Video Article

High-throughput Titration of Luciferase-expressing Recombinant Viruses

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Abstract

Standard plaque assays to determine infectious viral titers can be time consuming, are not amenable to a high volume of samples, and cannot be done with viruses that do not form plaques. As an alternative to plaque assays, we have developed a high-throughput titration method that allows for the simultaneous titration of a high volume of samples in a single day. This approach involves infection of the samples with a Firefly luciferase tagged virus, transfer of the infected samples onto an appropriate permissive cell line, subsequent addition of luciferin, reading of plates in order to obtain luminescence readings, and finally the conversion from luminescence to viral titers. The assessment of cytotoxicity using a metabolic viability dye can be easily incorporated in the workflow in parallel and provide valuable information in the context of a drug screen. This technique provides a reliable, high-throughput method to determine viral titers as an alternative to a standard plaque assay.

Video Link

The video component of this article can be found at http://www.jove.com/video/51890/

Introduction

Classical viral plaque assays continue to be a mainstay in virus research even though they can be notoriously time-consuming and constitute a significant bottleneck to obtaining results from experiments. More rapid indirect virus quantification methods have emerged including quantitative polymerase chain reaction (qPCR), ELISA, and flow cytometry[1-4]. Recent innovations such as the Virocyt virus counter can directly count viruses using advanced flow sorting technology and through a combination of protein and DNA/RNA dyes[5]. While all of these methods have undoubtedly quickened the pace of virus research, each method has its advantages and drawbacks. For example, qPCR can allow for quantification of specific viral genome sequence but cannot effectively discriminate infectious from defective virions[6]. ELISAs can be very specific however require a suitable antibody against the desired target viral protein and can be very expensive. While flow cytometry technology offers many advantages and has improved significantly, throughput and accessibility to highly specialized equipment nonetheless remains a hurdle. Importantly, all of these techniques are not ideally suited for high-throughput screening, for which the ease and time requirement of the virus quantification step is of critical importance.

Here we describe a high-throughput and easily automatable technique to titer viruses that express a Firefly luciferase (Fluc) transgene. This method generates approximate viral titers in a test sample based on luminescence signal reads through the parallel use of a standard curve of known amounts of virus. Samples containing unknown quantities of luciferase-expressing virus are transferred on to a permissive “plaquing” cell line in parallel with the standard virus dilution curve and virus-associated luminescence is read after a few hours incubation time. This allows for rapid, quantitative, often same-day generation of results, unlike classic plaque assay protocols which typically require several days of incubation in order to manually count visible plaques[7-9].

The protocol outlines the steps of our titration method using oncolytic Vesicular Stomatitis Virus encoding a Fluc transgene (VSVΔ51-Fluc) as an example and provides an overview of 1. Sample preparation 2. The plating of a permissive cell line for virus titration using an automated dispenser 3. The preparation of the viral standard curve 4. The transfer of the sample supernatants onto the permissive cell line using a 96-well manual pipettor 5. The assessment of sample cytotoxicity using a cell viability reagent 6. The preparation of the luciferin substrate 7. Reading of bioluminescence and 8. Data analysis.

Protocol

1. Sample Preparation

1. Obtain samples containing luciferase-expressing virus (herein VSVΔ51-Fluc as an example) for titration and transfer into 96-well plates. Alternately, perform infections in 96-well tissue culture plates and use supernatants directly.
2. Leave two columns untreated for the inclusion of standard curves. For experiments done directly in 96-well plates, titer at the end of the experiment (40 hr post-infection) or store at -80 °C and titer at a later date.

2. Preparation of Permissive Cells for Virus Titration

1. 24 hr prior to titering, prepare a suspension of Vero cells at a concentration of 2.5 x 10^5 cells/ml in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 30 mM HEPES, and 1% penicillin/streptomycin. NOTE: Although this protocol uses Vero cells, any suitable permissive cell line for VSV infection could be used.
2. Seed 2.5 x 10^4 cells (100 µl) in 96-well white solid flat-bottom plates using a microplate dispenser.
    1. Prepare 12 ml of cell suspension per plate plus 5 ml for priming.
    2. Clean microplate dispenser cassette by flushing the tubing with 50 ml of sterile water.
    3. Fill the microplate dispenser lines with cell suspension and let 5 ml of cell suspension flow through.
    4. Select a program that will dispense 100 µl in each well of a white-walled 96-well plate. Dispense, and repeat as necessary for additional plates. Also seed a few wells in a 96-well clear flat-bottom plate if opaque-bottom white-walled plates are used for verification of cell health and density.
    5. When finished, flush cells back into original container. Clean the cassette by running 50 ml of 70% ethanol followed by 50 ml of warm sterile water through the tubing.
    6. Make sure cassette and tubing are appropriately cleaned between uses.
3. Incubate cells for 24 hr at 37 °C in a humidified 5% CO_2 incubator.

