Integration of Fluorescence Detection and Image-Based Automated Counting Increases Speed, Sensitivity, and Robustness of Plaque Assays

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Plaque assays are used to measure the infectious titer of viral samples. These assays are multi-day and low-throughput and may be subject to analyst variability from biased or subjective manual plaque counting. Typically, on day 1, cells are adhered to plates overnight. On day 2, cells are infected with virus. After 3 additional days, plaques are fixed, stained with a horseradish peroxidase (HRP)-conjugated antibody and a HRP substrate, and counted by eye. Manual-based visual counting of plaques is time-consuming and laborious and may be subject to variability between analysts. Also, the assay must proceed for several days to allow the plaques to increase to sufficiently large sizes for manual identification. Here, we integrate fluorescent detection and automated plaque counting to increase the sensitivity and speed of the assay. First, we stain plaques with a fluorescent-labeled antibody. Second, we implement a plate-based cell imager to perform non-biased, non-subjective plaque counting. The integration of these two technologies decreases the assay length by 40%, from 5 days to 3 days, because plaque size, plaque signal to noise, and manual visualization are no longer limiting. This optimized plaque assay is sensitive, fast, and robust and expands the throughput and usage of this method for measuring plaque formation.

INTRODUCTION

Gene therapy is a promising platform for delivery of therapeutic genes in research and clinical trials. Adeno-associated virus (AAV) is an attractive vehicle for delivery of these therapeutic genes to target cells based on its effectiveness and favorable safety profile. AAV is a non-enveloped virus containing a single-stranded DNA (ssDNA) genome surrounded by a protein shell. AAV can be produced through various methods, including transient transfection of human embryonic kidney (HEK) cells, using mammalian producer cell lines, baculovirus infection of insect-derived SF9 cells, or a helper virus, such as HSV, to transfect a stable cell line.[1–5] Because recombinant AAV (rAAV) vectors are replication defective, they require either a plasmid containing helper functions or a helper virus, such as adenovirus or herpes simplex virus (HSV), to replicate. During infection, when AAV enters a cell's nucleus, the ssDNA becomes the double stranded (ds) form. This dsDNA is necessary for gene expression. Without a helper virus, the AAV remains in a latent form in the cell. In the presence of a helper virus, however, AAV can actively be produced with the gene of interest. Because optimal rAAV production can be dependent on the concentration of helper virus used, helper virus infectious titer is one of the critical factors in establishing an efficient and high-yielding rAAV production process.

There are many orthogonal approaches to measure viral genomes and infectious particle counting. Viral genome titer can be experimentally measured for DNA or protein using techniques such as RT-PCR, qPCR, and western blots.[6,7] Intact virus particles can be quantified using capsid titer ELISAs, flow cytometers, or commercially available virus counters.[8,9] Finally, virus, such as HSV, can be quantified using functional approaches, such as conventional plaque assays or cytopathic effect assays.[10] While protein and particle counters are rapid and quantitative, they do not yield information regarding the infectivity or functionality of the virus.

Typically, the infectious titer of a virus is measured through a plaque assay. The plaque assay was first developed in 1952 to calculate titers of bacteriophages in plant biology and was later adapted to measure the concentrations of viral samples.[11] In general, cells are seeded in multi-well plates to achieve a confluent monolayer. The following day, cells are inoculated with diluted viral samples for a specific amount of time (dependent on the helper virus being titered). The inoculum is removed and replaced with fresh medium, and cells are incubated for several days until large enough plaques form and can be visualized and counted by eye. The traditional plaque assay is multi-day, labor intensive and can be subjective due to manual plaque counting by different analysts.

