

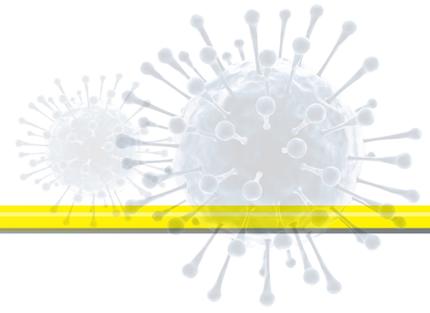
White Paper: An Overview of Virus Quantification Techniques

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 and manufacturing. 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 techniques have the advantages of being time-honored, and widely-accepted, though these methods are often both time- and labor-intensive. In addition, these methods are typically based on crude assessment methodologies and can produce results that vary widely between replicates and investigators obscuring precise quantitative values. Newer approaches frequently overcome many of these shortcomings as they are generally faster and give more precise data. In reviewing approaches commonly used to quantify virus, it is useful to distinguish between those methods that measure viral infection, those that measure viral protein antigens or expression levels of viral genes, and those that directly count viral particles. Plaque assays and the fluorescent focus (FFA) assay as well as median tissue culture infective dose (TCID₅₀) assays fall into this first category, being assessments of viral infectivity. Enzyme-linked immunosorbent assay (ELISA), hemagglutination assay (HA), single radial immunodiffusion assay (SRID), and quantitative polymerase chain reaction (qPCR) are associated with the second. Lastly, transmission electron microscopy (TEM) and flow cytometry via the Virus Counter 3100® sort into the final category.

Measurement of Virus Infectivity

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

Viral Plaque Assay. The Viral Plaque Assay is a biological method used for the quantification of infective viral particles. In this assay, viral inocula are serially diluted and used to infect confluent monolayers of adherent cells. After a short period of infection, the cell colonies, and subsequently the virus, are overlain with agar gel. This hinders lateral diffusion of viral particles. Infected cells are then incubated for several days until a cytopathic effect can be observed, either with the naked eye, or by stains designed to label only cells with compromised membranes. 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.

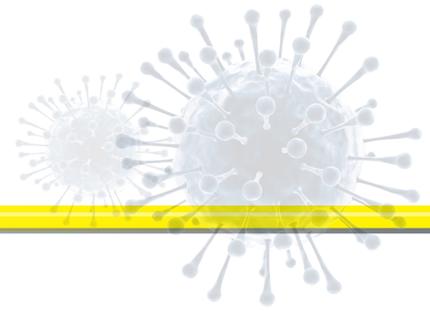


Plaque Titer Assay

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 labor-intensive, taking several hours of a researcher's time to prepare the cells, overlay with agar and count the infected colonies.

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 24-72 hours of infection time is necessary 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 traditional plaque assays, these assays can be quite expensive 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, which are 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 inoculated cell cultures 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 that are subject to high variability.



Assessment Based on Antigen Concentration or Gene Expression

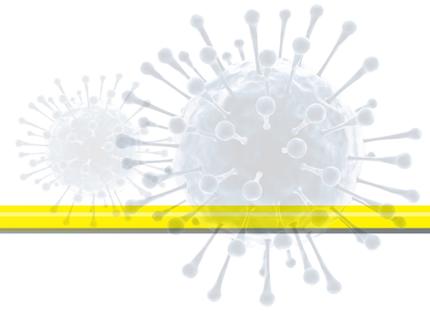
Relative assessments of viral production may be made through quantification of specific viral antigens or the expression levels of certain viral gene sequences. However, the values thus obtained may not be directly correlated with the numbers of viral particles. For protein-based assays, the presence of free, dissociated antigens in solution would be expected to skew results. For gene-based assays, artifacts created during amplification steps, quality of reagents and primers, as well as differences in the technical expertise of experimenters may likewise affect the measurement.