3. Preparation of Viral Standard Curve

1. Prepare a standard curve of VSVΔ51-Fluc in serum-free DMEM such that the final concentration of plaque forming units (pfu) per ml after transfer onto Vero cells is as follows: 10^8 pfu/ml, 10^7 pfu/ml, 10^6 pfu/ml, 10^5 pfu/ml, 10^4 pfu/ml, 10^3 pfu/ml, 10^2 pfu/ml, and 10^1 pfu/ml. Prepare 50 µl of each concentration per plate of Vero cells plus an extra 10%.
   NOTE: Titer of the luciferase virus stock will need to be assessed in a classical way[10] in order to generate a standard curve that will allow for absolute quantification. Otherwise relative quantification can be achieved without precise titer information by arbitrarily setting viral titer based on dilution steps. For example the first dilution of the standard curve may be set to 10^8 viral units and the following 1/10 dilution to 10^7 and so on. In this context, one should express values as a fold-change compared to a pre-determined sample as absolute quantification will not be accurate.

4. Transfer of Sample Supernatants onto Permissive Cells

1. Check Vero cells plated in the clear-bottom 96-well plate under a light microscope to confirm that monolayers are at least 95% confluent.
2. Transfer 25 µl of sample supernatant onto Vero cells seeded in white-walled plates. Do not transfer supernatants into the 2 columns designated for standard curves. NOTE: This can be done simultaneously for all wells on a single plate using a 96-channel liquid handler.
3. Using an 8- or 12-channel multi-channel pipettor, add 25 µl of each dilution of the standard curve prepared in Step 3 to the Vero cells in the 2 designated columns.
4. Centrifuge plates for 5 min at 430 x g at RT.
5. Incubate for 5 hr at 37 °C in a humidified 5% CO_2 incubator.

5. Assessment of Cell Viability

1. NOTE: When starting from supernatants obtained from infection experiments done in clear 96-well plates, sample viability can be assessed prior to quantification with a cell viability indicator dye such as resazurin.
2. Add resazurin in an amount equal to 10% of the volume in each well of the 96-well plate of samples containing virus and cells. Include cell-only controls as well as media-only control to determine values for 100% and 0% viability respectively.
3. After 2-4 hr (incubation time will vary depending on cell type and on the concentration of commercially available or reconstituted powder of the dye), read and record the signal using a fluorescence plate reader (530-560 excitation, 590 emission). Report cell viability for a sample according to the formula Relative Metabolic Activity = ((Test sample signal - negative control signal) / (Cell only control signal - negative control signal)) x 100%

6. Preparation of the Luciferin Substrate

1. 30 min before the end of the 5 hr incubation, prepare the luciferin to obtain a 2 mg/ml solution in sterile phosphate buffered saline (PBS). Prepare 2.5 ml per plate plus an extra 2 ml. Protect solution from light. NOTE: The luciferin may also be prepared earlier in the day and stored at 4 °C until use.

7. Reading Bioluminescence

1. After the 5 hr incubation period, add 25 µl of luciferin to each well of Vero cells in the white solid plates. Add luciferin manually or with an automated dispenser integrated in the luminometer. NOTE: The use of an instrument with only single and end point reads may require the addition of a luciferase compatible lysis buffer prior to adding the luciferin substrate to improve consistency.
2. Read the plates with the following parameters:
    1. Shake for 5 sec.
    2. Wait 30 sec.
    3. Read luminescence at an appropriate fixed sensitivity / exposure value. If the option is available on the instrument, use multi-point reads (*e.g.,* 3 x 3 matrix) to improve accuracy.
3. Record and save the quantified bioluminescent intensity.
4. If an automated dispenser was used to add luciferin purge the luciferin and prime the lines with warm sterile water.