Here, we describe the implementation of the Celigo imaging system from Nexcelom into the traditional plaque assay workflow to automatically identify, image, and count plaques. Automated plaque counting is not only consistent with manual plaque counting—it also improves the assay robustness by eliminating human bias. We also integrate fluorescence detection of plaques as a means of

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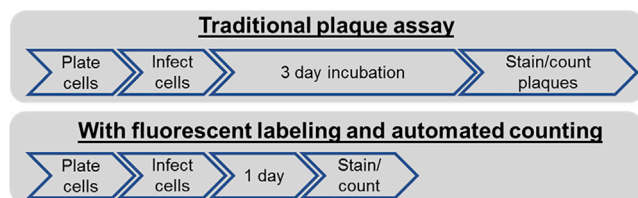**E-mail:** allyson.masci1@biogen.com

Figure 1. Flow Diagrams of Traditional and Improved Plaque Assay

By implementing a fluorescent-labeled antibody and an automated imager, the incubation time of this assay can be shortened from 3 days to 1 day, and the analysis can be more robust.

increasing assay sensitivity and therefore reducing the assay duration, since fluorescence allows earlier detection of very small plaques that are not normally visible by eye. Implementing such method improvements can potentially increase assay and analyst throughput to support critical process development timelines.

RESULTS

Manual Counting versus Automated Counting

The plaque assay is considered the gold standard for quantification of viral titer, despite its development in the 1950s.[11] Surprisingly, the method of counting plaques through manual visualization by analysts is still widely used. This method of counting can be subjective to analyst to analyst differences.

The Celigo imaging system from Nexcelom was evaluated for its ability to image and accurately count plaques for the helper virus used during process development. We compared the traditional counting method by eye versus the imaging and automated counting performed by the Celigo imager as outlined in Figure 1. The Celigo automatically detects and counts plaques in each well based on the established parameters. It highlights the plaques and includes a total plaque count per well. Figure 2A shows the 24-well plate as imaged on the Celigo. The total number of plaques counted per well is shown at the bottom of each well. This image also shows an individual well imaged on the Celigo. Plaques are filled in green.

The results in Figure 2B show that imaging and counting by the Celigo imaging system is similar to the traditional counting method by multiple analysts. Plaque counts for each sample are comparable, with the average standard deviation being 3 and the average RSD being 4% between manual and automated counting. There is also a strong linear correlation between the two methods of plaque counting, with an $R^2 = 0.9791$. Data from control samples run on different days have an average difference of 5.3%, showing the consistency of the results for the same samples over various assay days and runs. These results demonstrate that automated plaque counting by the Celigo imager is comparable to the traditional method of counting and highlights the potential to eliminate subjectivity in analyst to analyst bias. The implementation of the Celigo technology highlights the improvements in assay throughput and robustness by simple solutions to traditional plaque assays.

Automated Plaque Counting Using Fluorescence Detection

The HSV plaque assay is a laborious 5-day assay. The workflow of this assay is shown in Figure 1. A bottleneck of this assay is the method of manual plaque counting. This form of counting requires that the plaques be large enough to be visualized by eye. The hypothesis that an imaging system is just as accurate at identifying and counting plaques allowed us to assess whether alternative forms of detection could increase the sensitivity of this assay. In the method, plaques were visualized with the application of a viral antibody conjugated to horseradish peroxidase (HRP) and stained with diaminobenzidine tetrachloride (DAB). We tested whether fluorescence imaging using a fluorescent-labeled antibody could be used as an improved method of detection in this assay. As shown in Figure 3A, plaques stained with the fluorescent-labeled antibody, imaged, and counted using the Celigo imager were similarly visible after 3 days, or 72 hours post-infection. Importantly, both the fluorescent detection method and the traditional HRP-based method resulted in similar counts of plaques 3 days post-infection.

