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VIRUS QUANTIFICATION IN MINUTES, NOT DAYS.

White Paper: Rapid Quantification of Egg-Grown Influenza

Embryonated chicken eggs have been the primary means of producing influenza vaccines for many decades. Although there has been significant effort to move away from this system, it will continue to provide the bulk of the global vaccine supply during the current decade and possibly well into the next [1]. Until newer cell-based approaches are validated and scaled-up, there remains ample opportunity to improve egg-based methods. A notable bottleneck is the quantification of virus along the entire vaccine development and manufacturing process. Current approaches often require days or weeks to obtain a result, limiting their utility or potentially delaying the time-critical process of getting vaccines for seasonal and emerging pandemic strains to the public. Consequently, there is an urgent need for faster, more accurate methods of determining the amount of virus present during the creation of these life-saving products.

An Overview of Influenza Virus Growth in Eggs

Vaccination against harmful infectious diseases has been one of the most important global public health advances of the past 100 years. Due to surging demand for a wider range of vaccines, the industry is facing the challenge to updating from traditional, time-consuming methods to new, more efficient technology. An example of anticipated changes in the vaccine industry is the drive to transition away from egg-based production of influenza vaccine toward more nimble methods such as cell-culture, and baculovirus-based recombinant protein expression. Emerging technologies such as virus-like particles are also attracting attention as immunogenic agents. While there has been significant progress, top global producers of flu vaccine still rely heavily on eggs to grow the virus. As illustrated in Figure 1, egg-based production has a long timeline and requires close coordination between government agencies and vaccine producers to deliver safe and effective vaccines before the onset of flu season.

Virus quantification presents a rate-limiting step at many stages of vaccine development and production, for both egg and cell culture. Currently, one of the most widely used tools for the determination of virus concentration is the viral plaque assay, or variations such as tissue/egg culture infectious dose (TCID50/EID50). The viral plaque assay is a cumbersome, subjective and traditional biological technique that was originally applied to the quantification of viruses in the early 1950s [2].

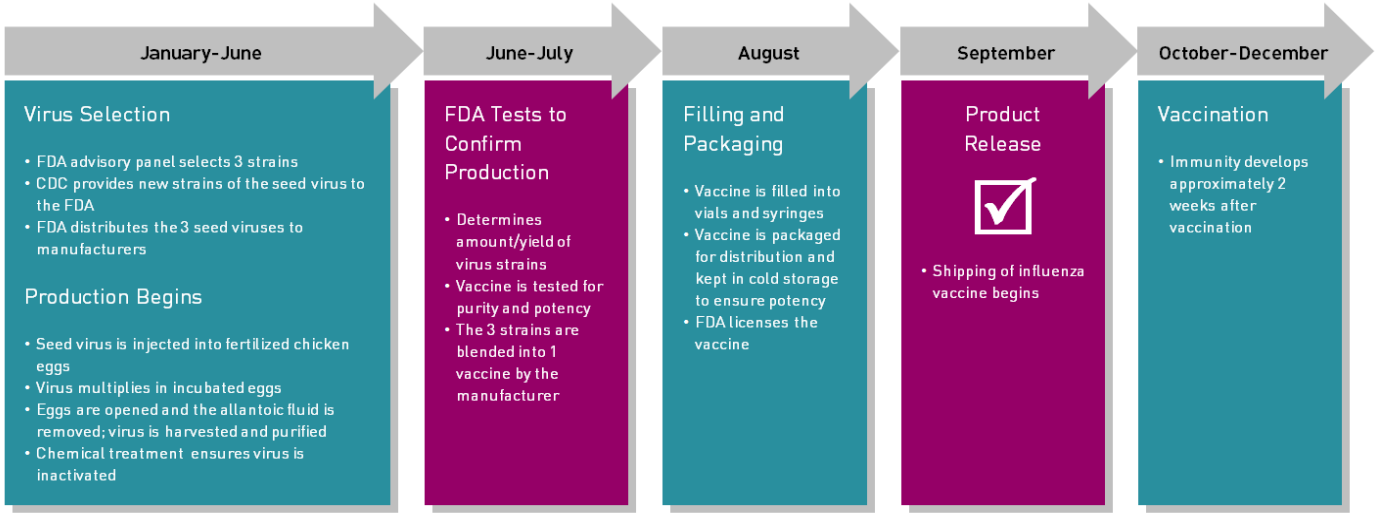

Figure 1. Influenza Vaccine Production Timeline

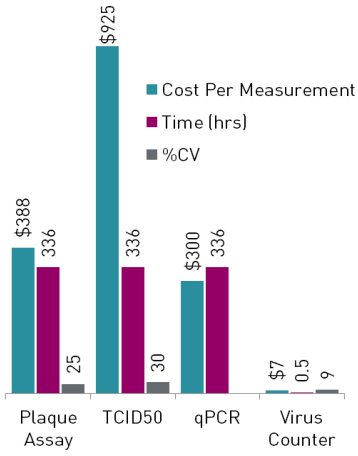

Figure 2. Contract Research Organization Estimates of Cost and Turnaround Time for Several Virus Quantification Methods Compared