HPLC

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, HA1, is chromatographically isolated and the UV peak height quantified[5-7]. The quantity of HA1 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. As is the case 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, HPLC is slow, not automated, has significant sample carryover following elution, lacks good control standards, requires the presence of a competent, well-trained instrument user; and, necessitates considerable expenditure for reference material for use as controls[8].

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.



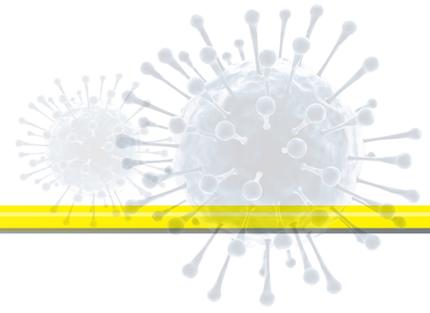
In 2009, this assay allowed researchers at the Center for Disease Control (CDC) to determine that some adults possess circulating cross-reactive antibodies against H1N1, making them less susceptible to severe infection[9,10]. However, while fast and easy to perform, interpretation of HA assays can be problematic in that various levels of partial agglutination may be difficult to class as positive or negative. 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 [11]. 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 [12-13]. In addition, considerable inter-laboratory variation has been reported, which has also raised concerns, particularly in light of recent pandemic influenza outbreaks[12]. Further, the cost of creating agarose gels with large quantities of polyclonal antisera 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. The assay utilizes agarose gel impregnated with concentrated mammalian RBCs and subsequent observation of radial lysis of these cells around wells into which viruses have been seeded[14-15]. This approach is used primarily to assess anti-HA antibody potency in seasonal vaccine development. Although time-honored, concerns have recently been raised due to a lack of standardization, considerable inter-laboratory variation, and the length of time necessary to complete[16].



ELISA

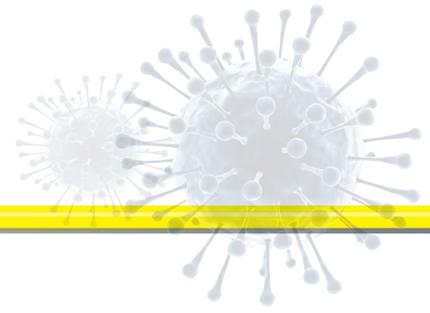
Enzyme-Linked Immunosorbent Assay (ELISA). ELISA assays may be performed in a number of ways, but all rely upon detection of binding between viral particles and/or viral antigens and antibodies. In the “sandwich ELISA” approach, the bottoms of multiwell plates are coated with antibodies against a specific viral antigen, and either viral inocula or samples bearing solubilized viral antigens are added. Following an incubation period 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 determined by colorimetric changes brought about by 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 less expensive [17]. 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 variation 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 inversely proportional to the colorimetric signal detected. While offering superior sensitivity for antigenic quantitation, ELISA assay development can be severely hampered by the absence of robust, specific antibodies with high affinities, a problem often 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 approach 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 permit analysis of multiple antigenic targets simultaneously. Unfortunately, reagents required for quantification of viral antigens are not readily available and the development of custom kits may prove to be prohibitively expensive. An added factor is the expertise required to run the system.

Flow Cytometry. Traditional flow cytometers have been employed to quantify the levels of viral proteins present on the surface of infected cells. One example is the use of anti-gp64 antibody conjugated to a fluorochrome to label baculovirus during the budding phase as it emerges from the host SF9 cell. This is a viral infectivity assay which measures the intensity of gp64 expression while baculovirus is still contained within intact host cells. The fluorochrome intensity is cross-referenced against an internal fluorescence control to determine the number of virus particles per host cell. This assay requires specialized flow cytometry equipment, antibodies specific to gp64, reference controls and a measureable and stable sample flow rate. Due to the complexity of this assay, there is high variability between replicate samples and the cost of the equipment and technical expertise can make this method not affordable to many labs.

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, then – 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. Quantitative PCR 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 the requirement for high-quality 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 or viral strain.



