AN OVERVIEW OF
VIRUS QUANTIFICATION TECHNIQUES
Viral quantification represents an important step at multiple points in viral-driven recombinant protein production, studies of the mechanisms of viral infection, and vaccine development. Accurate determination of viral concentration allows viral infections to be precisely normalized and viral expansion processes to be monitored, optimized, and altered to give maximum yields. Methods for viral quantification can broadly be divided into the older, traditional methods and more modern methods. Traditional methods have the advantages of being time-honored, and widely-accepted, though these methods are frequently both time- and labor-intensive. In addition, these methods are based on crude assessment methodologies and can produce results that vary widely between replicates, obscuring precise quantitative values. Newer methods frequently overcome many of these shortcomings; they are generally faster and give more precise data. In reviewing methods commonly used to quantify virus, we may distinguish between those methods which measure viral infection, those which measure viral protein antigens or expression levels of viral genes, and those which directly count viral particles. Plaque assays and the fluorescent focus (FFA) assay as well as TCID50 assays fall into this first category, being assessments of viral infectivity. ELISA, HA, SRID, and qPCR fall into the second category. Lastly, transmission electron microscopy (TEM) and viral flow cytometry via the Virus Counter 2100® fall into the final category.
Measurement of Virus Infectivity

There are several methods by which to measure the concentration of infectious virus in a sample. These assays are generally regarded as the oldest and most trusted methods of viral quantification. All are biological in nature and therefore somewhat time and labor intensive. In addition many require specialized training and specific reagents in order to perform.

Fluorescent Focus Assay (FFA)

To overcome the lengthy infection times inherent in plaque assays, FFAs were developed. The assay is performed in much the same way as a plaque assay. However, no agar overlay is required, and only 24–72hrs of infection time are required to generate results. Viral proteins expressed by infected cells are detected through the use of fluorescently-labeled antibodies, and fluorescent-forming units (FFUs) may be directly visualized and counted via fluorescence microscopy. While more sensitive and faster than the traditional plaque assays, these assays can become quite costly due to the costs of the antibodies used. Additionally, availability of suitable antibodies for detection of target antigens may become an issue, and variability may be introduced due to differences in the personnel doing the actual counting, high background signals generated by non-specific binding, or cross-reactivity with non-viral protein targets.

Tissue culture infective dose assay (TCID50)

As some viruses do not form plaques, end-point dilution was developed as an alternative technique. Briefly, numerous replicate cultures of confluent, adherent cells susceptible to infection are treated with a range of dilutions of viral inoculum, and scored according to whether or not cell death or detectable pathological effects have occurred. TCID50 values represent the viral concentration necessary to induce cell death or pathological changes in 50% of cell cultures inoculated and may be calculated using specific formulae [3]. Although TCID50 and plaque assay values would be expected to differ, it has been speculated that the number of plaque forming units (PFUs) would be approximately ½ or slightly more than ½ the value derived from TCID50 [4]. While time-honored, it must be pointed out that the number of total viral particles cannot be inferred from this method, only the infectivity of a specific viral inoculum. Also, as with plaque assays, considerable labor and time are involved in deriving results which may show high variability.

The Viral Plaque Assay

The Viral Plaque Assay is a biological method used for the quantification of viral particles. To perform a plaque assay, the researcher serially dilutes their virus containing samples. One or more of these dilutions are then used to infect cells grown on a nutrient medium such as agar gel. After a short period of infection where the virus binds to the cells, the cell colonies, and subsequently the virus, are immobilized with the addition of more nutrient medium. These cells are then incubated with the virus for several days until a cytopathic effect can be observed, either with the naked eye, or by stains designed to label only cells with a compromised membrane. The number of infectious particles in the original sample can be calculated from the dilution factor of the sample applied to the cell colonies, the volume applied, and the number of colonies on the plate producing a cytopathic effect.