8. Data Analysis

1. For accurate results, solve the non-linear regression to generate a Hill equation from the standard curves. Apply this equation to the titered samples to calculate viral expression units. Some viruses or situations may produce a standard curve with a linear relationship; in this case solve for a linear equation.

Representative Results

A summary of the workflow describing the high-throughput method is illustrated in **Figure 1**. **Figure 2** shows the results of a typical standard curve ofVSVΔ51-Fluc. Four-parameter non-linear regression analysis generated a Hill plot from which unknown input pfu (estimate of viral titer) can be interpolated. These estimated titers are termed viral expression units (VEU). **Figure 3A** shows VEUs and titers obtained by performing a standard plaque assay with the same samples[10]. Samples originated from an experiment where various chemicals were used to enhance the replication and spread of VSVΔ51-Fluc in 786-0 cells. VEUs interpolated from the standard curve must be multiplied by a factor which is based on the dilution of sample supernatants being transferred on to Vero cells (in this case, the dilution factor is 5). The linear correlation between titers and VEU is shown in **Figure 3B** with an R^2 of 0.8083 and a Pearson's r score of 0.899 ($p < 0.0001$). In **Figure 4**, typical cell cytotoxicity data obtained from a metabolic assay is shown for 786-0 cells treated with chemicals prior to infection with VSVΔ51-Fluc. Finally, **Figure 5** shows typical standard curves obtained with various viruses (Herpes Simplex Virus (HSV), Vaccinia virus, and Adeno-Associated Virus (AAV), all expressing Firefly luciferase) and describes incubation times and freeze-thaw cycles, as required.