During the assay, a series of dilutions of the virus of interest are used to inoculate a cell layer, which is subsequently incubated for a prescribed period of time. For "plaque forming" viruses, cells infected with the virus eventually burst and create plaques, which can be stained and counted to determine the concentration of infecting virus. This assay requires significant hands-on time by a trained technician, and has a time to result of several days or weeks depending on the virus. Other methods for virus quantification exist, including a variety of ELISA-type protein assays, transmission electron microscopy (TEM), and quantitative PCR (qPCR). However, each of these approaches has its own drawbacks, perhaps the most important being that they require well-trained technicians and have a significant time to result.

For influenza viruses, the hemagglutination assay (HA) is also widely applied. The HA assay is rapid but somewhat subjective, requires the use of animal red blood cells, and yields an HA titer value that is not readily translated into viruses per mL or pfu per mL. More on this topic can be found in the White Paper "An Overview of Virus Quantification Techniques." Figure 2 presents actual estimates obtained from Contract Research Organizations (CROs) that provide quantification services to the vaccine industry, of the cost and turnaround time for several of these traditional approaches, as well as the expected coefficients of variation (%CV). Please note the costs do not include set-up fees, which are often significant.

Figure 3. Virus Counter 3100 (top) and autosampler (bottom)

The ViroCyt® Virus Counter® (Figure 3) was developed in response to needed improvements in virus quantification. The instrument is a personal flow cytometer optimized for rapid virus enumeration (less than 5 min analysis time) at a per sample cost below $5. The Virus Counter assay utilizes a dual fluorescence staining approach. Viral genomes (and nucleic acids in general) are stained with a dye that emits in the yellow region of the visible spectrum and residual host cell envelope and viral capsid proteins are stained with a dye that emits in the red region of the spectrum. This "universal" approach to staining allows for the detection of a wide variety of viruses using a simple assay.

In the instrument, stained viral particles are hydrodynamically focused through a laser probe region and subsequently detected via fluorescence on two separate optical channels. Fluorescence bursts on each channel are counted as a function of time. However, when fluorescence bursts are simultaneously observed on both the nucleic acid and protein emission channels the "simultaneous event" is categorized as an intact virus particle. The dual stain approach provides some degree of discrimination from free protein aggregates (counted only on the protein channel) or free nucleic acids (counted only on the nucleic acid channel) in solution. The number of simultaneous events identified during sample analysis is used in combination with sample flow rate to calculate the volumetric concentration of virus particles. Because the 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 flow cytometric cell counting assays. Since the Virus Counter provides a direct, physical measurement of total intact virus concentration within minutes, it can have a significant impact at multiple stages during vaccine research, development and production, such as those indicated by a star in Figure 4 below.

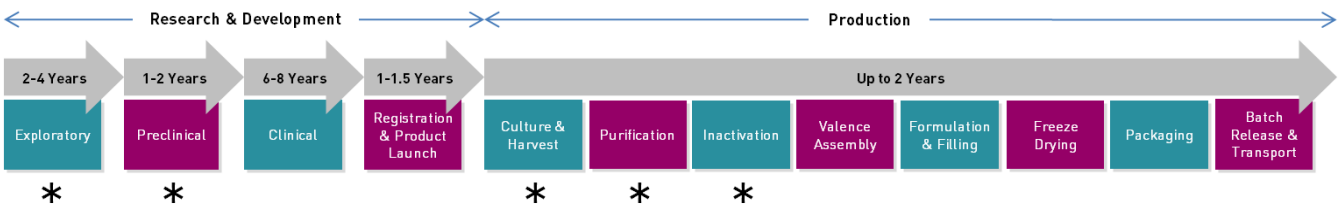

Figure 4. Opportunities for Improved Virus Quantification During Vaccine research, Development and Production

These area of potential impact include:

- The rapid selection of high-growth reassortants that have the appropriate antigenic properties for each type and strain to be used in the multivalent vaccine would significantly reduce the time to seed stock selection.
- Faster elucidation of virus yield as a function of various parameters during culture would speed time to scale up for production.
- Additional opportunities to track virus concentration during subsequent purification steps can identify points where virus is lost and will enable process improvements leading to greater efficiencies manufacturing the final product.
- The ability to track virus levels in real-time, which is not possible with traditional methods, will ensure optimal harvest time with maximal yields.