In addition, this method typically reports higher values than traditional particle counts since the number of viral genomes in a sample is often 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. Such standards can be difficult to obtain and may skew the results of analysis if they are not maintained properly.

Direct Counting of Viral Particles in Solution

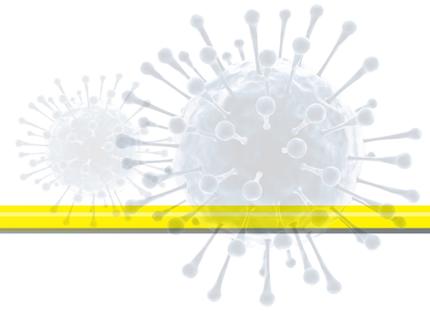
Direct counting of viral particles is an attractive approach to quantifying and characterizing viral cultures. However, a means of accomplishing this have–until recently–been very limited.

Transmission Electron Microscopy (TEM). Counting viral particles through TEM has long been considered to be a “gold standard” in absolute viral particle quantification. 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 label the background, creating contrast between viral particles and the underlying surface. In general, positive staining protocols provide information such as viral particle size and count to be obtained, while negative staining permits structural details to be resolved[18]. The preparation of samples for analysis using TEM can be extensive and tedious and often must be developed over time. In addition, because the method requires technicians to directly count viral particles, there is substantial variability between operators. Not surprisingly, TEM requires specialized equipment, extensive training and experienced technical support and resources.



Virus Counter 3100 (top) with
Autosampler (bottom)

The Virus Counter® 3100. The Virus Counter is a flow cytometer specifically re-engineered to detect virus particles by utilizing proprietary fluidics control systems and a dual stain labeling technology. It is optimized for rapid virus quantification with an analysis time of less than 5 minutes and can be converted to a fully automated analysis platform. With the Combo Dye system, viral genomes (and nucleic acids in general) and envelope proteins are stained with fluorogenic dyes that emit in the yellow and red regions of the visible spectrum. This approach to staining allows for the detection of a wide variety of viruses using a simple no wash assay. Using proprietary fluidics technology, stained viral particles are hydrodynamically focused into a narrow stream and passed through a laser interrogation point in the Virus Counter. Emitted fluorescence is detected on two separate optical channels where optical compensation hardware and software elements correct for any crosstalk between channels. Fluorescence bursts on each channel are counted as a function of time.



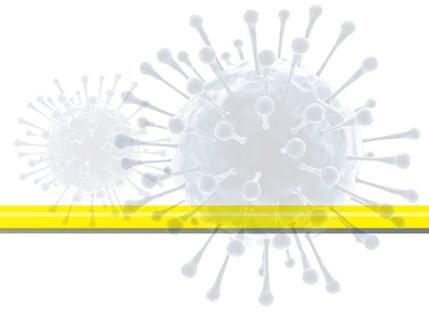
When fluorescence bursts are simultaneously observed on both the nucleic acid and protein emission channels, the “simultaneous event” is counted as an intact virus particle. The number of simultaneous events counted during the analysis time is used in combination with sample flow rate to calculate the concentration of virus particles per milliliter of sample. Since ViroCyt’s proprietary nanofluidics design ensures that sample flow rate is accurately and precisely measured in real-time, there is no need for an internal calibrant bead such as those used in typical counting assays. The addition of the optional 96-well plate autosampler allows overnight sample processing, dramatically reducing hands-on time.

Summary

Multiple methods have been developed over several decades to attempt to quantitatively characterize viral samples. Approaches for quantifying infective viral units, while useful, time-honored, and relatively unspecialized, frequently require considerable labor and time to produce results, which often show poor reproducibility. Methods for quantifying viral antigens or viral gene expression, while generally quicker and more reproducible, often have difficulty correlating to the number of viral particles actually present in solution. TEM-based counting, although highly accurate in total particle quantification, is often prohibitively expensive, time-consuming, and impractical for high sample numbers. Of the methods compared here, only the Virus Counter offers the advantages of absolute particle counting, speed, reproducibility, and ease of use.

