TECHNICAL MANUAL

Viral ToxGlo™ Assay

Instructions for Use of Products
G8941, G8942 and G8943

For Research Use Only. Not for use in diagnostic procedures.

Revised 2/15
TM393

Viral ToxGlo™ Assay

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Viral ToxGlo™ Assay[a,b,c] is a homogeneous, add-mix-measure method intended for use in research studies to identify cytopathic effect (CPE) induced by viral infection. The assay measures cellular ATP, a stable and tightly regulated surrogate of cell viability (Figure 1). Luminescent signals from the assay are proportional to viable cell number. When CPE occurs due to viral infection, ATP depletion can be measured and correlated with viral burden (1–6). Notably, this method is useful only for viruses that produce cytotoxicity and CPE.

The assay can be used in two principal ways. First, viral infectivity and the corresponding tissue culture infective dose (TCID) can be determined by serially diluting a virus preparation and applying it to target cell line(s) for a specified exposure period. The virus dilution that produces a cytotoxic endpoint effect (i.e., $TCID_{50}$ or 50% CPE) can be calculated and used for subsequent studies. Secondly, a known excess of virus (i.e., 100 $TCID_{50}$ or 100X the TCID50) can be delivered to cells with test compounds and incubated to determine potential antiviral effects. The latter application can be conducted in either single-dose high-throughput screens (HTS) or as part of a full-compound dose-response series (qHTS).

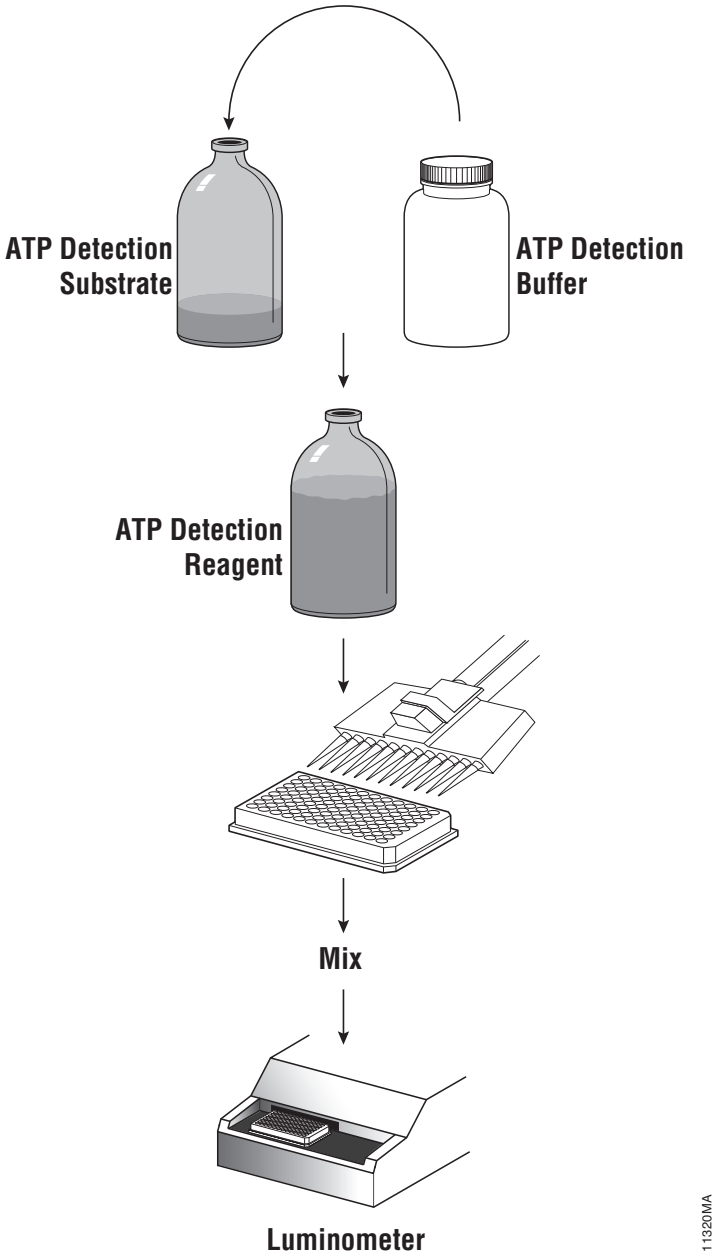

Figure 1. Flow diagram showing the preparation and use of Viral ToxGlo™ Assay reagents.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Viral ToxGlo™ Assay	**10ml**	**G8941**

