Improved Techniques for Staining and Enumerating Focus Formation in Viral Infectivity Assays

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Improved techniques consisting of glutaraldehyde fixation, Giemsa staining, and colony counter enumeration of foci in viral infectivity assays were compared to microscope scanning and lantern slide projection methods. These techniques broadened the scope of application to potentially hazardous viruses and increased the efficiency of assaying.

The use of focus formation for infectivity assay of oncogenic ribonucleic acid sarcoma viruses is well established. It has been widely used since Manaker and Groupe in 1956 recognized foci in cultures of chicken cells infected with Rous sarcoma virus, and Temin and Rubin introduced the quantitative assay in 1958 (2, 4). Nevertheless, it has proven tedious and time consuming, especially when performed on a large-scale basis. Enumerating the foci formed during the assay may present problems, also.

One reason that enumeration of focus formation in viral infectivity assays has remained a problem is the time-consuming, multiple-step procedures usually employed in fixation and staining of the cells (1, 3). We found the simplest and most satisfactory method to be one which fixes and stains the dishes in a single step. This consisted of simply adding 5 ml of fixative-stain solution directly onto the agar overlay. The fixative-stain solution was prepared by diluting commercial stock Giemsa blood stain (Matheson, Coleman and Bell GX 85 B527) 1:10 with 10% glutaraldehyde, buffered with 0.2 M sodium cacodylate adjusted to pH 7.4. The dishes were allowed to stand at room temperature overnight during which the fixative-stain solution diffused through the agar overlay, fixing and staining the cells simultaneously. The following morning the agar was discarded, the dishes were rinsed, and the foci were counted.

Several methods were used for enumerating foci formation in culture dishes (5, 1, 3). Three methods are compared in this report and include the initially developed microscope scanning (2, 4), the later reported modified slide projection technique (1, 3), and the colony counter technique reported herein. The microscope scanning method consists of placing the culture dish on a glass grid connected to the mechanical stage of an inverted microscope, and counting the foci seen in the 2-mm² squares of the grid using a 2.5× objective and 10× oculars. The modified lantern slide projection technique consisted of placing the stained culture dish in a slide carriage modified to hold a 60-mm culture dish and counting the foci seen when the image of the dish was projected onto a white, ruled background. The new colony counter technique consisted simply of placing the stained culture dish on the grid of a Quebec darkfield colony counter (American Optical model no. 3325) equipped with an auxiliary lens and counting the foci directly on the dish. A thin piece of white, ruled paper was placed between the dish and the grid to give better contrast.

The colony counter technique proved to be as precise and reliable as the other two methods. When a series of five dishes were counted three times each by two different technicians (Table 1). Moreover, it greatly increased the efficiency of foci enumeration over the other two methods tested. For example, whereas only approximately 10 dishes per hour could be counted accurately with the microscope, and approximately 60 dishes per hour with the modified lantern slide projection method, approximately 120 dishes per hour could be counted accurately with the colony counter technique.

The counts were generally lower with the colony counter due to the fact that the image with the projector, as well as with the microscope, resulted from a much greater magnification and allowed foci too small to be seen with the colony counter to be visualized with the other methods. Nevertheless, this did not adversely affect the reliability of the colony counter method, although the absolute titer of any given sample was lower.

The focus assay used in this laboratory was
performed basically as described by Vogt (5). Secondary leukemia-free chicken embryo fibroblasts were plated on 60-mm Falcon tissue culture dishes at $9 \times 10^6$ cells per dish in F-10 medium containing 0.002 g of Polybrene per ml (Aldrich Chemical Co.) and 1.6 ml of 50% beef embryo extract per liter (Gibco catalog no. 515), and then infected with the test virus such as Schmidt-Ruppin or Bratislavia-77 avian sarcoma virus. The fibroblasts were filtered through two layers of gauze to remove clumps and to assure a smooth monolayer for background contrast to reduce false-positive counts. Approximately 18 h after infection, the dishes were overlaid with 6 ml of 0.9% agar solution. On day 3 postinfection, the dishes were overlaid with an additional 4 ml of agar solution. On day 6 postinfection, the dishes were fed an additional 2 ml of medium. On day 9 postinfection, the dishes were fixed and stained simultaneously. On day 10 postinfection, the foci were counted on the colony counter.

The need for improvements in the evaluation of focus assays, such as the reduction of eye fatigue and boredom, which lead to decreasing accuracy and efficiency, was accurately described by Spahn et al. (3). The techniques described herein incorporate all the advantages of the projection method, plus several additional advantages. For example, the colony counter requires no screen, no focusing, less eye strain, less time, is more economical, and is commercially available without modifications. Also, the simplified fixing-staining technique requires a minimum number of manipulations and broadens the scope of application by inactivating potentially hazardous viruses. This increases the safety of working with such agents by reducing handling and exposure. As with the projection method, the elimination of linear scanning by the colony counter results in greatly reduced counting time.

The time saved by using the colony counter method, in conjunction with the time saved by the simplified fixing-staining technique, and the broadened scope of application to potentially hazardous viruses constitute a significant improvement in the focus assay for viral infectivity.

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**LITERATURE CITED**

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