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Accelerated $TCID_{50}$ Assay Using Live-Cell Analysis

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Abstract

The use of viruses and viral vectors in gene therapy, cell therapy, vaccines, or basic research necessitates an accurate assessment of infectious titers, a process that is often encumbered by tedious and lengthy infectivity assays. Assays like the tissue culture infectious dose 50 ($TCID_{50}$) assay are crucial, determining the dilution at which 50% of cell culture wells display a cytopathic effect (CPE) due to virus infection. While $TCID_{50}$ is widely used, its manual readout via visual CPE assessment is labor-intensive and prone to error, leaving scope for the introduction of an automated solution. Here we propose a staining-free evaluation of viral infectivity using the Incucyte Live-Cell Analysis System's automated confluence analysis to enhance accuracy, reduce manual steps, and shorten incubation time from 8 to 3.5 days. The system's efficacy is demonstrated using herpes simplex virus 1 (HSV1) and Vero cells as a model.

Introduction

Viruses or their modified forms (viral vectors) are efficient nucleic acid and gene delivery vehicles that play an important role in gene therapy, cell therapy products, and vaccine development and manufacture. The accurate assessment of infectious titer holds crucial significance, with the quality control of viral vectors presenting a substantial pain point in viral vector process development and production. To accelerate the development of the viral vector production process, or screen a variety of antiviral substances, there is a pressing need for reliable and efficient assays to quantify them. The $TCID_{50}$ assay is a well-known method. The $TCID_{50}$ assay can be performed to study the progression of virus infections, the effectiveness of therapeutic substances, or to determine the infectious virus titers, more precisely the number of infections the sample causes per unit volume.[1] The $TCID_{50}$ assay is used to identify the dilution at which 50% of the cell culture wells display a cytopathic effect (CPE) as a result of virus infection and replication. This is performed by adding serial dilutions of the virus sample onto a monolayer of susceptible cells and incubating until a cytopathic effect can be observed.[2] Various formulas exist to calculate the $TCID_{50}$/mL of a sample, including the Spearman-Kärber or the Reed-Muench methods.[3] A wide variety of virus species exist and not all viruses necessarily induce a cytopathic effect in infected cells. The cytopathic effect can manifest as rounding, detachment, or lysis of cells, and also depends on the cell line used. The $TCID_{50}$ assay is useful for quantifying viruses that cause a cytopathic effect in the infected cell line. These include but are not limited to: herpesviruses, e.g., herpes simplex virus[4], picornaviruses, e.g., poliovirus[5], and flaviviruses, e.g., hepatitis C virus.[6]

The $TCID_{50}$ assay is a commonly used, versatile assay applicable for a wide variety of viruses, however, the major disadvantage is that the readout is often performed by visually assessing the cytopathic effects caused by the infection of the cells by the virus. To facilitate a visual readout of the cytopathic effect, protocols often require performing an additional manual staining step. Obtaining this visual readout is not only laborious, resulting in low throughput, but is also more prone to user error and may present safety issues.

In this application note, we demonstrate an automated, stain-free evaluation of CPE as a replacement for visual analysis using the Incucyte Live-Cell Analysis System. This platform simultaneously reduces the potential for inaccuracy from user error, and accelerates the assay procedure by reducing the number of manual steps required and enabling accurate readouts after a shorter incubation period (3.5 days rather than 8 days). Note: the duration of the assay may vary for different viruses and target cell lines. As a model system, we used herpes simplex virus 1 (HSV1) and Vero cells.

Assay Principle

The Incucyte enables kinetic monitoring of cell cultures using HD Phase-contrast and brightfield images, that are automatically quantified via integrated software. Here, confluence analysis is used to evaluate the cytopathic effect of HSV1 on Vero cells and calculate $TCID_{50}$ values, enabling accurate prediction of viral titer after 3.5 days rather than 8 days, as well as visualization of assay progression over time.

The assay principle is shown in Figure 1.