Substrate resuspended with 10ml of ATP Detection Buffer is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 1 × 10ml ATP Detection Buffer
- 1 vial ATP Detection Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
Viral ToxGlo™ Assay	**10 × 10ml**	**G8942**

Each vial of substrate resuspended with 10ml of ATP Detection Buffer is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10 × 10ml ATP Detection Buffer
- 10 vials ATP Detection Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
Viral ToxGlo™ Assay	**100ml**	**G8943**

Substrate resuspended with 100ml of ATP Detection Buffer is sufficient for 1,000 assays at 100µl/assay in 96-well plates or 4,000 assays at 25µl/assay in 384-well plates. Includes:

- 1 × 100ml ATP Detection Buffer
- 1 vial ATP Detection Substrate (lyophilized)

Storage Conditions: For long-term storage, store the lyophilized ATP Detection Substrate and ATP Detection Buffer at –30°C to –10°C. For frequent use the ATP Detection Buffer can be stored at 2°C–10°C or room temperature (22°C–25°C) for 48 hours without loss of activity. See the product label for expiration date information. Reconstituted ATP Detection Reagent (ATP Detection Buffer plus ATP Detection Substrate) can be stored at room temperature for up to 8 hours with <10% loss of activity, at 2°C–10°C for 48 hours with approximately 5% loss of activity, at 2°C–10°C for 4 days with approximately 20% loss of activity or at –30°C to –10°C for 21 weeks with approximately 3% loss of activity. Reconstituted ATP Detection Reagent is stable for up to ten freeze-thaw cycles with less than 10% loss of activity.

Safety Information: The Viral ToxGlo™ Assay was designed to be used with potentially infectious viruses. Users should wear appropriate personal protective equipment (e.g., gloves and goggles). The lytic reagent supplied with this kit will not inactivate virus samples and should not in any way be considered a virucidal reagent. Users should adhere to their institutional guidelines for the handling and disposal of all potentially infectious materials when using this system.

3. Performing the Viral ToxGlo™ Assay

Materials to Be Supplied by the User

- opaque-walled multiwell plates compatible with cell culture
- multichannel pipette or automated pipetting station for reagent delivery
- plate shaker or device for mixing multiwell plates (recommended)
- luminometer, CCD camera or imaging device capable of reading multiwell plates
- viral stocks capable of inducing cytopathic effect
- virus-susceptible host cells
- water bath
- **optional:** antiviral compounds

3.A. Reagent Preparation

1. Thaw the ATP Detection Buffer and equilibrate to room temperature (22°C–25°C) prior to use. For convenience, the ATP Detection Buffer may be thawed and stored at room temperature for up to 48 hours before use.
2. Equilibrate the lyophilized ATP Detection Substrate to room temperature before use.
3. Transfer the appropriate volume of ATP Detection Buffer (10ml for Cat.# G8941 and Cat.# G8942, or 100ml for Cat.# G8943) into the amber bottle containing the lyophilized ATP Detection Substrate. This forms the ATP Detection Reagent (Figure 1).
4. Mix the ATP Detection Reagent by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution. The ATP Detection Substrate should go into solution easily and in less than 1 minute.

3.B. Protocol for Determining Tissue Culture Infective Dose (TCID)

The following method is intended for use as instructions for determining infective viral titers but assumes that host cell density, doubling times and culture medium parameters have been previously considered and optimized with respect to viral cytotoxicity. For additional guidance on how to address optimization activities, please refer to Section 4, Appendix, of this Technical Manual.

