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Accelerated Titering of Adenoviruses

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In recent years, adenovirus (Ad) vectors have become a popular tool, particularly for in vivo gene transfer applications. Following construction of a new Ad vector, quantification of the viral infectious unit by plaque titering is a standard step in the characterization of any newly generated virus stock. However, the management of the plaque assay is cumbersome and takes between 4 and 10 days. In addition, interpretation of the results is often complicated by the variability in size and shape of plaques. The fluorescent focus assay accelerates the speed necessary for determining adenovirus titers and is a common test in adenovirus applications (1,3,5,8). This assay takes only two days to obtain results. Although counting infected cells is easy, the procedure requires a suitable environment (dark) and expensive equipment (fluorescent microscopy for cell culture dishes). In addition, comparisons between the fluorescent focus assay and standard plaque assay in titering adenovirus are not available yet. We present a modified technique for an easy, reliable and fast determination of adenovirus titers, the "spot assay".

Viruses used for the titer experiments were AdCMV.Null (2), AdCMV.NLS- β gal (4), AdCMV.RiboI and AdCMV.RiboIV (both expressing ribozymes against α -galactosyl transferase) and AdCMV.antiGT (expressing an antisense structure against α -galactosyl transferase). Recombinant adenoviruses were prepared as previously described (2,6). For the plaque assay, 293 cells were grown as monolayers on 6-well culture dishes and maintained in DMEM with 5% fetal calf serum (FCS). Plates were set up at least in duplicate. At a density of 70%–80%, medium was removed and cells were infected with 100 μ L of six different virus dilutions (viral stock solution diluted 1:10⁷ to 1:10¹² in serum-free medium). Incubation at 37°C was maintained for 1.5 h with repeated rocking of the plates. Then complete medium with 5% FCS was added. After 24 h, culture medium was removed and replaced by 7.5 mL

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agar mixture prewarmed to 37°C. The agar mixture contained 0.9 g tissue culture agar (KC Biologicals, Lenexa, KS, USA) in 45 mL distilled water mixed with a solution of 50 mL 2× minimum essential medium (MEM), 2.5 mL FCS, 1 mL 1 M MgCl₂, 1 mL 100× L-glutamine 2.5% and 1 mL antibiotics (100 U/L penicillin, 100 mg/mL streptomycin). Plaques became visible five to six days later. Visibility of the plaques was enhanced if a second overlay of agar mixture containing 1 mL 100× neutral red stain was added after four days of incubation. The plaques were counted at each dilution, at which isolated plaques could be identified (Figure 1, panel A); the concentration of the original viral stock was calculated as pfu/mL.

For the spot assay, cells were prepared the same as for the plaque assay. Six different viral dilutions (viral stock solution diluted 1:10⁷ to 1:10¹² in serum-free medium), 100 µL each, were applied to the 293 cells in serum-free medium and incubated at 37°C. After 1.5 h, complete medium containing BSA at a concentration of 5% was added, and incubation was continued for an additional 48 h. The medium was aspirated, and cells were allowed to dry in a laminar flow hood. Ice-cold methanol (100%) was added, followed by incubation at -20°C for 15 min. Methanol was aspirated, and cells were washed twice with PBS containing 1% BSA (PBS/BSA). All the following steps were performed at room temperature.

A polyclonal antibody directed against adenoviral antigens (derived from Ad5 infected rabbits, provided by Professor Erik Falck-Petersen, Cornell University Medical Center, New York, NY, USA) was added to the cells (diluted 1:2000 in PBS/BSA, 50 µL/well). After incubation for 1 h, cells were washed 3× in PBS/BSA. Each washing step was performed under continuous rocking of the plate to ensure optimal clearance. A goat anti-rabbit antibody labeled with horseradish peroxidase (Dako A/S, Copenhagen, Denmark) was added (diluted 1:200 in PBS/BSA, 50 µL/well) and was followed by another wash step (3× PBS).

The infected (and therefore labeled) cells were detected using the ImmunoPure® metal enhanced DAB substrate kit (Pierce Chemical, Rockford,

IL, USA) with 3, 3'-diaminobenzidine (DAB) as substrate, which was applied according to the manufacturer's instructions. After developing color, the substrate was replaced with 200 µL PBS. Colored cell spots were counted microscopically (Figure 1, panel B). The concentration of infectious particles in

the original viral stock was calculated as spot-forming units (sfu).

To compare both methods, 22 individual titer determinations on five different types of viruses were performed. Figure 2 shows the results expressed as either an sfu or pfu. The linear correlation was $r = 0.86$ (significant, α

Figure 1. Microscopic appearance of plaques and spots in the titer assays. Both panels are photographs taken from 6-well plates of 293 cells that were infected with AdCMV.NLSβgal. (A) Plaque from a 6-well dish of 293 cells after infection with AdCMV.NLSβgal, nine days after infection. The 293 cells were incubated with increasing concentrations of virus in serum-free medium for 90 min before complete medium was added. Cells were overlaid with agarose 24 h after infection. Plaques began to appear about five days after infection. (B) Spots from a 6-well dish of 293 cells after infection with AdCMV.NLSβgal 48 h after infection. Cells infected in the same manner as in the plaque assay did not get an overlay but were fixed with methanol 48 h after infection. Detection of infected areas was performed using immunocytochemistry with a first antibody directed against viral proteins and a second antibody labeled with horseradish peroxidase. Development of color was obtained with DAB as substrate.

Figure 2. Comparison of results from the spot assay with results from the plaque assay in titrating of adenoviruses. The 293 cells grown in 6-well plates were infected with AdCMV.Null, AdCMV.RiboI, AdCMV.RiboIV, AdCMV.antiGT or AdCMV.NLSβgal. Titer of each virus batch was determined as described in the Materials and Methods section using the plaque assay and the spot assay. Results from both assays on the same virus batch are displayed on the graph. Correlation coefficient and trendline were calculated.

Table 1. Titer of AdCMV.Null Results from the Plaque Assay and Spot Assay (Nine Determinations Each)

	Plaque Assay	Spot Assay
Mean	3.6×10^9	3.3×10^9
Median	3.2×10^9	3.2×10^9
SD	1×10^9	3.9×10^8
Relative cv	9.9%	4.2%

<0.01%) (7). For one batch of virus, nine determinations of both titer methods were performed (Table 1). The mean of the titer was 3.3×10^9 in the spot assay. There was a lower standard deviation (3.9×10^8) and a lower variation coefficient (4.2%) with the spot assay than with the plaque assay (1×10^9 and 9.9%, respectively) (7).

Accurate titering is easier when using the spot assay because it allows counting of separate foci even at a high density. Similar to other applications of immunocytochemistry, it is important to retain an intact monolayer of cells that can be carried out by letting the cells dry after removing the culture medium and before adding methanol for fixation. The final visualization of cell spots containing adenoviral proteins is optimal with DAB used as substrate because it produces a clear, well-distinguished precipitate that is easily visualized by light microscopy. No background problems arose from the use of peroxidase-labeled antibody.

No spots of adenoviral proteins were detected soon after infection (for example, 12 h), which indicates that the spots observed later are newly synthesized after local adenovirus infection and do not represent residues from the adenovirus used for the infection. Thus, spots detected by immunocytochemistry are really representative of plaques in the plaque assay.

In summary, the new method described for adenovirus titering is reliable, simple to perform, requires only standard equipment and reduces the time for the titer to be available. The spot assay may be especially advantageous for the titering of E3-deleted viruses that display a delayed plaqueing phenotype since it does not rely on the formation of plaques.

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