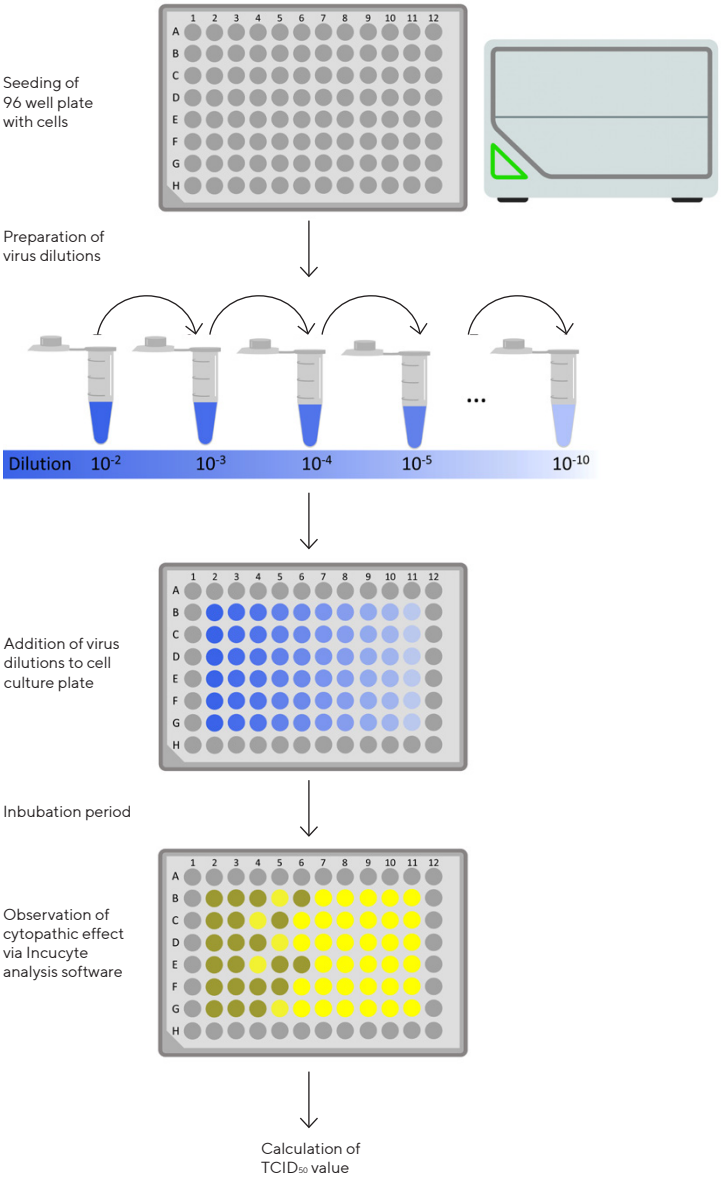

Figure 1: $TCID_{50}$ assay workflow.

Materials and Methods

Cell Culture

Vero monkey kidney epithelial cells were cultured in tissue culture flasks. At 70–90% confluence, they were harvested for seeding into the $TCID_{50}$ assay.

Incucyte $TCID_{50}$ Assay Workflow

1. **Seed 96-well plate with cells**
   The first step involves seeding the cells at 1×10^5 cells/mL (initial confluence of cells 20%) into a 96 well plate that is placed in the Incucyte inside a static incubator. Cells are allowed to attach to the well bottom and grow to 50–90% confluence, as evaluated using the integrated confluence analysis software. Image scans are scheduled in regular intervals and acquired images are analyzed with the integrated software.
2. **Preparation of serial virus dilutions**
   A dilution series of the virus sample is prepared. The serial dilution can be prepared in reaction tubes or in another 96 well plate using a multi-channel pipette.
3. **Infection of cells with virus**
   The diluted virus samples are added directly to the cell culture plate, without removing the media already present. The outside wells (row A and H and column 1 and 12) contain cell culture media or PBS and act as buffer to edge effects resulting from evaporation or other environmental factors in the incubator.
4. **Incubation period**
   The plate is incubated for several days in a static incubator at 36 °C ± 1 °C, 5 ± 1% CO_2 to maintain cells at an optimal temperature and allow the virus to infect cells and replicate.
5. **Observation of cytopathic effect**
   Throughout the incubation period (4–8 days), the wells of the cell culture plate are analyzed for signs of cytopathic effects. This is performed via the automated image analysis of the cell confluence.
6. **Calculation of $TCID_{50}$ value**
   Identification of the highest virus dilution that still causes a cytopathic effect in 50% of the cell culture wells and calculation of the $TCID_{50}$ value by using the Spearman-Kärber, Reed-Muench, or similar method.