Notes:

- Multiwell plates must be compatible with the luminometer used.
- If using host cells in suspension, proceed to Step 4.

1. For attachment-dependent host cell lines, we recommend adjusting the cell number in culture medium to the desired density, and seeding the cells in opaque-walled multiwell plates, 100µl per well for 96-well plates or 24µl per well for 384-well plates. For no-cell control wells, dispense only culture medium into the last column of either plate format.
2. Incubate the plates containing cells at 37°C in a humidified CO_2 incubator for at least 4 hours (and up to 24 hours) to facilitate attachment and allow cells to recover from initial seeding stresses. **Note:** This step pertains to pre-seeded, attachment-dependent cells only.

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.16	10	31.6	100	316	1,000	3,162	10,000	31,622	100,000	UT	Blank
B	3.16	10	31.6	100	316	1,000	3,162	10,000	31,622	100,000	UT	Blank
C	3.16	10	31.6	100	316	1,000	3,162	10,000	31,622	100,000	UT	Blank
D	3.16	10	31.6	100	316	1,000	3,162	10,000	31,622	100,000	UT	Blank
E	3.16	10	31.6	100	316	1,000	3,162	10,000	31,622	100,000	UT	Blank
F	3.16	10	31.6	100	316	1,000	3,162	10,000	31,622	100,000	UT	Blank
G	3.16	10	31.6	100	316	1,000	3,162	10,000	31,622	100,000	UT	Blank
H	3.16	10	31.6	100	316	1,000	3,162	10,000	31,622	100,000	UT	Blank

Figure 2. Example viral dilution series. Numbers represent half-log (3.16-fold) dilution series. UT = untreated (no-virus control); Blank = medium only (no-cell controls).

3. Dilute stock virus by making an initial dilution in culture medium (e.g., 1:10 or 1:100). Using a multichannel pipette, perform subsequent half-log (3.16-fold) serial dilutions by adding 46µl of initial virus dilution to cells in column 1 for a 96-well plate (add 11µl of initial virus dilution for a 384-well plate). Pipet this volume at least 5 times to facilitate mixing. Change tips to avoid viral carryover, then aspirate 46µl (11µl for a 384-well plate) from column 1, transfer to column 2, mix and repeat through column 10 (column 22 for a 384-well plate). From the last viral dilution column, remove 46µl (11µl for a 384-well plate) and dispense to waste. Column 11 serves as the no-virus control and column 12 as the no-cell control (columns 23 and 24, respectively, for a 384-well format). See Figure 2 for a 96-well template of a viral dilution series. Proceed to Step 5.

4. **Optional Suspension cell protocol:** Alternatively, viral dilutions can be prepared in a plate without host cells. Dilute stock virus by making an initial dilution in culture medium (e.g., 1:5 or 1:50). Add 50µl of culture medium to each well of 96-well plates (12µl per well for 384-well plates). With a multichannel pipette, perform half-log serial dilutions by adding 23µl of initial virus dilution to wells in column 1 (5.5µl for 384-well format). Pipet this volume at least 5 times to facilitate mixing. Change tips to avoid viral carryover, then aspirate 23µl (5.5µl for a 384-well plate) from column 1, transfer to column 2, mix and repeat through column 10 (column 22 for a 384-well plate). Remove 23µl from column 10 (or 5.5µl from column 22) and dispense to waste. Column 11 will serve as the no-virus control and column 12 as the no-cell control (columns 23 and 24, respectively, for a 384-well plate). Cells in suspension can now be added, in 50µl of culture medium, to columns 1–11 for 96-well plates (or in 12µl of culture medium to columns 1–23 for a 384-well plate). For no-cell control wells, add 50µl of culture medium (or 12µl for a 384-well format) to the last column of each assay plate.

