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Quantifying Adenoviral Titers by Spectrophotometry

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Adenoviruses have been studied for many decades following the realization they were able to transform rodent cells in culture. Since then, they have served as valuable models for the exploration of the cellular pathways involved in DNA replication, regulation of gene expression and cellular proliferation. In particular, they have played a major role in identifying the *Rb* and *p53* tumor suppressor genes as major regulators of cell growth and as key factors in the genesis of human tumors (1).

While most adenoviruses are relatively easy to grow, quantifying the

number of virus particles and the infectivity of a particular inoculum is much harder. Traditionally, those wanting to accurately quantify the titer of a virus used the plaque-forming assay (6). However, this assay requires rather lengthy incubations (up to 10 days) and the choice of cell lines that are appropriate for this assay is restrictive. While early passage 293 cells (3) can generally be used successfully, older passage 293 cells give variable results, in our experience.

Other lines such as HeLa cells can be troublesome and many cell lines are not appropriate for this assay. The fluorescent focus assay, while faster than the plaque-forming assay and less dependent on cell type, requires a number of reagents and substantial preliminary optimization (6). Titrating multiple viruses using this assay is both arduous and time consuming and subject to investigator bias because fluorescent foci must be visually identified and counted. Visual identification assumes that a confluent monolayer remains after numerous washes, subsequent to antibody binding steps, and hence is subject to considerable inaccuracies. One of the most widely used assays for quantifying viral titers is the cytopathic effect (CPE) assay (6). CPE assays rely on the ability of viruses to cause cells to detach from a monolayer or undergo lysis. Generally considered the most convenient assay and accurate enough for most needs, this assay can be performed on a wide variety of human cell lines.

The CPE assay involves seeding cells into 96-well plates and infecting them with serial dilutions of virus. At an appropriate time after infection, CPE (loss of cell attachment) can be estimated microscopically or, alternatively, the media can be removed and the remaining cells fixed and stained with crystal violet solution. The subsequent CPE is assessed and an estimate of virus titer can be made in terms of the dilution of virus, which generates CPE in a prescribed percentage of cells (e.g., 50%). Several problems associated with this method of quantitation are that fixing and staining of cells can result in loss of the monolayer and, as with fluorescent titration, quantitation by direct visualization is subject to investigator bias.

Here, we describe a variation of the CPE assay using an inexpensive protein stain and spectrophotometric analysis, which gives an accurate and reproducible quantitation of viral titer that is less subject to investigator bias. Virus is harvested from infected 293 or other cells by centrifuging both floating and trypsinized cells at 500× *g* for 5 min and then resuspending the pellet in 20 mM Tris, pH 7.4. Infectious virus is released by rapidly freezing (in dry ice) and thawing the cell suspension 3×, removing cellular debris by centrifugation at 500× *g* for 10 min and placing aliquots of the supernatant at -70°C until needed.

We describe here the titration of wild-type adenovirus type 5 (Ad5) and the Ad5 replication defective mutant dl 312, which contains a deletion of much of the E1a coding region (5). However, this technique is equally applicable to any lytic virus or viruses capable of causing detachment from the monolayer. We seeded 3×10^4 293 cells into each well of a 96-well plate and left them to attach in 100 μ L of DMEM + 0.5% FBS at 37°C, 10% CO₂. Serial dilutions of the virus inoculum are made in a further 100 μ L of DMEM + 0.5% FBS and added to the culture when cells have attached. Infected cells are incubated for 3 days at 37°C, 10% CO₂ (although this time should be determined empirically, depending on the cell type and virus used), and the media and dead or floating cells are then removed by aspiration.

Cell loss (CPE) is determined by measuring changes in total protein concentrations in each well. Protein concentration is obtained by adding 200 μ L of BCA™ protein assay reagent (Pierce Chemical, Rockford, IL, USA) to each well, followed by gentle shaking. This reagent contains 0.1 M NaOH and components of the Biuret reaction allowing solubilization of the cell membrane, release of cellular proteins and formation of a purple color proportional to the protein concentration. The plate is agitated for 1 min, the color is allowed to develop by incubating the plate at 37°C and absorbance ascertained by spectrophotometry at 570 nm on an Anthos microtiter plate reader. The average absorbance of several blank wells (containing no cells) is sub-

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tracted from the absorbance readings of the serial dilutions and then expressed as a percentage of uninfected controls. The mean of four replicate data points and the SEM are then plotted against virus dilution.

The graphs thus obtained (Figure 1) are used to determine the virus dilution at which a prescribed level of CPE is obtained. For adenovirus, we have arbitrarily set this CPE level at 50%. By constructing a line of best fit in the straight line region of the graph (Figure 2), the virus dilution that produces 50% CPE is ascertained and assigned a virus titer of 1 CPE U. Figure 1A shows a typical CPE curve using wild-type Ad5 to infect 293 cells. The virus dilution at which 50% CPE is apparent is 10^{-5} . The titer is calculated by multiplying the dilution factor (10^5) by the cells killed (50% of $3 \times 10^4 = 1.5 \times 10^4$) and dividing by the inoculum of 0.1 mL. Thus, this virus inoculum has a titer of 1.5×10^{10} CPE U/mL. An identical estimate of virus titer was obtained using A549 cells (Figure 1C). Using a standard plaque-forming assay, the same virus inoculum resulted in an average of 11.3 plaques per 100 μ L of a 1×10^{-8} dilution of virus. This equates to a titer of 1.1×10^{10} plaque-forming U/mL.