Scheduling Scans

Following cell seeding, the plate should be placed into the Incucyte and scans scheduled as follows.

1. Schedule vessel scans
2. Select "Schedule" and "Launch Add New Vessel" via the "+" icon
3. Select "Scan on Schedule" and create a new vessel
4. Choose "Whole Well" as a scan type
5. Select only the phase image channel
6. Select the type of the 96 well plate used, based on manufacturer and catalog number
7. Choose vessel location and which wells to scan
8. Add a plate map to the vessel notebook (this can also be done later)
9. Create a schedule with scans at intervals of e.g., one scan every hour for 7 days
10. Leave the plate to incubate within the Incucyte for four days

Launching Analysis

The assay can be analyzed in real-time once the plate scans have been scheduled. The analysis can be set up using the following steps.

1. Open the acquired images of the experiment under "View" and select "Launch Analysis"
2. Create a new analysis definition and select "Basic Analyzer"
3. Choose "Phase" as the image channel
4. Select representative images to setup analysis
5. Define image analysis parameters to create the confluency mask. In our example (HSV1 and Vero cells) we chose:
   a. AI Confluence
   b. Hole fill of 3 microns²
      Size adjustment of 2 pixels
6. Apply this analysis to all scan times in all wells and select "Analyze future scans" to enable real-time analysis of the assay plate

Experimental Data

Comparison of $TCID_{50}$ Using Conventional Assay Procedure or Live-Cell Confluence Analysis

To evaluate the accuracy of confluence analysis in determining the $TCID_{50}$ titer, two identical plates were seeded with Vero cells and incubated with a serial dilution of HSV1. One was incubated as normal and examined visually at daily intervals. The other was placed into the Incucyte for monitoring by the instrument. The plate map showing the location of each viral dilution is displayed in Figure 2.

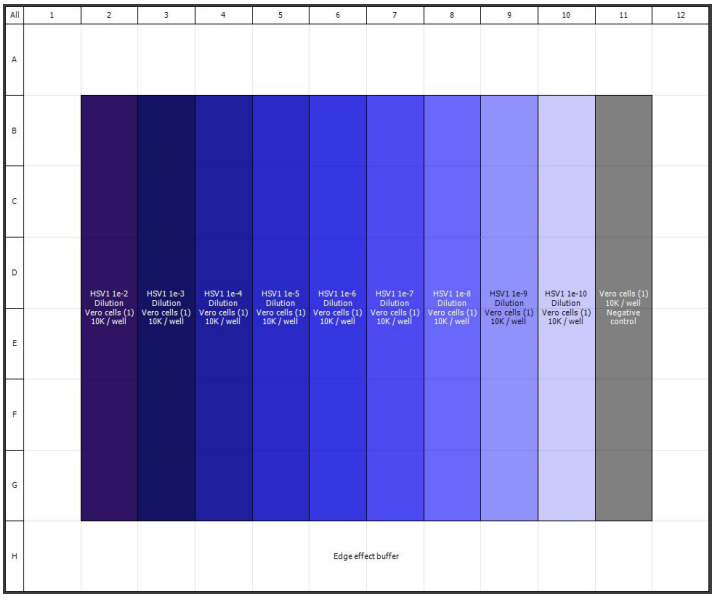

Figure 2: Plate map layout for $TCID_{50}$ assay set-up.