5. Incubate the plates under conditions determined to be optimal for both the cells and for viral cytotoxicity.

   Note: The amount of time needed for development of cytopathic effect in host cells can vary greatly and is dependent on the replication rates of the virus used and the intrinsic host cell susceptibility to that virus. Typical incubation periods range from 48–144 hours and should be optimized for the greatest signal window (i.e., greatest signal difference between healthy untreated cells and maximal cytopathic effect).

6. Prepare ATP Detection Reagent as described in Section 3.A, Step 3.

3.B. Protocol for Determining Tissue Culture Infective Dose (TCID; continued)

7. Add 100µl of ATP Detection Reagent to each well of a 96-well plate (25µl to each well of a 384-well plate). Wait at least 10 minutes before measuring luminescence. Avoid introducing bubbles, which can adversely affect luminescent signal detection.

   Note: Mixing the plate for 2 minutes on an orbital or electromagnetic shaker (for 96-well or 384-well plates) may increase reproducibility of results in replicate wells. Alternatively, homogeneous mixing can be accomplished by adjusting liquid handler dispensing speeds or waiting for an additional time period (15–30 minutes) prior to measuring luminescence.

8. Calculate $TCID_{50}$ values by plotting net RLU (relative luminescence units) values after subtracting average of blank wells against viral dilution. Remember to include initial viral dilution (e.g., tenfold or 100-fold) in the calculation of the final dilution achieved. The $TCID_{50}$ value is the reciprocal of the dilution that produced a 50% decline in ATP levels compared to untreated controls. Although it may be possible to grossly approximate viral $TCID_{50}$ using standard plotting techniques, we suggest using commercially available software packages (GraphPad Prism®, SigmaPlot, etc.; Figure 3), which greatly aid in statistical treatment of the data through validated regressions and fits.