A potential benefit of using fluorescence detection of plaques is eliminating the bottleneck of visualization of plaques that must be large enough to the eye. Plaques stained by the traditional HRP-labeled approach are only visible by the eye 3 days post-infection. Using the Celigo imager, the readout from the traditional assay can be reduced to 2 days post-infection, since the Celigo imager can detect and count plaques that are harder to visualize by eye. Plaques stained using the HRP-labeled method cannot be detected with the Celigo 1 day post-infection. In contrast, integration of fluorescence detection of plaques and automated imaging increased the sensitivity and speed of the traditional plaque assay. Fluorescently labeled plaques can be visualized 3, 2, and even 1 day post-infection. Although the fluorescent plaques are much smaller to the eye 1 and 2 days post-infection, the plaques can still be detected, imaged, and counted by the Celigo, as shown in Figure 3A. Though plaque size is different over different days, all samples have a consistent plaque count over the different post-infection periods, as shown in Figure 3B. These results demonstrate that fluorescent detection results in similar plaque count accuracy as the traditional method of detection. In addition, integration of fluorescence detection of plaques and automated counting can increase speed of the plaque assay from 5 days to 3 days, as outlined in Figure 1.

Though plaque morphology may be slightly different, plaques are still detectable by analysts and the Celigo imager.

DISCUSSION

The automated plaque counting method with fluorescence detection described here assists in the speed of detection of plaques in a plaque assay. There are several types of infectious viral titer assays, however, that do not form plaques. 50% tissue culture infectious dose ($TCID_{50}$) is another commonly used assay to detect infectious viral titer. $TCID_{50}$ is an endpoint dilution assay that determines what dilution of a viral sample is needed to infect 50% of inoculated cells. After infection, infectious titer is measured by qPCR.[12,13]
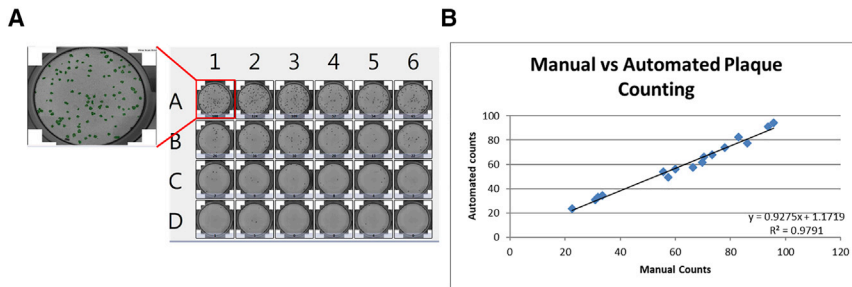
Figure 2. Manual Counting versus Automated Counting

(A) An overview of a 24-well plate demonstrates automated plaque counting with Celigo. A zoom-in picture shows A1 well from the plate. (B) Results from automated counting shows correlation with manual counting. The results are comparable.

Since both plaque and TCID$_{50}$ assays are multi-day and low-throughput, alternative methods to determine infectious viral titer are desirable. Flow cytometry has been used to detect adenoviral infectivity. Using adenoviral vectors that are tagged with a GFP, infected cells can be detected by flow cytometry. This method generally takes about a day, and samples tested on both this method and the plaque assay have comparable infectious titer results, making this an attractive alternative to the plaque assay.[14] In addition to adenovirus, flow cytometry has been used to measure the viral infectivity of measles virus and baculovirus.[13,15]

High performance liquid chromatography (HPLC)-based methods using anion exchange columns has been shown to quantify viral particles in baculovirus. After an incubation using SYBR green dye to label viral DNA, the virus can be eluted from the anion exchange column in less than 25 min.[16] Although this method quantifies the amount of viral particles present, it does not necessarily measure the amount of infectious virus in a sample. Additionally, a new technology, droplet digital PCR (ddPCR), quantifies the absolute amount of target DNA in a reaction. This technology has been shown to quantify infectious titer in lentivirus.[17]

While the automated plaque counting method described here is a step forward from the traditional plaque assay, it will not be helpful in infectious titer assays that do not form plaques, such as any of the methods listed above. It could be useful, however, in plaque-like assays.

