

A Useful Quantitative Semimicromethod for Viral Plaque Assay (37202)

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This paper presents a general method for rapid, easy, and convenient quantitation of virus titers which we have utilized in applications varying from measurement of virus distribution in infection *in vivo* to assay of neutralizing antibody or antiviral agents. Basically, this technique utilizes disposable multicompartamental plastic plates with 1-ml volume, flat bottomed wells for cell culture, together with a carboxycellulose (CMC) overlay and a stain which permanently fixes the cell sheet. This then retains the quantitation inherent in plaque assays as conducted in larger plates, but introduces an increased ease and speed of handling because of the bulk processing possible. As well a sharp reduction in cost is introduced because of savings in reagents and plasticware.

Materials and Methods. Viruses and cells. Viruses in current use in the Divisions of Cell Pathology and Communicable Diseases were used in these studies. Primary and continuous cell lines were used as listed in Table I.

Growth medium. L-15 (Leibowitz) medium was supplemented with 10% tryptose phosphate broth (TPB). In addition penicillin (100 U/ml) and streptomycin (100 U/ml) were used in all experiments. MEM (minimal Eagle's medium) or BME (basal medium Eagles) were also used with certain cells as indicated.

Plates. Disposable plastic plates and covers (Linbro Chemical Co., New Haven, CT, Disposotray *96CV-TC and *912, cover, sterile) containing 96 wells, diameter 1.6 cm (Fig. 1). Before use plates and covers were sterilized by exposure for 30 min to ultra-

violet light. Normally, monolayers were prepared in a horizontal laminar-flow cabinet. A covered cabinet with a glass barrier between the operator's face and the plates was used for virus challenge.

Preparation of cell monolayers. One milliliter of an appropriate cell concentration was distributed into each well. Plates were then put in closed polyethylene bags and incubated at 37°. These bags did not require any special sterilization procedures. Where a well-humidified incubator was available plates were incubated without the bags. Furthermore, plates were incubated in 5% CO₂ atmosphere only when media containing Tris buffered systems could not be used.

Virus challenge. Infection of monolayers. Growth medium was removed by suction from the well immediately prior to inoculation. To avoid dehydration no more than 24 wells were evacuated at any one time. 0.2 ml of each virus dilution was added to each well, this unit volume was found to cover the whole area and give a good distribution of plaques. Following virus inoculation, or each time when the plates were handled, they were carefully inspected for free excess drops, to avoid cross contamination. Plates were then put into polyethylene bags to retain a high degree of humidity and placed in an incubator at 37°. After allowing one hour for virus adsorption (employed for all viruses except for yellow fever virus and varicella zoster which were allowed 4 hr) unabsorbed virus was removed with a sterile pasteur pipette and wells were overlaid with 1 ml of CMC overlay medium. Then plates in polyethylene bags were returned to the incubator for further incubation. At this stage, care was taken not to move assay plates until the end of the incubation period.

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TABLE I. Cell-Virus Systems Studied.

Cell type ^a	Cells/well	Growth medium	Days to confluency	Challenge virus/0.2 ml	Incubation period (days)	Special requirements ^b
CEF	2×10^8	L-15 + 10% CS	2	Herpes simplex Vaccinia (CL)	3 3	
VERO	2×10^5	L-15 + 5% CS	2	Coxsackie B-3 (Nancy) Echo 11	3 3	
MK	5×10^4	BME + 10% CS	7	Polio 2 Influenza B/Hannover/1/70	3 4	Monolayer washed 3 times with serum free media prior to virus challenge. Haemadsorption end points were detected with guinea pig red blood cells.
L-cells	2×10^5	L-15 + 3% CS	2	Encephalomyocarditis virus (EMC) Vesicular stomatitis (Indiana) (VSV)	1½ 2	
HNF	1×10^5	MEM + 10% FCS	7	VSV (Indiana) Varicella zoster	2 7	
PS	2×10^5	L-15 + 3% CS	2	17D Yellow fever	5	Stained with naphthalene black
HeLa	5×10^5	BME + 10% CS	3	Rhinovirus 2	4	Overlay medium: 0.5% agarose in BME with 30 mM MgCl ₂ , 5% TPB and 100 mg/ml DEAE-dextran.