Thus, the CPE assay described here results in a similar estimate of virus titer to that obtained by the plaque-forming assay. Perhaps this is not surprising given that both assays use the ability of viruses to generate cellular damage to estimate their titer. The minor differences between the estimations are unlikely to have a major effect on biological assays in which the inherent variation is greater than that described here. However, it is possible to increase the sensitivity of this assay by using twofold instead of tenfold dilutions (Figure 2). Nonetheless, in our hands, this increased sensitivity rarely leads to major differences in the estimates of virus titer (see Figure 2 legend) and may not warrant the extra time and effort involved.

The method described here also allows the quantitation of titers for virus mutants that are unable to induce CPE on their own, but whose lytic ability can be complemented by virus proteins expressed in various cell lines. Figure 1D demonstrates that the adenovirus

mutant dl 312 (a replication incompetent adenovirus described above) was unable to induce CPE in A549 cells. However, dl 312 was able to induce CPE in 293 cells that express the adenoviral E1a and E1b proteins (3) and therefore complement the defect in the dl 312 virus, allowing virus propagation and lysis (Figure 1B). This means that the viral titer of the dl 312 inoculum can be estimated by its ability to generate CPE on 293 cells, which in this case is 1.5×10^9 CPE U/mL (Figure 1B). We have used this method to estimate the viral titer of several aden-

oviral mutants that would not normally generate CPE. This method should be just as effective in estimating the titers of other virus mutants by using cell lines that constitutively express the proteins not expressed by the mutant virus.

There are several issues that the prospective user should take into account when using this assay. The linear range of the BCA reagent falls between 20 μ g/mL and 2000 μ g/mL. In the assay outlined here, uninfected 293 cells and A549 cells seeded at 3×10^4 cells per well and cultured for 3 days have protein levels that fall well within this

Figure 1. CPE assay using BCA reagent. 3×10^4 293 cells (A and B) and A549 cells (C and D) were seeded into 96-well plates in 100 μ L DMEM + 0.5% FBS. Then, tenfold dilutions of wild-type Ad5 and mutant dl312 inoculums were made and 100 μ L of each dilution were used to infect the appropriate wells. Infected cells were incubated for 3 days, after which media and dead and floating cells were removed. BCA protein assay reagent was added and agitated for 1 min to allow color to develop. Absorbance was ascertained at 570 nm on a microtiter plate reader and the mean of four replicates, and their standard error was plotted against virus dilution. The virus dilution that induced 50% CPE was arbitrarily chosen to determine viral titer. (A) 100 μ L of wild-type Ad5 virus diluted to 10^{-5} induced 50% CPE in 3×10^4 293 cells. This equates to a viral titer of 1.5×10^{10} CPE U/mL. (B) 100 μ L of dl 312 virus diluted to 10^{-4} induced 50% CPE in 3×10^4 293 cells. This equates to a viral titer of 1.5×10^9 CPE U/mL. (C) 100 μ L of wild-type Ad5 virus diluted to 10^{-5} induced 50% CPE in 3×10^4 A549 cells. This equates to a viral titer of 1.5×10^{10} CPE U/mL. (D) 100 μ L of dl 312 does not cause CPE in A549 cells at the range of virus dilutions tested as this virus is replication incompetent in cells that do not express the complementing viral proteins.

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Figure 2. Comparison of the sensitivity of twofold and tenfold dilutions of wild-type adenovirus. The CPE assay outlined in Figure 1 was used to generate CPE curves for twofold dilutions (A) and tenfold dilutions (B) of wild-type Ad5 on 293 cells. The solid line represents a line of best fit through the straight-line portion of the graph. The dotted lines indicate the dilution of virus at which 50% CPE is generated. Since the dilution axis is a log scale, we estimate virus titer to be $(10^{4.9} \times 1.5 \times 10^4/0.1)$ or 1.3×10^{10} CPE U/mL using twofold dilutions and $(10^{4.8} \times 1.5 \times 10^4/0.1)$ or 9.4×10^9 CPE U/mL using tenfold dilutions.

linear range (data not shown). However, protein levels should be determined for each cell type because cells grow at different rates and express proteins at

variable levels. Seeding densities may need to be adjusted to remain within the working range of the assay. In addition, the time taken for CPE to develop should be kept constant because an increase in incubation time following infection could result in increased CPE and an overestimation of the viral titer. Alternatively, increased incubation times may mean that cells in control wells will overgrow and so cause CPE, independent of virus infection. Or they may generate protein levels outside of the linear range of the assay. Finally, some adenovirus mutants result in small plaque phenotypes because of an inability to lyse cells and therefore to shed virus (2). Others result in fusion of cells rather than overt CPE (2). This assay, as with all CPE assays and the plaque-forming assay, is only effective if virus infection results in either destruction of the cell monolayer or detachment of the cells.

This method has been used successfully to determine adenovirus titer in a large number of human cell lines in our laboratory (4). We believe it will be similarly effective at titrating any virus capable of causing cells to lyse or lift from a culture plate. The procedure is extremely simple and easily performed, lending itself to high-throughput laboratories. Further automation is also possible by linking computer analysis to the curves thus generated and thereby

decreasing the likelihood of error. The described procedure is a rapid, easy and inexpensive method for determining virus titer that circumvents many of the problems of previous CPE and other assays. It has been extensively tested in our laboratory.

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