An example of the cytopathic effect (CPE) caused by the replication of the virus in infected cells is shown in Figure 3.

CPE negative well

CPE positive well

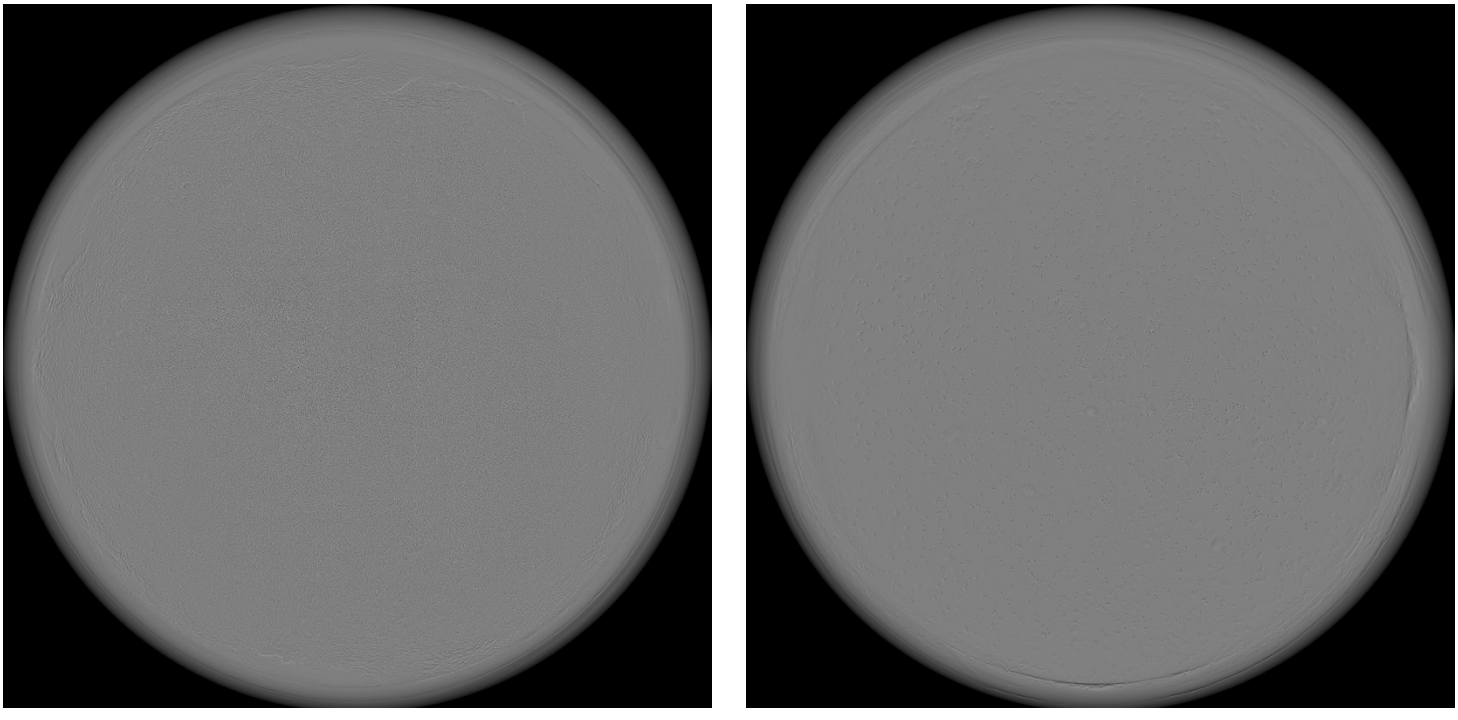

Figure 3: Cell culture wells showing no cytopathic effect (left) and showing cytopathic effect (right).

