Method Paper

A simple and highly repeatable viral plaque assay for enterovirus 71

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The classic plaque assay is a method for counting infectious viral particles, however its complexity limits its use in a variety of virological experiments. To simplify the operation and to improve the repeatability, we employed an improved plaque assay procedure based on Avicel to make the whole experiment easier and optimize the results on a model of Vero cells infection with Enterovirus 71 (EV71). Clear plaques visible to the naked eyes can be formed on a 24-well plate or a 96-well plate without immunostaining. Following further improvement, this plaque assay procedure could be applied to other viruses, being both simple and repeatable.

Abbreviations: EV71 – Enterovirus 71; TCID50 – 50% Tissue Culture Infective Dose

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Classic plaque assays have been employed for the quantification of infectious viral particles in a variety of virological experiments, such as cell-based viral titration test, drug-susceptibility assay and plaque inhibition assay [1, 3, 7]. Plaque assay, with its accuracy for counting infectious particles, is still considered to be an essential experiment, though many researchers prefer to use 50% tissue culture infective dose (TCID50). In classic plaque assays, overlay media are thought to play a role in confining a single live virus and its progenies (or its copies) to a local area known as the plaque. Appropriate viral plaque assay can result in countable plaques. The most frequently used overlay media are agarose/agar and methylcellulose [9], but the tedious preparation limits their use and also leads to low repeatability (Supporting Information Table S1). Thus, a more convenient and highly repeatable method of plaque assay for most viruses is still required in present virological studies.

Recently, comet and comet reduction assays have been used as alternative methods for plaque assay in drug-susceptibility test [11, 4]. By using an imaging-based technique, comet reduction assays have shown to be more sensitive than traditional plaque-reduction assays. Nevertheless, comet assays may not be applied to a number of viruses as some viruses cannot be efficiently released from host cells into the extracellular fluid. Furthermore, the liquidity of overlay media in the comet assay will be influenced by several factors, such as vibration and occasional moving of plates.

Avicel (microcrystalline cellulose) has been previously used as an overlay medium for influenza virus plaque assays, providing a convenient, low cost and high throughput method to researchers [5, 8]. This method is limited by the high cost of immunostaining agents and possible antigenic variation of viruses, or the alternative staining procedure using crystal violet has yielded plaques of low visualization due to low color contrast [5]. A method including fixation by using formaldehyde saline and staining by using neutral red in distilled water is supposed to form clearer plaques [6], yet the whole procedure including the use of agarose as the overlay media is still complicated. In this study, we combined the advantages of these two methods to create an improved simple plaque assay procedure.

In this study, Vero cells (ATCC CCL-81) were used. We ensured that monolayers of cells reached 90–100%
confluence at the bottom of wells prior to experimentation. To obtain viral stocks, monolayers of Vero cells were infected with EV71 (GZ223A) at a multiplicity of infection (MOI) of 0.5 and cultivated in DMEM medium (1% fetal bovine serum) for 4 days. EV71 (GZ223A) was clinically isolated from Guangzhou Children’s Hospital and identified by RT-PCR and sequencing. To prepare a 2.4% suspension of Avicel (R-591, FMC), we referred to the methods described by Matrosovich [5]. R-591 (2.4 g) was dispersed in 100 ml distilled water and stirred on a magnetic stirrer for 1 h. The suspension was sterilized by autoclaving for 20 min at 121 °C and stored at room temperature. 2 × DMEM media was prepared by dissolving 13.5 g DMEM powder (high glucose, L-glutamine) and pyridoxine hydrochloride, 110 mg/L sodium pyruvate, and 0.3% streptomycin in 500 ml distilled water. To make up a 1.2% Avicel suspension (in distilled water) with the same volume of 2 × DMEM. The 0.6% Avicel overlay media was made up by mixing 1.2% Avicel suspension with the same volume of 1 × DMEM, then 0.6% Avicel was diluted to 0.3%, 0.15% (Supporting Information Fig. S1).

The procedures of the improved plaque assay based on Avicel overlay medium (EV71 and Vero cell) are described in Table 1. Cells in logarithmic growth phase were seeded in plates (Cell concentration is 2.5 × 10^5/ml). The volume of cell suspension depended on the size of the wells, usually, 3 ml for 6-well plate, 1 ml for 24-well plate and 0.1 ml for 96-well plate are appropriate for each well. To minimize the damage to cells, Pasteur pipettes instead of tips were used to transfer cell suspensions. The volume of overlay media (1.2% Avicel suspension) for each well was equal to the half of each well’s volume.

Three diluted concentration levels (1.2%, 0.6% and 0.3%) of R-591 dispersed in 1 × DMEM were applied in this comparative experiment. A simple 1:10,000 dilution of the EV71 stock solution provided countable plaques. The plaque size showed no obvious differences among the three diluted concentrations (data not shown).