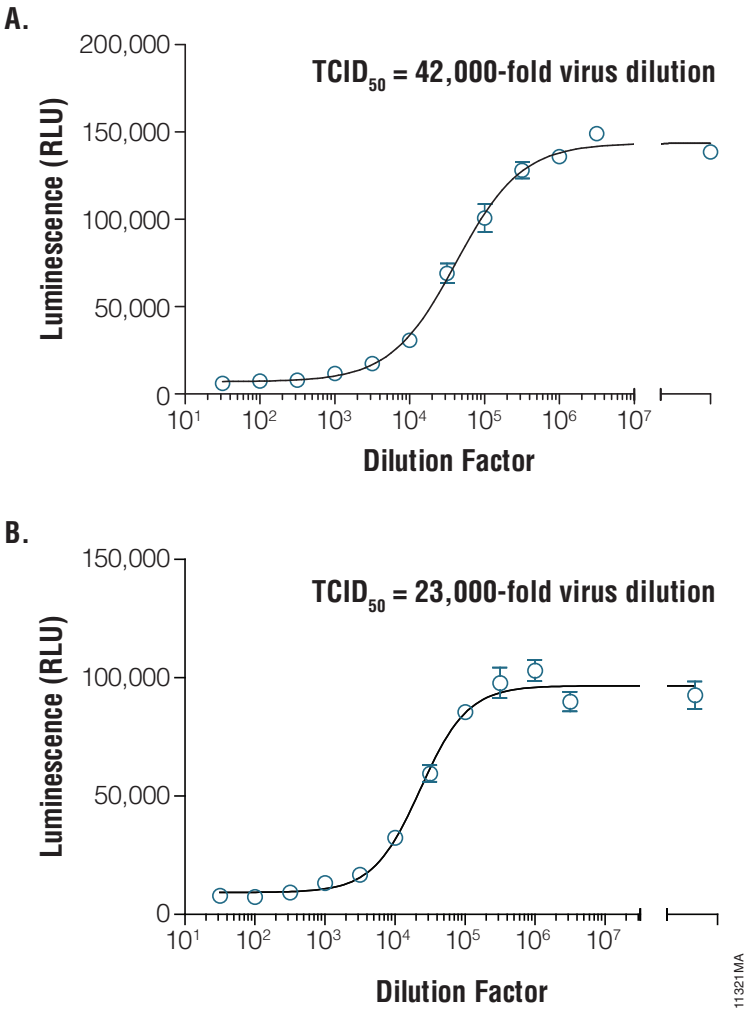

Figure 3. Calculation of $TCID_{50}$ values. Panel A. Half-log (3.16-fold) serial dilutions of influenza virus H1N1 were added to replicate wells of a 96-well plate containing MDCK cells for 72 hours. **Panel B.** Half-log serial dilutions of Dengue virus (Serotype 2) were added to replicate wells of a 96-well plate containing BHK-21 monolayers, for 96 hours. Subsequently, the ATP Detection Reagent was added to wells and luminescence measured. $TCID_{50}$ was calculated by plotting raw luminescence versus dilution factor using GraphPad Prism® and fitted by a sigmoidal dose-response (variable slope) algorithm. Experimental data was provided by Southern Research Institute, Birmingham, AL, and is used with permission.

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3.C. Protocol for Determining Antiviral Potency

Notes:

- Multiwell plates must be compatible with the luminometer used.
- If using host cells in suspension, proceed to Step 3.

1. For attachment-dependent host cells lines, we recommend adjusting the cell number in culture medium to the desired density and seeding the cells in opaque-walled multiwell plates, 50µl per well for 96-well plates (12µl per well for 384-well plates). For no-cell control wells, dispense only culture medium into the last column of either plate format.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NV	20µM	10µM	5µM	2.5 µM	1.25 µM	0.625µM	0.312µM	0.156 µM	0.078 µM	NC	Blank
B	NV	20µM	10µM	5µM	2.5 µM	1.25 µM	0.625µM	0.312µM	0.156 µM	0.078 µM	NC	Blank
C	NV	20µM	10µM	5µM	2.5 µM	1.25 µM	0.625µM	0.312µM	0.156 µM	0.078 µM	NC	Blank
D	NV	20µM	10µM	5µM	2.5 µM	1.25 µM	0.625µM	0.312µM	0.156 µM	0.078 µM	NC	Blank
E	NV	20µM	10µM	5µM	2.5 µM	1.25 µM	0.625µM	0.312µM	0.156 µM	0.078 µM	NC	Blank
F	NV	20µM	10µM	5µM	2.5 µM	1.25 µM	0.625µM	0.312µM	0.156 µM	0.078 µM	NC	Blank
G	NV	20µM	10µM	5µM	2.5 µM	1.25 µM	0.625µM	0.312µM	0.156 µM	0.078 µM	NC	Blank
H	NV	20µM	10µM	5µM	2.5 µM	1.25 µM	0.625µM	0.312µM	0.156 µM	0.078 µM	NC	Blank

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Figure 4. Example compound dilution series and experimental controls. NV = no-virus; NC = no-compound; Blank = no-cell control (medium only).

2. Incubate plates containing cells at 37°C in a humidified CO_2 incubator for at least 4 hours (and up to 24 hours) to facilitate attachment and allow cells to recover from seeding stresses.

   Note: Step 2 pertains to pre-seeded, attachment-dependent cells only.

3. Prepare initial dilutions of test compound and reference antiviral inhibitor in cell culture medium (e.g., 40µM) and mix vigorously to ensure homogeneity. In a separate dilution plate, add 50µl of culture medium to the wells in columns 3–10 for a 96-well plate (12µl to columns 3–22 for a 384-well plate).

   Add 100µl (24µl for a 384-well plate) of test compound to column 2, rows A–D, and 100µl (24µl for a 384-well plate) of reference antiviral to column 2, rows E–H. Begin serial dilutions of test compound and reference antiviral by transferring 50µl (12µl for a 384-well plate) from column 2 wells to the corresponding wells of column 3. Pipet this same volume at least 5 times to facilitate mixing. Change tips to minimize compound carryover.