^a CEF = chick embryo fibroblast cells; VERO = continuous vervet monkey kidney cells; MK = primary rhesus monkey kidney cells; L-cell = continuous mouse embryo fibroblasts; HNF = human neonatal foreskin fibroblasts; PS = continuous pig kidney cells; HeLa = continuous human tumor cells.

^b If not stated otherwise all plates were stained with crystal violet.

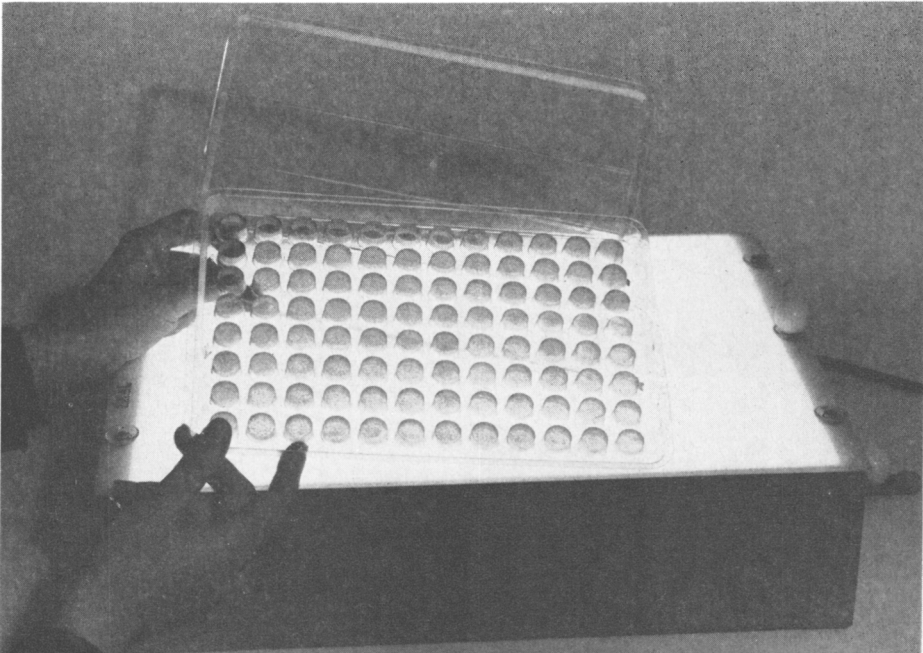


FIG. 1. The multicompartimental plate and cover used in this technique.

Preparation of CMC overlay medium. This was the same as the growth medium used for the particular cell line, but contained in addition 0.75% CMC which was prepared as follows:

Stock CMC - tyrosine. Since it is impossible to prepare concentrated L-15 medium without tyrosine crystallizing out upon storage at 4°, tyrosine solution was prepared and added to CMC powder. 61.8 mg of L-tyrosine was dissolved overnight at 37° in 100 ml ion-exchange purified water and used on the following day. Quantities employed for 100 ml total volume of overlay medium include: 0.75 g of CMC (sodium salt low viscosity, BDH Chemicals, Poole, England, product No. 27649) and 25 ml of L-tyrosine solution. This mixture was left overnight at +4° and later autoclaved (15 lb for 15 min), and was kept as stock CMC-tyrosine at room temperature until use. Just before use CMC-tyrosine stock solution was dissolved in 25 ml, 2 × L-15 and 50 ml 1 × L-15. The complete overlay was made by adding 10% TPB, 3–10% calf serum (C. S.), 2 mM glutamine and antibiotics.