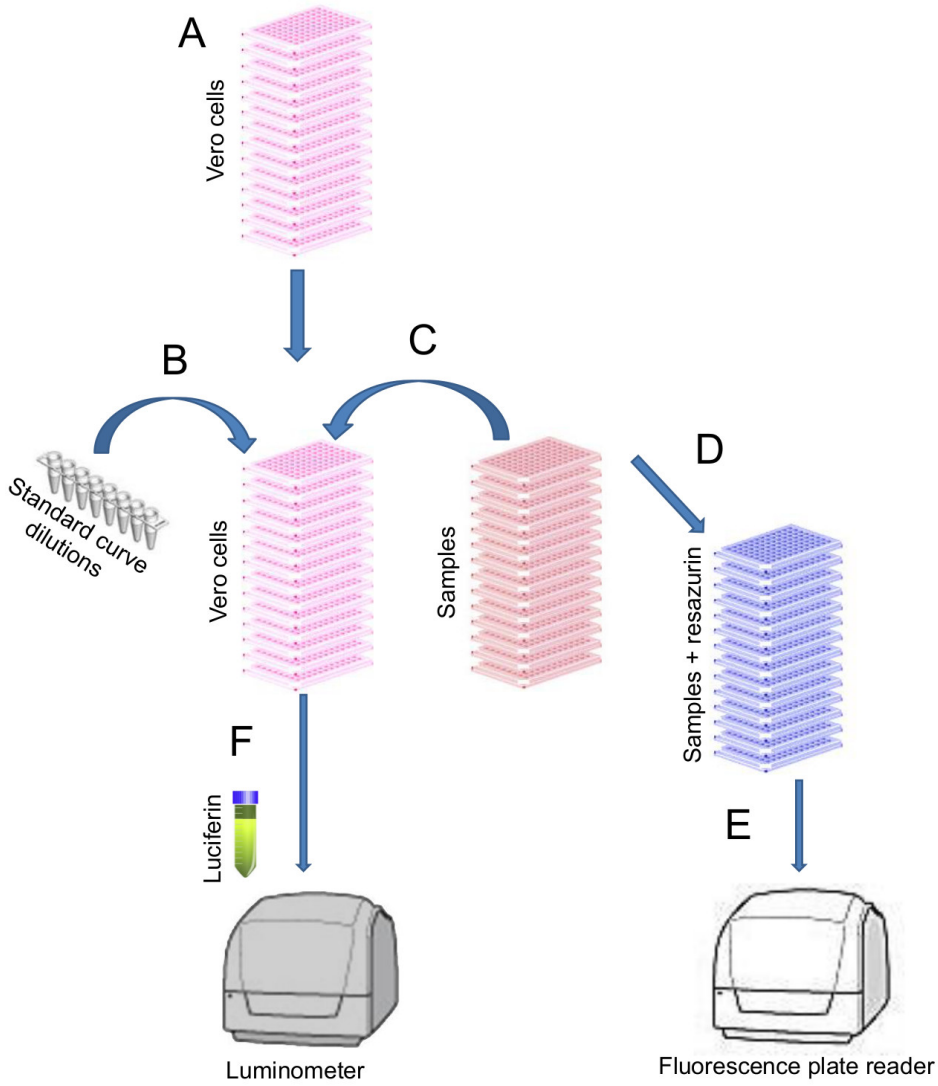

Figure 1. Workflow of high-throughput virus titering and cytotoxicity assay using VSVΔ51-Fluc. (A) Seed 2.5 x 10^4 Vero cells per well (100 µl) and incubate at 37 °C. **(B)** 24 hr later transfer 25 µl of standard curve on to Vero cells (2 columns per plate). **(C)** Transfer 25 µl of samples to be titered on to remaining Vero cells. Centrifuge plates and incubate at 37 °C. **(D)** In an amount equal to 10% of the volume in the well, add resazurin reagent to the plate containing the original samples. Incubate plates at 37 °C. **(E)** After 3 hr, read and record fluorescence and assess cytotoxicity. **(F)** After 5 hr, add 25 µl of 2 mg/ml solution of luciferin to each well of Vero cells. Read luminescence and calculate Viral Expression Units.

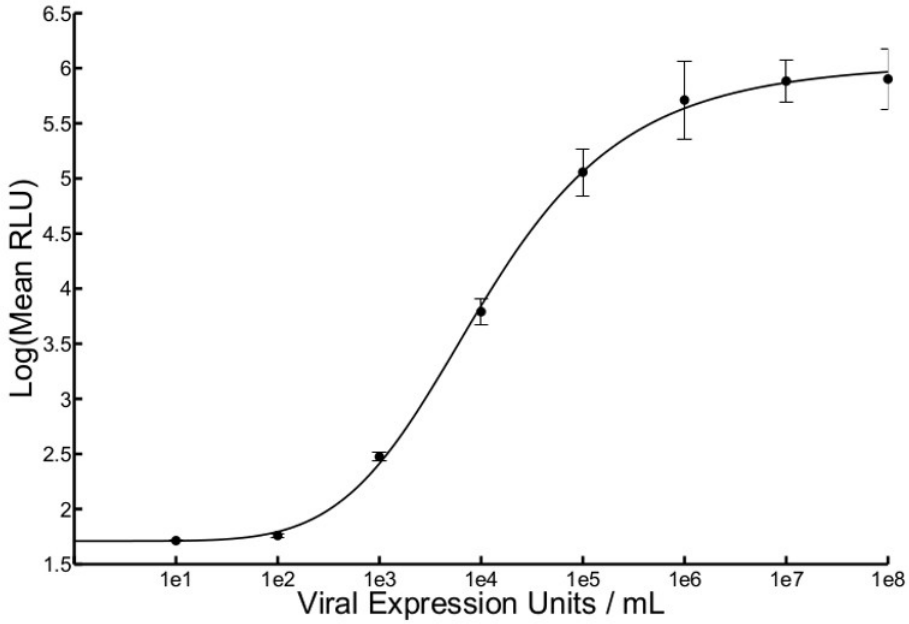

Figure 2. Expected standard curve of VSV∆51-Fluc. Luciferase expression was measured 5 hr post supernatant transfer at five different points within a well using a luminometer and bioluminescence was expressed in mean relative light units (RLU). Mean RLU was plotted against known input pfu/ml to solve the non-linear regression and generate a Hill equation. The average of two replicate curves and standard error bars are shown (r^2= 0.9993).