   Transfer 50µl (12µl for a 384-well plate) from column 3 to column 4, mix and repeat through column 10 (column 22 for a 384-well plate), diluting twofold at each successive column. At column 10, remove 50µl and dispense to waste (12µl from column 22 for a 384-well plate). After completing compound serial dilutions, add 50µl of medium to columns 1, 11 and 12 (12µl to columns 1, 23 and 24 for a 384-well plate). Column 1 is a no-virus control, column 11 is the no-compound control and column 12 is a no-cell control (columns 1, 23 and 24, respectively, for a 384-well plate).

4. Transfer 25µl (6µl for a 384-well plate) of serial dilutions of compounds and controls to corresponding wells of the attachment-dependent host cell plate.

   For host cells in suspension, transfer 25µl (6µl for a 384-well plate) of serial dilutions of compounds and controls to a cell-free plate. Suspension host cells can then be delivered to this same plate in 50µl (12µl for a 384-well plate) to columns 1–11 for a 96-well plate (or columns 1–23 for a 384-well plate).

5. Dilute stock virus to a concentration known to produce optimal cytopathic effect at standard host cell parameters over a predetermined incubation. This dilution should be empirically determined for each virus and study design but often is a substantial excess of $TCID_{50}$ (e.g., 100 $TCID_{50}$) ensuring maximal cytopathic effect in the absence of antiviral agents. Add 25µl of virus dilution to columns 2–11 (6µl to columns 2–23 for a 384-well plate) of assay plates prepared in Step 4. Add 25µl of culture medium to columns 1 and 12 (6µl of medium to columns 1 and 24 for a 384-well plate).

   Note: Final compound concentrations in assay wells are fourfold less than that in the compound dilution plate due to the addition of virus and host cell culture volumes.

6. Incubate plate at standard host cell parameters for a predetermined period that allows for optimal viral cytotoxicity.

   Note: The emergence of cytopathic effect in host cells can vary greatly depending upon viral replication rates and intrinsic host susceptibility and should be optimized for the greatest signal window (i.e., signal difference between healthy untreated cells and maximal cytopathic effect). Typical incubation periods range from 48–144 hours.

7. Prepare ATP Detection Reagent as described in Section 3.A, Step 3.

8. Add 100µl of ATP Detection Reagent to each well of a 96-well plate (25µl to each well of a 384-well plate) and wait at least 10 minutes prior to measuring luminescence. Avoid introducing bubbles, which can adversely affect luminescent signal detection.

   Note: Mixing the plate for 2 minutes on an orbital or electromagnetic shaker (for a 96-well or a 384-well plate) might increase reproducibility in replicate wells by more rapidly facilitating complete cell lysis. Alternatively, homogeneous mixing can be accomplished by adjusting liquid handler dispensing speeds or waiting for an additional time period (15–30 minutes) prior to measuring luminescence.

9. Calculate EC_{50} values by plotting net RLU values (subtracting average of blank wells) versus compound concentration. The EC_{50} value is the compound concentration that produced a 50% increase in ATP levels compared to virus and no-virus controls. Although it may be possible to grossly approximate compound EC_{50}, we suggest using commercially available software packages (GraphPad Prism®, SigmaPlot, etc.), which greatly aid in statistical treatment of the data through validated regressions and fits. Figure 5 is an example of such data treatment.

3.C. Protocol for Determining Antiviral Potency (continued)