Evaluation of $TCID_{50}$ Using Visual Inspection

The plate kept in the incubator was examined visually for CPE at regular intervals and the individual wells were scored as either positive or negative (Figure 4).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		X	X	X	X	X						
C		X	X	X	X							
D		X	X	X	X							
E		X	X	X	X	X						
F		X	X	X	X							
G		X	X	X	X							
H												

Figure 4: Scoring of wells as either positive (marked with X) or negative for CPE following 4 or 8 days of incubation (both readout times showed the same results).

Evaluation of $TCID_{50}$ Using Automated Confluence Analysis

After launching the confluence analysis, the plate or selected wells can be examined with the confluence mask (Figure 5). The confluence mask paints yellow all areas of the plate occupied by cells. Thus, when cells are present the color is yellow, while no cells are seen as black or gray background. At a high confluence (cell monolayer) of approximately 100% the well is completely yellow, showing no CPE. A CPE is observed when the cell confluence is low, showing only a few yellow spots with cells. The empty wells around the periphery of the plate are not shown as they contain no virus.

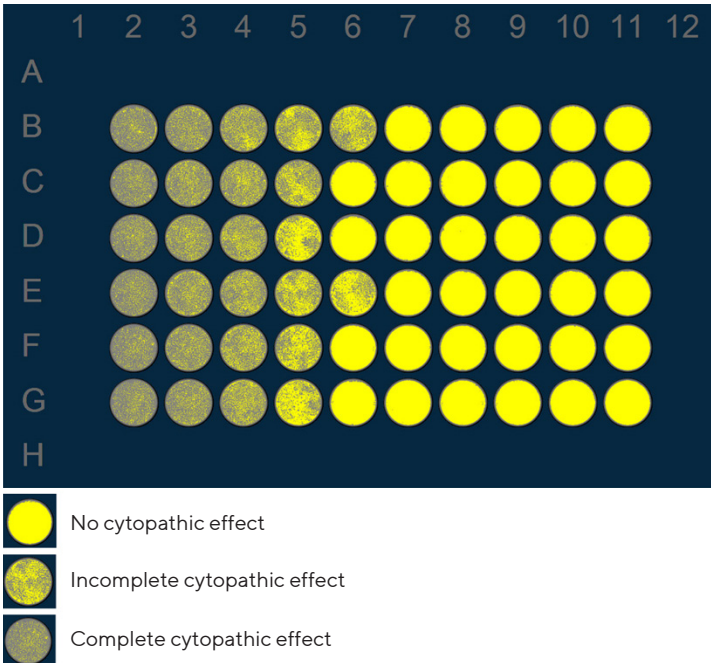

Figure 5: Plate overview with the confluence mask shown in yellow.

This CPE can then be quantified using time versus confluence graphs. These depict the confluence of cells at each virus concentration and replicate over time (Figure 6). When no cytopathic effect occurs, the confluence increases steadily over time until reaching a plateau of 100%. When CPE occurs in a well the confluence decreases after a certain time (the point when cell death overtakes cell proliferation) and stays below 100% for the entire assay period. The stronger the cytopathicity, the lower the final confluence observed. The continuous acquisition of data over time enriches the information obtained from the assay regarding the time needed for virus infection and replication until CPEs are visible.