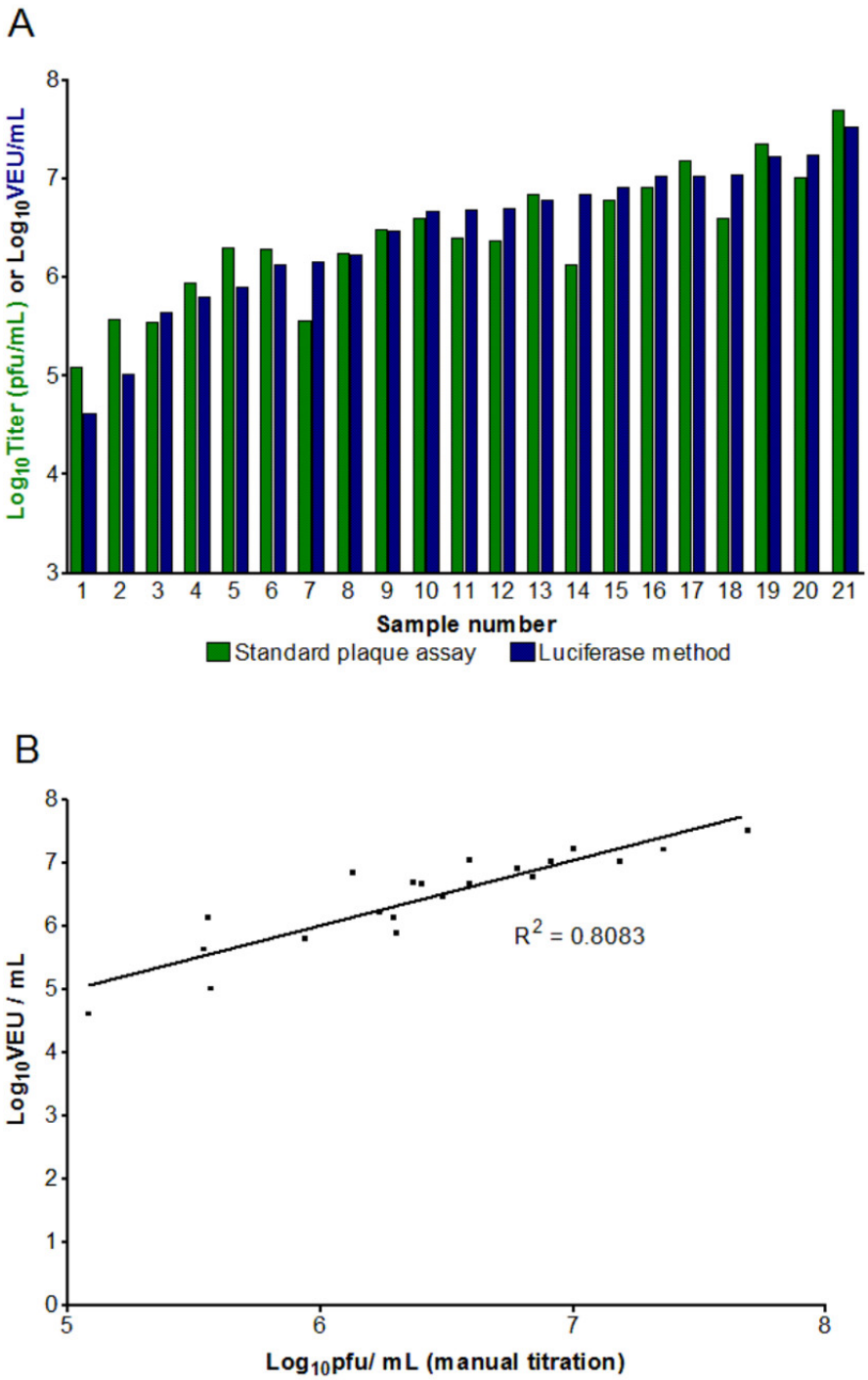

Figure 3. Comparison of standard plaque assay titers with those obtained by high-throughput method. (A) Viral titers in pfu/ml obtained by standard plaque assay on Vero cells were compared with calculated viral titers (VEU/ml) from the same samples titered using the high-throughput luciferase assay. **(B)** Linear relationship between VEU/ml and titer obtained via standard plaque assay. Linear regression curve and coefficient of determination (R^2) are shown.