Avicel, a type of microcrystalline cellulose, is often used as a matrix in pharmaceutical industries. Recently its use as overlay media has made it an alternative to agarose/agar and methylcellulose in plaque assays. Its low viscosity is an advantage for plaque assays, allowing the whole procedure to be highly simplified. Matrosovich proved that obvious large plaques can be formed under Avicel-containing media, and even extremely diluted Avicel overlays (0.3%) ensured formation of localized plaques, while plaque assay under 0.25% methylcellulose could only form large comet-shaped foci [5]. Why different concentrations of Avicel (for example 0.6%, 1.2% and 2.4%) can form almost the same number of plaques still remains unclear. Our study demonstrated that 1.2%, 0.6% and 0.3% Avicel in DMEM were less stable than 2.4% Avicel in water. Avicel can form suspension in distilled water, but the state of this suspension is not stable in form of ink colloid. If the suspension is stable, in theory, different concentrations of Avicel in DMEM should form different densities of suspensions and finally form a homogeneous liquid colloidal dispersion system like ink colloid. However, the Avicel suspensions would precipitate after standing at room temperature or 37 °C for more than 1 h (Supporting Information Fig. S1). As a matter of fact, the density of deposit (formed after the sedimentation of Avicel particles) in 1.2%, 0.6%, 0.3% Avicel suspension was almost equal to that of 2.4% Avicel suspension, which suggests the flow of the culture media could be limited by deposited Avicel particles and the same deposit density means the same ability to limit the liquid flow. Before cell fixation, the monolayers of cells were not stable and easy to exfoliate from the bottom of the wells. Thus, in the experiment of McKimm-Breschkin, agarose overlay was removed after overnight fixation [6]. However, the removal of solid overlay is not an easy

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**Table 1.** EV71 plaque assay protocol in 24-well plate.

| 1. | Seed Vero cells (approximately 2 × 10^5 cells per well) in standard 24-well plate with 1 ml growth medium (DMEM, 10% FBS) per well |
| 2. | After formation of the cell monolayer, remove growth medium carefully and add 150 μl serial dilutions of EV71 stocks to each well (virus stocks was serially diluted in PBS by 1:10) |
| 3. | Incubate the 24-well plate at 37 °C with gentle shaking every 20 min for 2 h |
| 4. | Directly add overlay medium (1.2% R-591, a total of 1 ml per well) |
| 5. | Incubate the 24-well for a week at 37 °C, 5% CO_2 |
| 6. | After 7 days, add 1 ml 8% neutral formaldehyde (dissolved in PBS) to each well gently |
| 7. | Leave the fixative to mix naturally with the liquid overlay medium for 2 h |
| 8. | Pipette the overlays gently, never touch the bottom of the wells. Wash the wells gently with PBS twice |
| 9. | Add 1 ml 0.5% neutral red (dissolved in distilled water) to each well. Stain at room temperature overnight |
| 10. | Discard neutral red carefully (do not wash the wells), and dry at room temperature |
| 11. | The 24-well plate can be visualized with naked eyes directly, or be scanned by a scanner. |
thing, especially in 96-well plates, not only because this step was laborious, but also because some cells of the monolayer can still detach from the bottom of wells during the complex operation. The final concentration of formaldehyde in our experiments was also 4%, which is consistent with Matrosovich’s method [5]. Because of the fluid state of the Avicel overlay, it was easier for the fixative to reach the monolayers of cells, thus the fixation can be completed in 2 h.

After being autoclaved for 20 min at 121 °C, the degree of Avicel’s dissolving in distilled water (2.4% R-591) is stable in sterile conditions at room temperature for several months (Supporting Information Fig. S1 A). In fact, the suspension in this concentration is near saturation. However, 1.2% R-591 suspension (dispersed in 1 x DMEM solution) became unstable at 37 °C or room temperature. It would form sediments after standing for several minutes and the sediment layer dispersed homogeneously just like that of 2.4% Avicel (Supporting Information Fig. S1 B). The state of sediment layer in 0.6% and 0.3% R-591 were similar to that in 1.2% R-591 suspension (Supporting Information Fig. S1). This could explain why the different concentrations of Avicel did not affect the formation of plaques. In traditional viral plaque assay, overlay media consisting of different concentration of agarose will form solid gel in different sizes of aperture, which means different ability to limit the liquid flow. Low viscosity allows Avicel particles to

Figure 1. Improved EV71 plaque assays on Vero cell monolayer using plates (Cornings) of different sizes. EV71 viral stocks (223A) were serially diluted by 1:10. All experiments were carried out according to our improved procedure, except that C(1) was crystal violet staining in the last step. A(1) are 96-well pictures scanned by MiraScan 6.3 (specular light), and A(2) are the same group of wells photographed by OLYMPUS DP71 at low magnification (transmission light). B and C were photographed by a conventional camera (transmission light).
easily spread on the monolayer of cells in a short time and form liquid gel.

The advantage of neutral red over crystal violet had been described by McKimm-Breschkin [6], and this was also confirmed in our study (Fig. 1C). In our experiments, the washing step following neutral red staining was avoided by placing the plates upside down on dry paper.

EV71 is a member of the enterovirus genus belonging to the picornaviridae family. Although RD cells (derived from rhabdomyosarcoma) are more sensitive to EV71 infection, Vero cells were more frequently used in plaque assays or TCID50 test [2, 10], as they have a higher cell survivability than RD in vitro. Thus, a cell monolayer of Vero cells is easier to form and plaque assays on Vero are relatively less affected by other adverse factors. EV71 has a lower cytopathic effect (CPE) than HSV on cells, consequently our experiments have demonstrated that assays using HSV on Vero cells formed clearer plaques than when using EV71 (Supporting Information Fig.S2). Because of more convenience and higher repeatability than the traditional methods, our improved procedure of plaque assay based on Avicel has the potential for use in a wide range of viruses.

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References