The plaque assay is widely regarded as the gold standard for virus quantification and is heavily used in the field of virology. The plaque assay is attractive because it requires few specialized resources, and directly measures infectious viral particle titer. Often it is useful for the researchers to know the number of particles that retain the ability to infect and replicate in cells. There are several drawbacks to the assay that affect its utility. First, the assay requires a significant amount of time to perform. The cells, once infected with virus, must be incubated for sufficient time to cause cytopathic effect that can be observed visually. Second, many viruses do not cause a sufficient level of cellular damage to be visualized using this method. Third, there can be ambiguity as to the state of a given plaque and certain researchers will count different morphologies as positive. The result is user to user variability in the measurement. This effect as well as the native variability of the assay can result in coefficients of variation ranging from 5% [1] - 44% [2] or even higher. In addition the practice is quite laborious taking several hours of a researcher’s time to prepare the cells, overlay with agar and count the infected colonies.
Assessment Based on Antigen Concentration or Gene Expression

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High Performance Liquid Chromatography (HPLC)

The concentrations of specific viral antigens may be quantified through UV analysis of fractions generated during HPLC. For example, influenza virus has been assessed using a novel reverse phase chromatographic method in which the hydrophilic subunit of hemagglutinin, HA(1), is chromatographically isolated, and the UV peak height quantified [5-7]. The quantity of HA(1) has been found to be directly proportional to total HA concentration in crude, partially-purified, and fully-purified samples. Although convenient, the results of this assay may not correlate to viral particle concentration as it fails to account for free HA in solution. Likewise, for other viral antigens, detected and measured levels may or may not correlate to viral particle numbers as many proteins may be “shed” into solution, leading to “false positives”. Moreover, although capable of being largely automated, HPLC use does require the presence of a competent, well-trained instrument user.

Hemagglutination Assay (HA)

Developed in the 1940s, the HA assay is specifically used to assess influenza viruses based upon their ability to aggregate red blood cells (RBCs). Briefly, samples of mammalian blood are distributed into round-bottom wells of a multiwell plate, and dilutions of viral inoculum are added. Viral particles bearing the hemagglutinin protein will bind to red blood cells in solution, creating foci of aggregated RBCs which grow into networks incapable of normal precipitation. Thus, wells can be scored according to whether or not RBCs precipitate into the bottoms of the wells, forming visible spots. A common variation of this assay is the HA Inhibition Assay (HI Assay), which allows for the presence of anti-viral antibodies to be assessed in sera. By binding to the hemagglutinin protein on the surfaces of viral particles, the presence of antibodies effectively inhibits hemagglutination. This assay recently aided researchers at the Center for Disease Control (CDC) in finding that some adults possess circulating cross-reactive antibodies against H1N1, making them less susceptible to severe infection [8,9]. However, while fast and easy to perform, interpretation of HA assays can be problematic in that various levels of partial agglutination may be difficult to class as + or -. Moreover, HA assays still do not achieve a reasonable level of viral quantification as only relative assessments can be made.

Single Radial Immunodiffusion (SRID)

SRID assays rely upon the radial diffusion of purified viral antigens (standards) and viral particles through agarose gel seeded with polyclonal antisera against a viral antigen [10]. In this assay, known standards and viral samples are allocated into small holes punched in the antisera-impregnated gel, and outward radial diffusion occurs over a timeframe of 10 hours to several days. By comparing the sizes of the rings formed around the sample wells and normalizing them to the rings formed by known standards, the approximate amount of that viral antigen can be inferred. In particular, this assay seems to have gained popularity within the influenza community, where it is used as an orthogonal method to HA. However, concerns have been raised about the length of time required to perform these assays [11-12]. In addition, considerable inter-laboratory variation has been reported, which has also raised concerns, particularly in lieu of recent pandemic influenza outbreaks [11]. Further, the cost of creating agarose gels with large quantities of polyclonal antiserum incorporated into their matrices may be a barrier to widespread use. A related assay, the single radial hemolysis (SRH) assay, is also in common use. This assay relies upon the use of agarose gel impregnated with concentrated mammalian RBCs and the observation of radial lysis of these cells around wells into which viruses have been seeded [13-14]. This assay is used primarily to assess anti-HA antibody potency in seasonal vaccine development. Although time-honored, recently concerns have been raised due to a lack of standardization, considerable inter-laboratory variation, and the length of time necessary to complete [15].
Quantitative Polymerase Chain Reaction (qPCR)