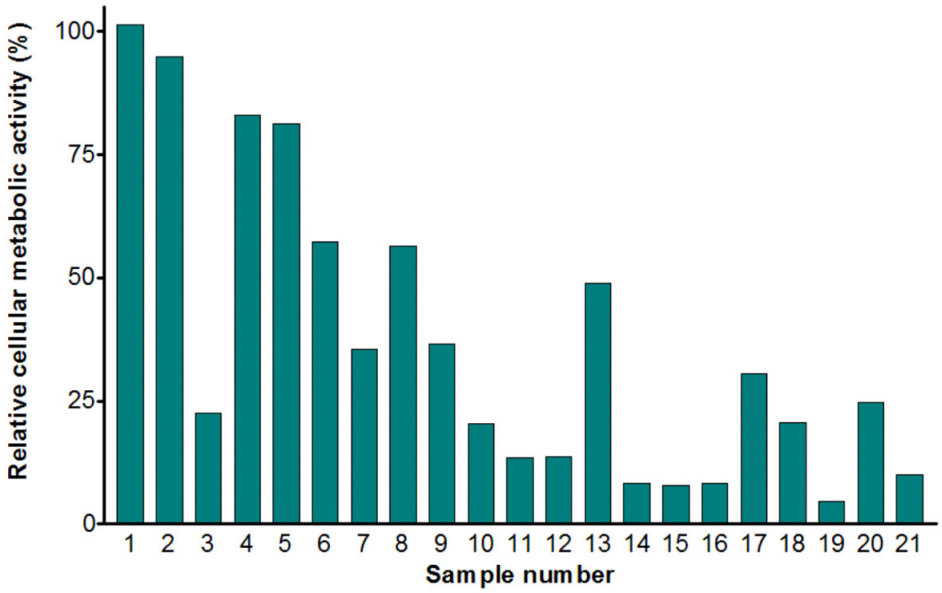

Figure 4. Sample viability. Sample viability prior to supernatant transfer onto Vero cells was determined by assessing cellular metabolic activity using a commercially available resazurin solution. Raw fluorescence values were normalized to that of untreated, uninfected wells.

