Chapter 11

Quantification of Coronaviruses by Titration In Vitro and Ex Vivo

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Abstract

Several techniques are currently available to quickly and accurately quantify the number of virus particles in a sample, taking advantage of advanced technologies improving old techniques or generating new ones, generally relying on partial detection methods or structural analysis. Therefore, characterization of virus infectivity in a sample is often essential, and classical virological methods are extremely powerful in providing accurate results even in an old-fashioned way. In this chapter, we describe in detail the techniques routinely used to estimate the number of viable infectious coronavirus particles in a given sample. All these techniques are serial dilution assays, also known as titrations or end-point dilution assays (EPDA).

Key words  Titration, TCID$_{50}$, CD$_{50}$, Plaque-forming units, End-point dilution assay (EPDA)

1 Introduction

With the new-generation technologies progressing at a fast pace, high-throughput techniques are becoming available straight onto the bench, providing fast and accurate methods for routine tests in the lab. Detection of virus particles or selected antigens and their quantification are made possible in a short time in an easy way, thus establishing new laboratory standards [1, 2]. Detection of viral particles based on physical properties is commonly performed using specialized techniques that combine advanced optics and microfluidics, such as flow cytometry [1, 3], light scattering [4], capillary electrophoresis [5], or fluorescence correlation spectroscopy [6], providing fast results with high sensitivity. However, questions regarding the biological properties of viral particles analyzed through physical detection methods or nucleic acid amplification techniques (NAAT) remain a conundrum when identifying infectious particles is key. Combining analysis of physical particles and biochemical properties may answer this question, but this is not widely applicable. Classic virological techniques are therefore still needed to quantify virus infectivity. These techniques exploit the
fact that viruses propagate in biological systems, such as cell culture, embryonated eggs, or organ cultures, and replication is generally accompanied by morphological or functional changes dictated by the number of infectious particles [7]. In this chapter we provide protocols for the quantification of coronaviruses using different methods that are applicable depending on the sample, virus, and, inevitably, laboratory capability. The tissue culture infectious dose/50 (TCID$_{50}$) or plaque assay titrations are described in detail for coronaviruses that have been adapted to grow in cultures of primary cells or continuous cell lines, providing results respectively in tissue culture infectious dose/50 (TCID$_{50}$) or plaque-forming unit (PFU) per volume of sample. Additionally, we describe a titration method for avian infectious bronchitis virus (IBV) adapted from Cook et al. [8], using trachea organ cultures (TOCs). This is applicable for viruses that cause ciliostasis, providing results in ciliostatic dose$_{50}$ (CD$_{50}$), and representing an alternative to titrations carried out in embryonated eggs (EID$_{50}$) for those viruses not adapted to cell culture, thus limiting the required number of animals in compliance with the 3R principle.

All these methods provide accurate titrations; however, they are not always applicable for every virus. Although propagation of a virus in cell culture is generally accompanied by changes in cell morphology (referred to as cytopathic effect or CPE), which can be visualized using a microscope, some viruses do not induce CPE. In addition, use of TOCs for titration of respiratory viruses relies on observation and scoring of the cilia beating, as viruses replicating within the epithelia generally cause ciliostasis. However, some virus strains may be poorly ciliostatic; therefore, other techniques need to be used in these cases to quantify the virus.

To assess viral titer, a sample containing virus is diluted tenfold or twofold, depending on the expected virus concentration, and is used to infect tissue cultures or TOCs. Several days later or during the course of the infection, the cytopathic effect or the ciliostasis is recorded. From these data, the virus titer is calculated using the methods described by Spearman and Kaerber [9, 10] or Reed and Muench [11]. Importantly, these calculations apply to mismatched group sizes, as may happen when TOCs are lost due to bacterial infections or aspecific death. The virus titer is defined as the reciprocal of the dilution at which 50% of the inoculated tissue cultures show CPE or at which 50% of the inoculated TOCs show no residual beating of cilia.

Current limitations in adopting cell-based techniques are related to strain specificity in terms of host range and tropism. As many field strains of coronaviruses do not grow in cell culture, this limits the application of some techniques in principle. However, most isolates can be adapted to propagate in vitro in primary cells or continuous cell lines upon serial passage, selecting for the fittest subpopulations, eventually acquiring mutations related to
cell-culture adaptation and as a result altering virus characteristics in vivo [12–14]. Here we describe protocols for titrating IBV by plaque assay or CD50 and porcine deltacoronavirus (PDCoV) by TCID50. These protocols can easily be adapted for use with different coronaviruses and different cells, depending on the culture conditions required for the virus in use.

2 Materials

2.1 Titration of PDCoV by Tissue Culture Infective Dose (TCID50)

1. Ninety-six-well plates containing 80–100% confluent LLC-PK1 cells.
2. Medium –trypsin: EMEM supplemented with 1% HEPES, 1% nonessential amino acids, and 1% antibiotic antimycotic.
3. Medium +trypsin: Medium –trypsin supplemented with 10 μg/ml trypsin (see Note 1).
4. Microfuge tubes.
5. Multichannel aspirator.
6. Multichannel pipette.
7. 37 °C cell culture incubator with 5% CO2.
8. Inverted microscope.