Comparison of Virus Quantification Methods

Technique	Assessment	Precision	Time	Labor	Cost Per Sample
Plaque Assay	Infective Units	Poor	Days/Weeks	High	Low
Fluorescence Focus	Infective Units	Poor	Days	High	High
TCID50	Infective Units	Poor	Days/Weeks	High	Low
HPLC	Viral Antigen	Excellent	Days	High	High
Hemagglutination	Viral Antigen	Good	Hours	Low	Low
SRID	Viral Antigen	Good	Days	Moderate	High
ELISA	Viral Antigen	Good	Hours	Moderate	High
Flow Cytometry	Viral Particles	Poor	Days	High	High
qPCR	Viral Gene Expression	Excellent	Hours	High	High
TEM	Viral Particles	Good	Days/Weeks	High	High
Virus Counter	Viral Particles	Excellent	Minutes	Low	Low



References

1. Shurtleff, A. (2012) Standardization of the filovirus plaque assay for use in preclinical studies. *Viruses*. 4:3511-3530.
2. Bae, HG. (2003) Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay. *Journal of Virological Methods*. 110:185-191.
3. Reed, LJ, and Muench, H. (1938) A Simple Method of Estimating Fifty Percent Endpoints. *American Journal of Hygiene*. 27:493-497.
4. <http://www.protocol-online.org/biology-forums/posts/1664.html>
5. Kapteyn, JC, et al. (2006) Haemagglutinin quantification and identification of influenza A&B strains propagated in PER.C6 cells: a novel RP-HPLC method. *Vaccine*. 24(16):3137-3144.
6. Kapteyn JC, et al. (2009) HPLC-based quantification of haemagglutinin in the production of egg- and MDCK cell-derived influenza virus seasonal and pandemic vaccines. *Vaccine*. 27(9):1468-1477.
7. Girard, M. (2011) http://www.who.int/vaccine_research/diseases/influenza/18_feb_2011_Michel_Girard_ws.pdf
8. Transfiguracion, J. et al. (2011) Development and validation of a HPLC method for the quantification of baculovirus particles. *Journal of Chromatography*. 879(1):61-68.
9. Racaniello, V. (2009) <http://www.virology.ws/2009/05/27/influenza-hemagglutination-inhibition-assay/>
10. Racaniello, V. (2009) <http://www.virology.ws/2009/05/22/adults-have-pre-existing-antibodies-a-california-04-2009-h1n1/>
11. http://www.virapur.com/assay_services/srid.php
12. Taylor, N. (2011) <http://www.in-pharmatechnologist.com/Processing/Influenza-pandemic-prompts-EMA-vaccine-quality-rethink>
13. Schmeisser, F, et al. (2010) An alternative method for preparation of pandemic influenza strain-specific antibody for vaccine potency determination. *Vaccine*. 28:2442-2449.
14. Russell, SM, McCahon, D, and Beare AS. (1975) A single radial haemolysis technique for the measurement of influenza antibody. *Journal of General Virology* 27:1-10.
15. Farrohi, K, et al. (1977) Evaluation of the single radial hemolysis test for measuring hemagglutinin- and neuraminidase-specific antibodies to H3N2 influenza strains and antibodies to influenza B. *Journal of Clinical Microbiology*. March:353-360.
16. European Medicines Agency. (2011) http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/10/WC500115612.pdf
17. Lee, SH, et al. (2008) Enzyme-linked immunosorbent assay, single radial immunodiffusion, and indirect methods for the detection of failure of transfer of passive immunity in dairy calves. *Journal of Veterinary Internal Medicine*. 22(1): 212-218.
18. Brum, J. (2011) http://www.eebweb.arizona.edu/faculty/mbsulli/protocols/Positive_and_Negative_Staining_of_Viruses_on_TEM_Grids.pdf