For example, one method for determining infectivity of Adenovirus 5 (Ad5), a helper virus, is a focus formation assay. Similar to a plaque assay, cells are infected with a serial dilution of virus-containing samples, and, days later, they are fixed, incubated with an HRP-conjugated antibody to the virus, and stained with a DAB substrate. Cells infected with Ad5 form countable foci, and results are reported in infectious units per milliliter (ifu/mL).[18,19] With optimization of parameters, the Celigo can be used to recognize and count individual foci, thus aiding in the speed of the readout of the assay.

A similar fluorescent focus formation assay has been shown to replace a plaque assay in murine norovirus (MNV). In this plaque assay variation, the MNV antigen is stained with a fluorescent tag. Since viral antigen expression precedes plaque formation, this assay can quantify infectious titer in less time than a plaque assay.[20] The Celigo could be useful in counting focus-forming units in this assay.

Recent studies have similarly assessed and implemented new technologies to improve upon the traditional plaque assay. Near infrared fluorescence coupled to detection by the LI-COR Biosciences In-Cell Western system demonstrated similar increases in assay sensitivity and speed in traditional plaque assays as to what we have described here.[21] Unlike the LI-COR system, the Celigo imager that we have implemented can also be used for imaging non-fluorescent plaques.

Electronic impedance technology was also implemented to assess virus infection of cells.[22] This study demonstrated that impedance technology could be used for real-time monitoring of virus-cell interactions and detecting transient effects of certain anti-viral drugs. However, this technology workflow does not calculate a plaque-forming unit (PFU) value of the traditional plaque infectivity assay.

Though there are new methods to quantify infectious viral titer, the plaque assay and TCID$_{50}$ assay remain the gold standards. The automated plaque counting method described here is an improvement to a well-established method. By utilizing automated counting and a computer algorithm for counting plaques, analyst subjectivity is removed. Using the Celigo imager also significantly reduces plaque counting time and improves data consistency. This technology, when applied to 48- or 96-well plates can significantly increase the sample throughput to support a high volume of samples from process optimization. Also, by integrating fluorescence detection of plaques, we demonstrate that the assay time duration can be decreased by 40% while maintaining similar plaque counting performance to the traditional plaque assay.

Another advantage of using a computer-based imaging system is the improved data capture, documentation of results, and traceability. The ability to save and review images may also allow more clarity around setting assay acceptance criteria and provide guidance and examples when training new analysts. In quality control or good manufacturing practice (GMP) environments, the process of running assays requires both an analyst and a reviewer. By implementing a plate imager with automated plaque counting, the processes of analyzing the results and data review are well-documented, and images can be saved for potential filings or audits by regulatory agencies.

Our goal was to assess technologies that could be easily implemented into the current workflow of the plaque assay, yield the PFU value,
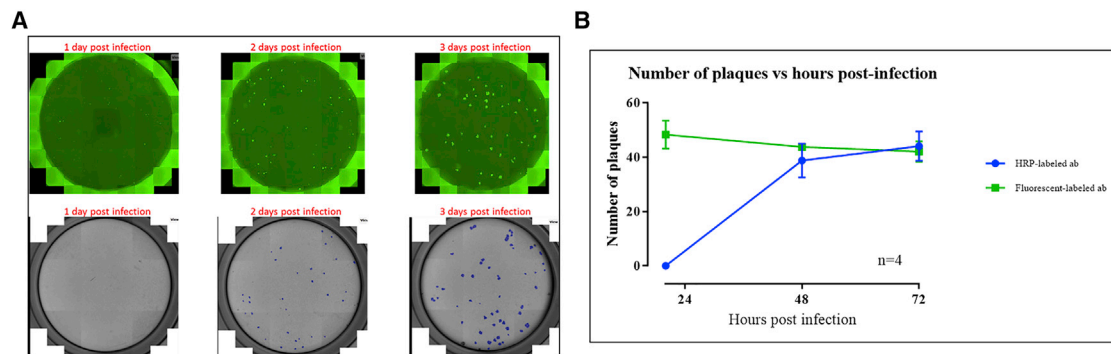
Figure 3. Plaque Counting and Visualization Using the Fluorescent Staining versus HRP Staining