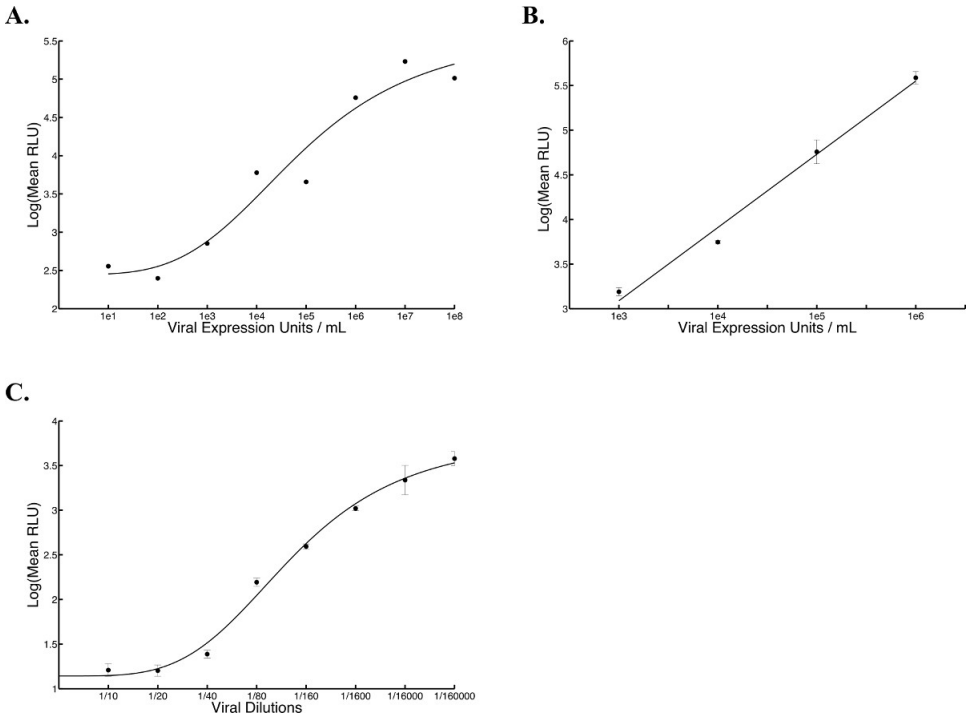

Figure 5. Expected standard curves with HSV, Vaccinia, and AAV viruses. Luciferase expression was measured at five different points within a well using a luminometer and bioluminescence was expressed in mean relative light units (RLU). **(A)** HSV standard curve was added onto Vero cells plated 24 hr earlier at a density of 2.5 x 10^4 cells per well (100 µl) and luciferase measurement was made 17 hr post supernantant transfer (R^2= 0.9489, n=1). A Hill equation was generated by solving the non-linear regression. **(B)** Vaccinia standard curve was added onto Vero cells plated 24 hr earlier a density of 2.5 x 10^4 cells per well (100 µl) and incubated for 2.5 hr at 37 °C, after which luciferase measurements were taken (R^2= 0.9892). A linear equation was generated by solving for the linear regression. **(C)** AAV standard curve was added onto human lung carcinoma cells (A549) plated 24 hr earlier at a density of 2.5 x 10^4 cells per well (100 µl) and luciferase measurement was made 24 hr post infection. A Hill equation was generated by solving the non-linear regression. The average of five replicate curves and standard error bars are shown (R^2= 0.9926).

Discussion

The luciferase-based approach described here provides a number of advantages over other existing methods including its ease, quickness, minimal equipment need, and relatively low cost. A key contributor to this is the avoidance of a serial dilution step. Nonetheless, serial dilution-

based derivatives of this protocol are certainly feasible and were recently used to assess luciferase-expressing Ebola titers in a high-throughput antiviral screen[11]. While inherently more time consuming and more expensive, such adaptations may provide greater dynamic range for viral quantification when necessary. In addition to being particularly well suited for evaluating viral titers in the context of high-throughput screens, our one-step luciferase based viral quantification method generates accurate estimates of infectious virions in the case of replicating viruses. Furthermore, readouts of luminescence along with cytotoxicity data from the same experiment give a more complete picture of the effect of the experimental conditions on target cells, which is particularly useful in the context of oncolytic viruses and drug screens.

The example illustrated here uses a replicating negative single strand RNA virus; however, this protocol can be adapted to a number of replicating and non-replicating viruses with a few minor protocol adjustments. This includes DNA viruses such as Vaccinia, HSV, and AAV (see **Figure 5**). Samples infected with intracellular viruses, such as Vaccinia virus for example, require a virus-release step prior to quantification (*e.g.*, at least one freeze-thaw cycle). When applying this technique to other viruses, it is necessary to optimize the incubation time from the transfer of the viral supernatant or lysate to the reading of the plates by the luminometer. This parameter will depend mainly on the replication cycle of the virus in the permissive cell line and the strength of the promoter driving luciferase expression. This is best done by using the full standard curve in the optimization step. To do this, one must infect the appropriate permissive cell line with various replicates of the prepared standard curve and read each replicate at a different time points post-transfer. Ideally, an incubation time point is chosen that leads to a linear relationship between LOG(RLU) and LOG(titer) spanning the expected sample titer range. For VSVΔ51-Fluc, this is typically from 10^4 pfu/ml -10^7 pfu/ml for a 5 hr incubation time. If lower or higher titers are expected from samples, one can simply increase or reduce the incubation time respectively. Alternately, samples may be diluted to fall within the range much as is done typically for ELISA.

As mentioned above, this method is well suited to perform high-throughput drug screens using drug libraries as most of the steps can be automated. Cells can be plated efficiently using an automated microplate dispenser, the drug library can be added using a 96-channel liquid handler, virus can be added using a microplate dispenser and plates read using an automated luminometer. In theory, this can also be adapted to 384-well or smaller formats; however, the limitation to this end is number of cells that can be plated, given fewer cells leads to a narrower range in the linearity of the LOG(RLU) to LOG(Titer) relationship. Finally, assessment of cell viability using resazurin or other metabolic dyes can be easily incorporated in the workflow, allowing for discrimination of cytotoxic compounds in antiviral screens or identification of compounds that lead to synergistic killing in combination with viruses[12]. Nevertheless, limitations of this method include the requirement of a luciferase transgene expressing virus, which is not always possible, and the availability of a sufficiently permissive cell line. However, it is likely possible to adapt the method for use with other reporter genes (*e.g.*, GFP) provided the reporter quantification method has a suitable linearity and signal to noise ratio. Overall, the described high-throughput method can be modified to suit many different viruses and tailored to diverse applications.

Disclosures

The authors have nothing to disclose.

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