay robust enough for HTS.

- Validation included testing against three different viruses with different kinetics and different cell lines. Evaluation metrics were: 1. Robustness with a high degree of reproducibility and low deviation, 2. High sensitivity, low background, and capable of evaluating a broad range of compounds, and 3. Adaptability for screening large compound libraries. The use of Viral ToxGlo™ gave results consistent with the use of MTT, but with a greater range of sensitivity, larger S/B, and greater rapidity and ease of use.
- HTS of a small diversity library produced excellent data with Z values above 0.7 and good reproducibility from day to day and from screen to screen. This reagent can be used to evaluate inhibition of CPE in antiviral assays and compound toxicity as a counter screen in parallel.

Acknowledgments

This work was partially funded by Southern Research Institute and Promega Corporation.

References

1. Noah, J. W., et al. (2007) A cell-based luminescence assay is effective for high-throughput screening of potential influenza antivirals, *Antiviral Res 73*, 50-59.
2. Zhang, J.H., Chung, T.D. Oldenburg, K.R., 1999. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen*. 4, 67-73.
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