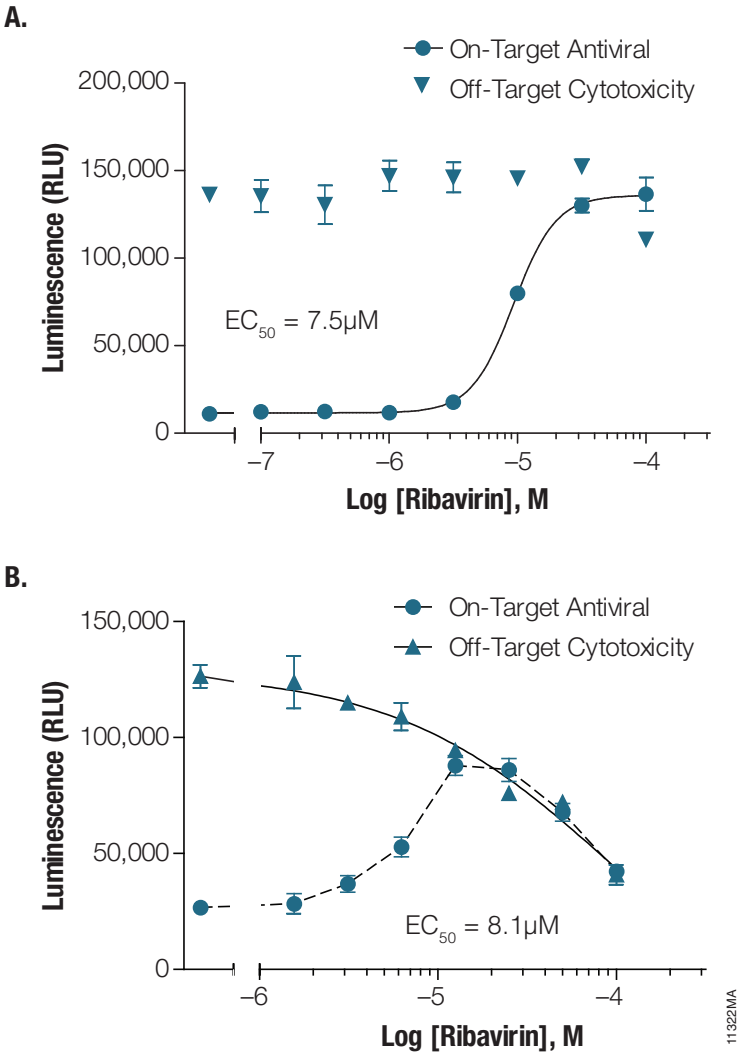

Figure 5. Calculation of antiviral potency. Panel A. Half-log (3.16-fold) dilutions of Ribavirin were added to either MDCK cells with 100 $TCID_{50}$ of H1N1 (on-target) or MDCK cells only (off-target) for 72 hours. **Panel B.** Twofold serial dilutions of Ribavirin were added to replicate wells of a 96-well plate containing BHK-21 cell monolayers. Either 100 $TCID_{50}$ of Dengue virus (Serotype 2) or medium alone were immediately added to two series of replicates to determine antiviral efficacy (on-target) and cytotoxicity (off-target), respectively, for 96 hours. For both panels, after incubation, ATP Detection Reagent was added and luminescence measured. Raw luminescence was plotted versus compound concentration, using GraphPad Prism® for both data sets. The antiviral dose-response plot was generated by a point-to-point fit, whereas the cytotoxicity plot was generated using a sigmoidal fit. The reported EC_{50} is the predicted compound concentration corresponding to a 50% inhibition of the viral cytopathic effect. Experimental data was provided by Southern Research Institute, Birmingham, AL, and is used with permission.

4. Appendix

4.A. Virus and Cell Model Considerations

Cell Type and Density

Viral infectivity is greatly affected by intrinsic host cell receptor expression and distribution. Efforts to define a susceptible host cell and optimal conditions for susceptibility (often linked to cell cycle, passage level and culture conditions) can improve observed CPE and in vitro results. Therefore, virus titration against multiple host cell types is recommended to optimize the CPE infection model. It is also important to conduct host cell titration studies without virus to establish the linear response range of the ATP detection chemistry.

Once host cell density and linear response range are established, it is important to define the exposure period for viral/host cell contact. Too short of an exposure period might lead to sub-optimal CPE. Too long of an exposure might adversely affect the cell health of uninfected or untreated control cell populations. Both scenarios would adversely affect the obtainable assay window.