Cell monolayer stain. After an appropriate

incubation period, plates were stained to demonstrate plaques. Monolayers were fixed by adding formolsaline (10% formaldehyde, 40% w/v, in saline) to each well without previously removing the overlay. After allowing penetration and fixation through the CMC, the plate was placed on its side within a perspex box allowing the supernatant to drain off the cells. Then the whole box was filled with stain and left for 15–30 min, depending on the cells. After the stain was removed and collected to be reutilized, the plates were rinsed in tap water and dried. Plaques were counted on an illuminated X-ray viewing box (Fig. 1).

Two different stains were used, and were made up as follows: *Crystal violet:* 1 g of crystal violet colour index number 42555 (R. A. Lamb, North Acton, England) was dissolved in 200 ml 70% alcohol and 800 ml of water. *Naphthalene black:* 1 g of naphthalene black (naphthol blue black, color index number 20470, R. A. Lamb, North Acton, England), 13.6 g of sodium acetate dissolved in 60 ml of glacial acetic acid and water added to a volume of 1000 ml.

Results. Cell suspensions were obtained

from stock cultures grown in bottles of freshly trypsinized chick embryos. Cell concentrations were the same as used in 60-mm petri dishes or tubes, but the volumes used were smaller. Confluent monolayers of various cell types were obtained with no difficulty providing high degree of humidity was maintained. The cell concentrations and growth media used for the various types of cells are summarized in Table I. Length of incubation periods varied with the cell; generally 2-3 days was sufficient time for all cells to form confluent monolayers except for MK and HSF which required 7 days. Table I further shows the different viruses which we have found produce plaques with this method, and the time of appearance of plaques. The

overlay media usually contained CMC in the appropriate growth media, but monolayers of HeLa cells under overlay containing agarose and additives were several orders more susceptible to rhinoviruses than similar monolayers under CMC. Monolayers were stained with crystal violet except for PS (a continuous pig kidney cell line) cell monolayers, which required naphthalene black. As Porterfield and Madrid (1) have demonstrated, PS cells can be utilized for a wide variety of arboviruses.

We have also used this technique for measuring infection by less cytopathic viruses such as influenza. Virus-infected wells were detected macroscopically after the addition of indicator guinea pig red blood cells because