(A) The increased sensitivity of the fluorescent-labeled antibody enables plaques to be visualized as early as 1 day post-infection. The HRP-labeled antibody allows plaques to be seen starting at 2 days post-infection. While plaques cannot be seen at 1 day with this antibody, detection using the fluorescent antibody shows that plaques have already begun to form. As time increases, the plaque sizes increase in all images. (B) The graph shows that the fluorescent-based method detects plaques as early as 1 day post-infection, while the HRP-based method is not sensitive enough to detect plaques at this time point. However, the number of plaques over time does not significantly increase; therefore, detection at 2 days post-infection can be considered for both fluorescent and HRP-labeled plaques. Error bars represent the SD of the data points.

and improve assay robustness, sensitivity, and speed. This study highlights improvements in classic assays by simple solutions like automated imaging technologies.

MATERIALS AND METHODS

Cell Line

V27 cells, VERO cells (kidney epithelial cells from African green monkey) that have been stably transfected with the infected cell protein 27 (ICP-27)-expressing gene of HSV-1, were cultured in DMEM without sodium pyruvate (Hyclone SH30022.02) supplemented with 10% fetal bovine serum (FBS) (Hyclone SH30071.03). For the plaque assay, cells were used at or above passage 3 from thaw, but not beyond passage 50.

Antibodies

Polyclonal rabbit anti-HSV-1 antibody and HRP-conjugated anti-HSV-1 antibody were purchased from Dako. The unlabeled antibody was fluorescently labeled using the Alexa Flour 488 Labeling Kit from Thermo Fisher (catalog number A20181).

Viral Plaque Assay

HSV infectious titer was measured using a modified viral plaque assay with some modifications.[23] On day 1, V27 cells were seeded into 24-well plates at 2.5×10^5 cells/mL. On day 2, cells were infected with 10-fold serial dilutions of rHSV-1 vector stocks. Cells were incubated with the virus for 6 h at 37°C, 5% CO_2. At the end of the incubation, the infection medium was removed and replaced with medium containing 0.2% human gamma globulins. Cells were incubated for 1, 2, or 3 days post-infection. On the day of plaque staining, the medium was removed from the 24-well plates, and wells were dried for approximately 2 h. Ice-cold methanol was used to fix the cells for 20 min at −20°C. After fixation, methanol was removed, and cells were washed with PBS containing 1% BSA (Thermo Fisher Scientific) and then blocked for 30 min with the same buffer on an orbital shaker at room temperature.

After blocking, cells were incubated with a polyclonal rabbit-anti-HSV-1 antibody (Dako, 1:350) in PBS containing 1% BSA for 1–2 h. Cells were then washed three times with PBS, and viral plaques were visualized by application of a VIP vector substrate staining with diaminobenzidine tetrachloride (DAB, Pierce). Viral plaques were identified as dark purple spots by visual analysis. PFU per milliliter were calculated by (average PFU/well) × (dilution factor)/ (volume of inoculum).

Automated Plaque Counting and Data Analysis

The plaque assay was run conventionally as described above. However, after cells were fixed and stained, plates were scanned on the Nexcelom Celigo imager. Using the brightfield setting, viral plaques in each well were detected and counted using the "single colony verification" application on the instrument.

Fluorescence Detection and Automated Plaque Counting

This assay was run conventionally, as described above; however, after blocking, cells were incubated with an anti-HSV-1 Alexa Fluor 488-labeled polyclonal antibody (1:350). In both cases, the antibodies were diluted 1:350 in PBS containing 1% BSA, and the cells were incubated for 1–2 h on an orbital shaker. After the antibody incubation, cells were washed three times with PBS, and plates were left to dry in the dark. Fluorescent viral plaques in each well were detected on the Celigo imager using the fluorescence setting.

AUTHOR CONTRIBUTIONS

CONFLICTS OF INTEREST

All authors were employees of Biogen at the time of the study.

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