2.2 Titration of IBV by Plaque-Forming Units

1. Six-well or twelve-well plates containing 70–90% confluent CK cells.
2. 1 × BES medium: EMEM, 10% w/v tryptose phosphate broth, 0.2% w/v bovine serum albumin (BSA), 20 mM N,N bis (2-hydroxethyl)-2-aminoethanesulfonic acid (BES), 0.4% w/v sodium bicarbonate, 2 mM L-glutamine, 250 U/ml nystatin, 100 μg/ml penicillin, 100 μg/ml streptomycin.
3. 2 × BES medium: 2 × EMEM, 20% w/v tryptose phosphate broth, 0.4% w/v BSA, 40 mM BES, 0.8% w/v sodium bicarbonate, 4 mM L-glutamine, 500 U/ml nystatin, 200 U/ml penicillin, 200 μg/ml streptomycin.
4. Sterile phosphate-buffered saline (PBS).
5. 2% w/v agarose in water (autoclaved).
6. 10% w/v formaldehyde in PBS.
7. 0.1% w/v crystal violet in water.
8. 37 °C cell culture incubator with 5% CO2.
9. Microwave.
10. Water bath.
11. Small spatula.
2.3 Titration of IBV by Ciliostatic Dose (CD50) in Tracheal Organ Cultures (TOCs)

1. Tissue culture roller drum capable of rolling at approximately 8 revolutions/hour at 37 °C.
2. Associated rack suitable for holding 16 mm tubes on roller drum.
3. Sterile, extra-strong rimless soda glass tubes 150 mm long, 16 mm outside diameter, suitable for bacteriological work.
4. Sterile silicone rubber bungs 16 mm diameter at wide end, 13 mm diameter at narrow end, and 24 mm in length.
5. Inverted microscope.
6. TOCs culture medium: MEM, 40 mM HEPES buffer, 250 U/ml penicillin, and 250 U/ml streptomycin.
7. Sterile PBS.

3 Methods

3.1 Titration of PDCoV by Tissue Culture Infective Dose (TCID50)

1. Seed LLC-PK1 cells into 96-well plates 1 or 2 days before the titration. At the time of titration, the monolayer should be nearly confluent. Each virus to be titrated requires ½ a 96-well plate (see Note 2).
2. Prepare two- or tenfold serial dilutions of the samples in medium –trypsin (see Note 3).
3. Aspirate the medium from the 96-well plate.
4. Add 100 μl of medium –trypsin to the wells in row H including five replicates. These provide a negative control.
5. Add 100 μl of diluted virus including five replicates with the most dilute in row G and the most concentrated in row A (Fig. 1).
6. Incubate the plate at 37 °C for 1 h.
7. After 1 h, add an additional 100 μl of medium +trypsin.
8. Incubate the plate at 37 °C for 5 days.
9. Quantify wells as positive or negative for CPE.
10. Calculate the viral titer using the Reed and Muench equation, as follows:

\[
\text{log } \text{ID}_{50} = (\text{Log dilution above 50\%}) + (\text{proportionate distance} \times \text{log dilution factor})
\]

where the proportionate distance is calculated as follows:

\[
\text{Proportionate distance} = \frac{(%\text{positive above 50\%}) - 50\%}{(\text{positive above 50\%} - \text{positive below 50\%})}
\]
3.2 Titration of IBV by Plaque-Forming Units

3.2.1 Infection of Cells

1. Seed CK cells into 6- or 12-well plates 3 days prior to titration. When performing the titration, the monolayer should be 70–90% confluent.

2. Prepare tenfold or twofold serial dilutions of virus in 1× BES.

3. Remove media from cells and wash once with sterile PBS.

4. Remove PBS from the cells and add 500 or 250 μl of diluted virus per well for 6- or 12-well plate, respectively. Duplicate wells should be inoculated for each dilution in a six-well plate or triplicate wells in a 12-well plate.

5. Incubate cells at 37 °C for 1–2 h to allow virus attachment.

6. Melt 2% agar in a microwave and then transfer to a 42 °C water bath. Allow the agar to equilibrate in temperature.

7. Mix the partially cooled agar with 2× BES pre-warmed to 37 °C to generate 1× BES + 1% agar. Keep at 42 °C until needed to prevent premature setting (see Note 4).

8. Remove virus inoculum and overlay cells with 2.5 or 2 ml of the 1× BES/agar mix for the 6- or the 12-well plates, respectively.