Virus dilution: 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10} NC

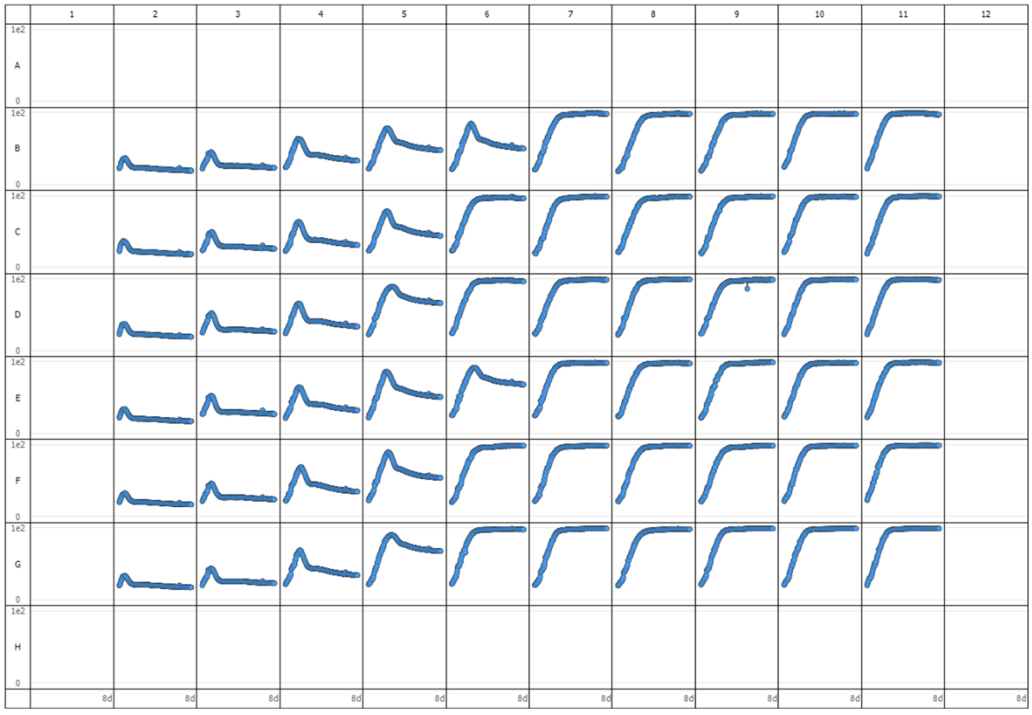

Figure 6: Plate view of confluence graphs over time of cells treated with the different virus dilutions indicated above the plate . Column 11 is a negative control (NC) containing no virus.

Calculation of the $TCID_{50}$

For calculation of the $TCID_{50}$ value different calculation methods exist.

Reed-Muench method:
In most cases there is no single virus dilution which results in exactly 50% of infected cells. Thus, the proportionate distance (PD) between two dilutions is calculated that show CPE just above and below 50%.

$$PD = \frac{\%\text{positive above 50\%} - 50\%}{\%\text{positive above 50\%} - \%\text{positive below 50\%}}$$

Then the dilution corresponding to 50% endpoint (ID_{50}) is calculated with the following equation:

$$\log(ID_{50}) = \log(\text{dilution with} > 50\% \text{ positive}) + PD \times (-\log(\text{dilution factor of dilution series}))$$

According to the Spearman-Kärber method the $TCID_{50}$ is calculated with the following equation:

$$\log(ID_{50}) = \log(\text{highest dilution giving 100\% CPE}) + 0.5 - \frac{\text{total number of wells showing CPE}}{\text{number of well replicates per dilution}}$$

In addition to these commonly used calculation methods, other modified calculation methods also exist and are compared in detail elsewhere.[3]

Summary

In this application note, we have developed an automated image-based method to determine the $TCID_{50}$ of viruses that cause cytopathic effects during cell infection using the Incucyte Live-Cell Analysis System. We have demonstrated:

- A rapid, stain-free, and simplified approach for high-throughput functional quantification of viruses.
- Real-time, kinetic readouts and integrated software enable tracking of cytopathic effects in infected cells over-time.
- Reduction of manual steps and shortened incubation time to 3.5 days (compared to 8+ days for visual readout). Note: the duration of the assay may vary for different viruses and target cell lines.
- Reduction in hands-on time and removal of the requirement to manipulate potentially harmful virus-containing plates on a daily basis.
- Applicability of assay: accelerate virus titration to determine optimal processing conditions for improved viral vector development, or screening of antiviral substances among others .

The data was generated at the Sartorius Glasgow, Scotland, UK Site.

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