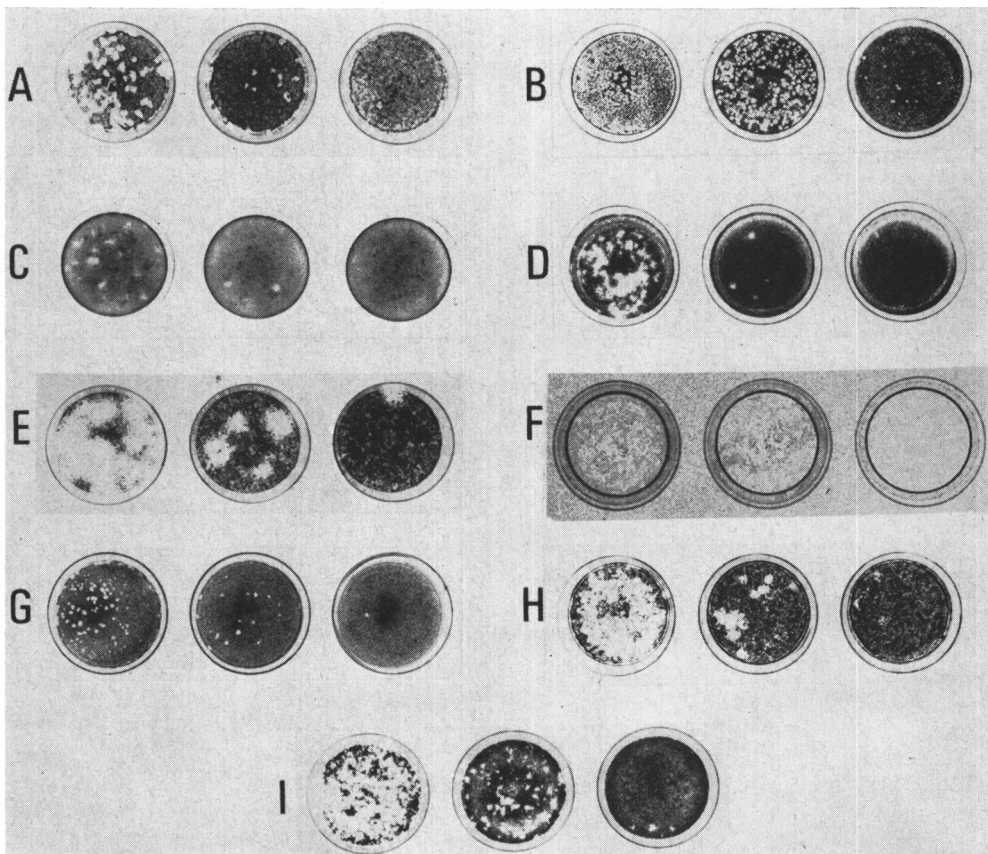


FIG. 2. A series of typical monolayers showing (left to right) the effects of tenfold dilutions on virus plaque numbers. (A) Herpes simplex—CEF; (B) Vaccinia—CEF; (C) Coxsackie B-3—Vero; (D) ECHO 11—Vero; (E) Polio 2—HK; (F) Influenza B/Hannover/1/70—HK; (G) Vesicular stomatitis virus—L cells; (H) Vesicular stomatitis virus—HNF and (I) Rhinovirus 2—HeLa.

of their red color as a result of hemadsorption. In this technique infected areas in any one well could be identified following the pattern of red blood cell adsorption and could also be scanned microscopically for further quantitation.

In this assay system at least 400 wells could be manipulated in two hours. Figure 2 illustrates the appearance of the various types of plaques. Depending upon the size, as many as 30 plaques can be counted in each well without difficulty.

A comparison between herpesvirus plaque assays on chick embryo fibroblasts monolayers grown in 60-mm petri dishes and in the described multicompartmental plastic plates showed a linear relationship between plaque numbers obtained in both assay systems (Table II).

Figure 3 illustrates a plaque assay of varicella-zoster virus using dispersed infected cells as an inoculum. This assay is much more

rapidly and easily performed than the usual plaque assay (2). Furthermore, it is approximately 8-fold more sensitive than a micro foci assay (P. Slack and T. C. Merigan, personal communication, 1972). Figure 2 also demonstrates the advantages of lateral illumination and a dark background over a transmitted light for the precise counting of these plaques.

Discussion. Plaque assays for quantitation of virus are widely used and are the most accurate techniques available at present. However, they are time consuming. The versatility and ease of performance of the method described in this paper are shown by our results. The savings in time and expense in comparison to standard plaque assay are obvious. We have found airborne or cross-contamination to be very rare, despite periodically uncovered tissue culture wells, as long as adequate care is employed and work is conducted in a tissue culture hood.