Antiviral "Hits" and Rescreening Activities

It is a reasonable and advisable experimental practice to rescreen compounds that demonstrate antiviral activity. Such rescreening can either confirm antiviral activity or reveal false determinations and artifacts. Rescreening can be conducted in single point concentrations or in full dose-response titrations. Confirmation of efficacy can be conducted with compound, virus and cells, whereas compound-specific cytotoxicity can be revealed using only cells exposed to compounds. Compound-specific cytotoxicity can be determined using the Viral ToxGlo™ Assay or other orthogonal viability and cytotoxicity assays available from Promega. See Section 6, Related Products.

4.B. Reagent Chemistry Considerations

Temperature

The intensity and decay rate of the luminescent signal from the Viral ToxGlo™ Assay depends on the luciferase reaction rate. Environmental factors that affect the luciferase reaction rate will change the intensity and stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate the assay plates to a constant temperature before performing the assay.

Chemicals

The chemical environment of the luciferase reaction affects the enzymatic rate and thus luminescence intensity. Differences in luminescence intensity have been observed using different types of culture media and sera.

Solvents for the various test compounds may interfere with the luciferase reaction and thus the light output from the assay. Interference with the luciferase reaction can be detected by assaying a dose titration of compound or solvent in medium with exogenously spiked ATP.

Plate Recommendations

We recommend using standard opaque-walled multiwell plates suitable for luminescence measurements. In addition, be sure to use multiwell plates that are compatible with your luminometer.

5. References

1. Noah, J.W. *et al.* (2007) A cell-based luminescence assay is effective for high-throughput screening of potential influenza antivirals. *Antiviral Research* **73**, 50–9.
2. Heil, M. *et al.* (2007) Development and validation of a high-throughput screen for inhibitors of Respiratory Syncytial Virus. *Antiviral Research* **74**, A59. Abstract #77.
3. Severson, W.E. *et al.* (2008) High-throughput screening of a 100,000-compound library for inhibitors of influenza A virus (H3N2). *J. Biomolec. Screening* **13**, 879–87.
4. Rothwell, C. *et al.* (2009) Cholesterol biosynthesis modulation regulates dengue viral replication. *Virology* **389**, 8–19.
5. Li, Q. *et al.* (2009) Assay development and high-throughput antiviral drug screening against Bluetongue virus. *Antiviral Research* **83**, 267–73.
6. Phillips, T. *et al.* (2011) Development of a high-throughput human rhinovirus infectivity cell-based assay for identifying antiviral compounds. *J. Virological Methods* **173**, 182–8.

6. Related Products

Detection Instrumentation

Product	Size	Cat.#
GloMax®-Multi+ Detection System with Instinct™ Software:		
Base Instrument with Shaking	1 each	E8032

Cytotoxicity Assays

Product	Size	Cat.#
CellTox™ Green Cytotoxicity Assay	10ml	G8741
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260

Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
CellTiter-Blue® Cell Viability Assay	20ml	G8080

Multiplexed Viability and Cytotoxicity Assays

Product	Size	Cat.#
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200

Mitochondrial Toxicity Assay

Product	Size	Cat.#
Mitochondrial ToxGlo™ Assay	10ml	G8000

Oxidative Stress Assays

Product	Size	Cat.#
GSH-Glo™ Glutathione Assay	10ml	V6911
GSH/GSSG-Glo™ Assay	10ml	V6611

7. Summary of Change

The following change was made to the 2/15 revision of this document:

Removed a duplicate step in Section 3.C Protocol for Determining Antiviral Potency that resulted in the user adding virus twice.

[a]U.S. Pat. Nos. 6,602,677, 7,241,584 and 8,030,017, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

[b]U.S. Pat. Nos. 7,083,911, 7,452,663 and 7,732,128, European Pat. No. 1383914 and Japanese Pat. Nos. 4125600 and 4275715.

[c]U.S. Pat. Nos. 7,741,067, 8,361,739, 8,603,767, Japanese Pat. No. 4485470 and other patents pending..