9. Leave cells at room temperature for approximately 5 min until agar has solidified.

10. Incubate at 37 °C and 5% CO2 for 3 days to allow plaques to develop.

3.2.2 Staining Cells and Determining Titer

1. After 3 days, overlay agar with 1 ml of 10% formaldehyde in PBS per well.

2. Incubate at room temperature for 1 h.

3. Remove formaldehyde and ensure disposal according to local regulations.

4. Using a small spatula, flick off the agar from the cells (see Note 5).
5. Wash cells by shaking the plate upside down in a sink full of water.

6. Add 0.5 ml of 0.1% crystal violet to each well or the minimum volume to just cover each well.

7. Incubate at room temperature for 10 min.

8. Remove crystal violet and dispose of according to local regulations.

9. Wash the plate by shaking upside down in a sink of water.

10. Pat plate dry and leave upside down at room temperature to fully dry.

11. Plaques should be clearly visible as holes in the monolayer varying in size and morphology based on the IBV strain (Fig. 2). Count the number of plaques per well at the dilution with clearly defined, individual (not over-lapping) plaques (typically 10–50 plaques/well). Ensure duplicate wells are counted and an average taken.

12. Determine titer using the following equation:

\[
\text{Titer (PFU/ml)} = \frac{\text{average number of plaques}}{\text{dilution factor} \cdot \text{inoculum volume (ml)}}
\]

13. For the most accurate results, the plaque assay should be repeated three times and the average titer determined.
3.3 Titration of IBV by Ciliostatic Dose Method

3.3.1 Screening and Selection of TOCs

1. After 2 days recovery after preparation, TOCs are screened individually for vitality and any tubes in which less than 60% of the luminal surface has clearly visible ciliary activity are discarded.

2. The selected tubes are used for the titration, calculating a number of five tubes per dilution per virus and an additional group of mock infected TOCs as negative control. At least one virus of a known titer should always be included as positive control.

3.3.2 Infecting TOCs

1. Prepare tenfold serial dilutions of virus in TOC culture medium.

2. Remove media from the tubes and wash once with sterile PBS.

3. Remove PBS from the tubes and add 500 μl of diluted virus to each tube selected for that dilution (see Note 6).

4. Incubate the tubes at 37 °C for 6 days before assessing the titer by scoring the ciliary activity under the light microscope. TOCs are scored positive for infection when cilia activity is completely abrogated with a tolerance of 5% cilia still beating, whereas negative when residual activity is recorded up to 95% (see Note 7).

5. Determine the titers using the Reed and Muench calculations looking at the log dilutions and TOCs scores as follows:

\[
\text{Log ID}_{50} = \log \left( \frac{\text{Log dilution above } 50\% + \text{proportionate distance} \times \text{log dilution factor}}{50\%} \right)
\]

where the proportionate distance is calculated as follows:

\[
\text{Proportionate distance} = \frac{\left( \frac{\text{positive above } 50\%}{\text{positive above } 50\% - \text{positive below } 50\%} \right)}{\text{positive above } 50\% - \text{positive below } 50\%}
\]

The log ID$_{50}$ represents the end-point dilution at which the 50% of the TOC score positive. The dilution factor is finally applied accordingly to what applied, generating the final log CD$_{50}$/ml.

6. For most accurate results, the CD$_{50}$ titration should be repeated three times and the average titer determined.

4 Notes

1. Trypsin/EDTA used for dissociating cells for passage can be used.

2. Titrations are performed using five replicate wells over eight rows (seven virus dilutions plus mock), requiring ½ and 96-well plate. If additional virus dilutions or additional replicate wells are preferred, plate cells accordingly.
3. If the likely titer of the virus is not known, use tenfold serial dilutions to identify the best range. If required, subsequently using twofold serial dilutions can provide a more accurate titer.

4. Alternative methods also exist for mixing media and agar. If there is concern regarding the overlay setting too quickly or risk of contamination from the water bath, hot agar can be mixed directly with cold media (4°C). Once the mixture feels warm to the touch, rather than hot, it can be added to cells.

5. The simplest method for removing agar from the cells is to hold the plate upside down with the lid removed. The small spatula is inserted between the agar and the wall of the well. Once the base of the well is reached, a small amount of pressure is applied to remove the agar, being careful not to scrape off the cells. The whole agar plug should then fall out easily.

6. A quick and easy way to speed up the washing step during TOC titration is to add a few milliliters of PBS without removing the media in the tube, then with a rapid and confident rotation of the hand, pour the media/PBS mix onto a stack of tissues being careful not to lose the TOC ring. Finally, remove the excess PBS by aspiration or using a pipette. This step speeds up the procedure taking into account that many tubes, often more than 100, may need to be processed.

7. Assessing cilia activity during TOC titration may seem a subjective interpretation; however, the main effect on the TOC lumen is in reality quite striking at 6 days postinfection for viruses that are ciliostatic, usually leaving no doubt about the results. However, if a virus is poorly ciliostatic, this test should not be the first choice for quantification unless virus is detected by other techniques, such as antibody-based assays or probe-based techniques.

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