qPCR can be used to quantify the amount of viral DNA or RNA present in a given sample. Briefly, viral nucleic acids are purified from a sample and using a virus specific primer PCR is performed in the presence of either a dsDNA nucleic acid stain, or a sequence specific reporter like TaqMan. As the DNA amplification increases, the signal from the reporting fluorophore increases. The speed of this increase is proportional to the quantity of initial nucleic source material present. The number of thermocycles needed to produce a minimum level of fluorescence (called the Ct value) is compared to that of a serially diluted standard. This comparison yields the number of genomes compared to a viral standard. This method has many advantages; it is relatively quick, quite specific, and uses equipment readily available in a molecular biology laboratory. It does, however, have several drawbacks. One is that it requires virus specific primers to carry out the PCR reaction. If one is dealing with many virus types, or seasonally different strains, primers must be specifically designed for each virus. In addition, this method typically reports higher values than a traditional particle count because the number of viral genomes in a sample is typically much higher than the number of intact virus particles. Also, a viral sample of known quantity is necessary to determine the absolute quantity of virus in a sample. These known standards can be difficult to obtain and can skew the results of analysis if they are not maintained properly.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA assays may be performed in a number of ways, but all rely upon detection of binding between viral particles and/or viral antigens and antibodies. In the “sandwich ELISA” approach, the bottoms of multiwall plates are coated with antibodies against a specific viral antigen and either viral inocula or samples bearing solubilized viral antigens are added. Following an incubation time to allow for binding to occur, secondary detection antibodies conjugated to enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) are used to label the antibody-bound targets. The quantity of bound target is then quantified by colorimetric changes brought about the addition of substrate which acquires color when acted upon by the enzymes. The intensity of a colorimetric change is proportional to the quantity of enzyme, which, in turn, is proportional to the number of bound detection antibodies. This approach has recently been shown to be a good alternative to SRID, being more sensitive, quicker, and cheaper [16]. An alternative strategy is the “Indirect ELISA”, in which viral particles are immobilized to the well bottom and labeled directly with enzyme-linked detection antibodies. Yet another approach is the competitive ELISA, in which known quantities of enzyme-linked antigen create “competition” with native antigens present in biological samples for binding to immobilized antibodies, and the concentration of antigen present in the biological sample is seen to be inversely proportional to the colorimetric signal detected.

While offering superior sensitivity for antigenic quantitation, ELISA assay development can be severely hampered by absence of robust, specific antibodies with high affinities, a problem that can be encountered in viral strains showing significant mutations from one season to the next (i.e. Influenza). Moreover, development of ELISAs for rare or novel viral types may be impossible.

Recently, bead-based ELISA-like assays in which capture antibodies are conjugated to polystyrene or magnetic beads which, following incubation with fluorescent-labeled detection antibodies, are analyzed on a flow cytometer or Luminex™ instrument have also emerged as an alternative. Through differential coloring of bead species, these assays offer the ability of analyzing multiple antigenic targets simultaneously. However, commercially-available kits for quantification of viral antigens are not readily available, and the development of custom kits could prove to be prohibitively expensive in addition to the expertise in running the required instrumentation.
Transmission Electron Microscopy (TEM)

Counting of viral particles through TEM has been long considered to be a “gold standard” in absolute viral particle counting. In TEM, electron beams are transmitted through biological samples which have been aldehyde-fixed and either positively- or negatively-stained. Positive stains label the viral particles themselves, while negative stains rely on staining the background, creating contrast between viral particles and their background. In general, positive staining protocols allow general information such as viral particle size and count to be obtained, while negative staining is able to allow for structural details to be acquired [17]. The preparation of samples to be analyzed via TEM can be extensive and tedious and often must be developed over time. In addition, because the method requires technicians to directly count the viral particles there can be some technician to technician variability. While data on viral particle numbers can be obtained using this method, the technique requires specialized equipment, extensive training, and experienced technical support and resources.

The Virus Counter 2100 is a novel new technique for quantifying the total number of viral particles in a sample. The technology behind the Virus Counter 2100 is based on a specialized version of flow cytometry developed specifically for use with nanometer scale particles. When using the Virus Counter each sample is stained with two different fluorescent dyes, one specific for nucleic acid, and the other specific for protein. After a thirty minute incubation time the stained sample is then analyzed on the Virus Counter 2100 instrument. Using a proprietary thresholding algorithm the computer analyzed the instrument output for events where there is coincident fluorescent output. These events are counted and combined with an extremely accurate flow rate measurement to arrive at a volumetric virus particle concentration.

There are many advantages to the Virus Counter 2100 system chief among them is time to result. By reducing a measurement which previously took hours or days into a process which takes less than an hour the labor and time involved in quantifying virus is dramatically reduced. In addition, because the process is wholly controlled with a computer and does not rely on user input to calculate the result, the reproducibility is dramatically increased, while inter-laboratory variability is significantly decreased. The Virus Counter has been shown to accurately measure many different varieties of virus using the same procedure, thus eliminating any virus specific procedures and reagents. Dramatic time and cost savings combined with excellent reproducibility and a universal assay make the Virus Counter 2100 a potent tool for virus production and investigation.
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