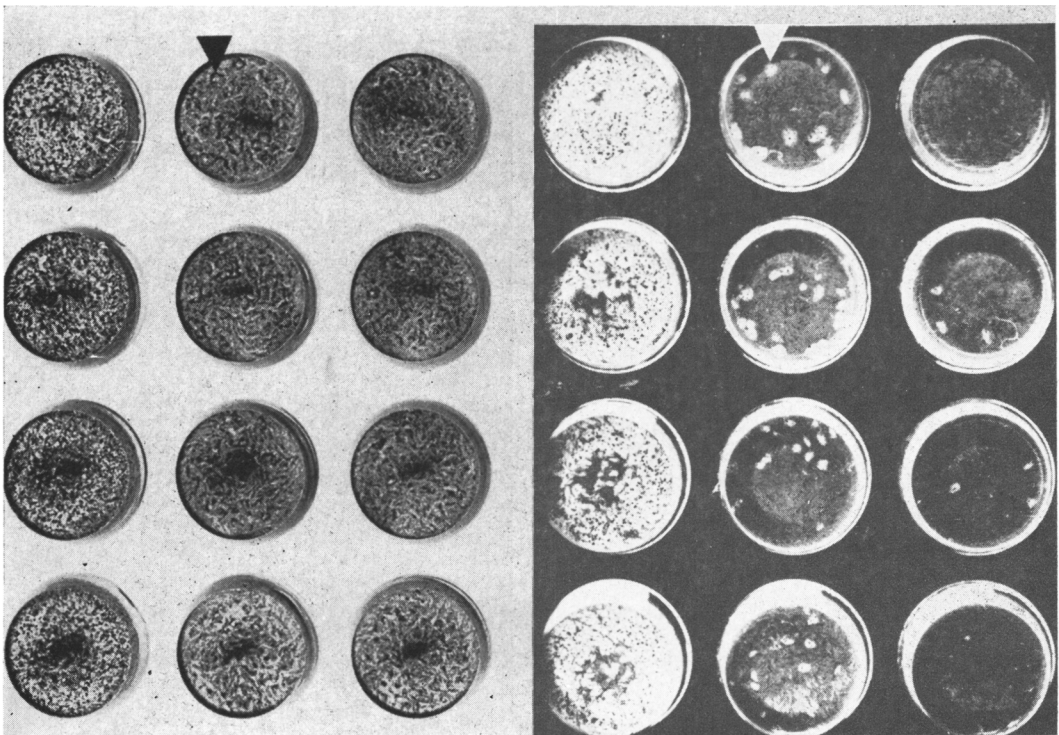


FIG. 3. Four replicate wells of each of three serial tenfold dilutions of varicella-zoster virus. Left half of figure showing standard transmitted illumination and right half showing some wells with oblique illumination against a black background. The triangular marker at the top of each panel shows the same plaque with both kinds of illumination.

TABLE II. Comparison Between Herpes Virus Titers in Two Systems.^a

Virus dilution	60 mm plastic dishes pfu/1 ml ^b	Multicompartamental plastic trays pfu/0.2 ml
10 ⁻³	TMTC ^c	TMTC
10 ⁻⁴	252	18
10 ⁻⁵	26	2
10 ⁻⁶	3	0
Virus titer/1 ml	3 × 10 ⁶	1 × 10 ⁶

^a Confluent monolayers of chick embryo fibroblasts were infected (CEF) with herpes virus in the given volume and dilution. Plates were stained and plaques counted following 3 days incubation at 37°. Each number is the average count of four plates.

^b pfu: plaque-forming units.

^c TMTC: too many to count.

The most relevant previous attempts to decrease efforts in plaque assays without compromising accuracy were those of De Madrid and Porterfield (1) using WHO haemagglutinating plates. Significant improvements offered by our method over their technique include the use of flat bottomed wells in our technique allowing visualization and quantitation of all plaques in the monolayer and the use of disposable plastic trays which will support the growth of a wide variety of cells. A technique involving metal racks containing glass vials on which cell monolayers were grown was described by Baron and associates (3) for use in titrating virus yields obtained in interferon experiments. This technique has not been used with as many cells and viruses as ours. In addition, the racks and tubes take up substantially more space than the plastic plates described here.

When one compares this technique to the microtiter plate tissue culture approach several differences are apparent. The usual

microtiter virus tissue culture assay depends on a diffuse cytopathogenic effect of viruses rather than plaque formation as conditions for macroscopically visible plaquing have not been worked out (4). This technique therefore loses the precision of a plaque assay. The multicompartamental type assay we use employs wells which are smaller than conventional petri dishes yet plaque can easily be counted (with at most the aid of a hand lens in counting) while fluids can be added or removed with standard laboratory equipment. The latter point most likely contributes to the low frequency of contamination referred to above. The larger volume of tissue culture media in each well compared with the microtiter plates minimizes the effect of dehydration and allows us to work without sealing the plates. Since 20 times more inoculum can be added, lower titer virus preparations can be assayed than by the microtiter technique. Therefore, we feel this method offers real advantages over the microtiter method and is likely to be useful to others.

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