Using Eggs to Produce Flu Vaccine: Primary Unmet Needs

Chicken eggs have been utilized since the mid-20th century to grow a variety of viruses (Figure 5), which are then inactivated and injected into the individual to provide protective immunity. Although the bulk of the seasonal flu vaccine is still produced in this manner, there are a number of issues associated with egg-based systems. Chief of these are the variability in growth characteristics of each viral strain and batch to batch differences between eggs. In addition, even seemingly minor inconsistences in incubation times can have a significant impact. Consequently, providing as close to "real time" quantification of total viral particle count during the growth cycle is a primary unmet need in the vaccine industry. Of utmost importance is the ability to take samples from a large representative number of eggs, and rapidly prepare and analyze the samples to allow for adjustments in incubation conditions or immediately harvest.

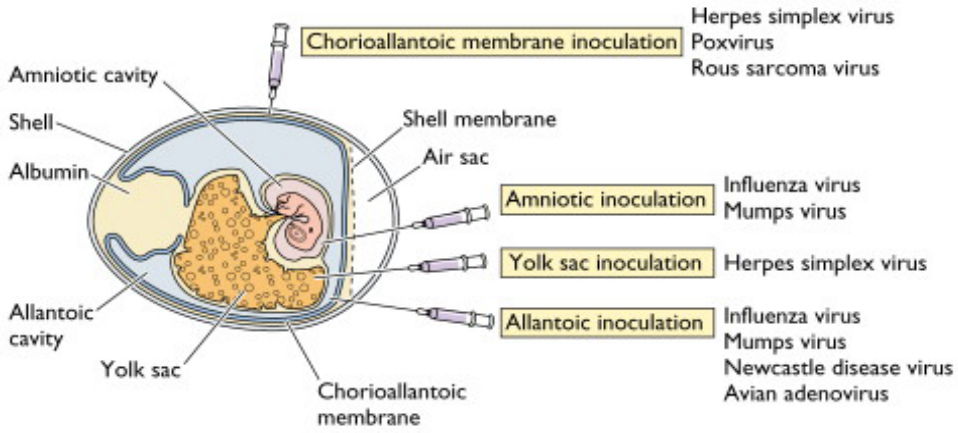

Figure 5. Examples of Viral Vaccines Produced in Chicken Eggs

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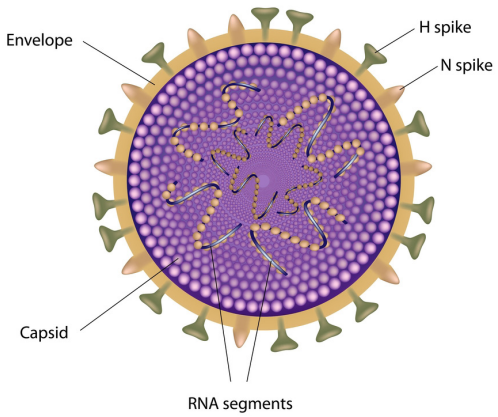
Not All Influenza Viruses Are Created Equal

There is a growing body of literature that describes the variability of influenza viruses, and that – more specifically – a significant portion are not infective. Each class, including defective interfering particles (DIPs), noninfectious cell-killing particles (niCKP), interferon- inducing particles (IFPs), and interferon induction-suppressing particles (ISPs), play significant biological roles without causing viral infection. The observation that these non-infectious particle types actually make up the majority of particles in active influenza infections raises the question of whether these particles should be ignored, as they would be if infectivity assays alone are used. In addition to their effect on biological systems, monitoring non- infectious particle numbers can be important for other applications as well. For example, following purification from chicken eggs, influenza virus particles are split apart using a specialized reagent and the immunogenic HA proteins are harvested from this solution. Non-infectious particles that are known to have a protein capsid and a partial genome will also contribute the immunogenic HA protein after being split. It is therefore essential during this process to have a rapid method for accurately measuring total particle concentrations. Given the extensive biological role of these non-infective particles, as well as their impact on the development and manufacture of viral vaccines, infective titers and total particle numbers are both essential for accurate viral characterization. For additional information, please see the White Paper "Why Viral Particle Quantification Matters."

The Influenza Virus

- 80nm – 120nm in diameter
- Typically spherical, although filamentous forms can occur
- Enveloped
- Segmented, single stranded, negative sense RNA,
- Approximatelt 13,500 bases
- Traditionally produced using embryonated chicken eggs
- New approaches include cell based manufacturing, recombinant proteins, VLPs



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Same Day Determination of Virus Concentration in Allantoic Fluid

Now that we've defined the unmet need for a method that provides rapid, quantifiable and reproducible results, what is the proof that the Virus Counter is the solution, specifically with regards to egg-grown influenza? The following are just a small sample of results obtained by the Virus Counter in this setting.

- Serial Dilution Curve. An essential first step in validating any quantification method is to perform a serial dilution analysis, whereby a concentrated sample is diluted by known fractions and analyzed. In this example, samples of three different influenza strains were obtained from allantoic fluid and diluted. The expected result is to see a linear relationship between the logarithm of the dilution and the logarithm of the result. The results in Figure 6 show good linearity across the dilution series, with slopes near 1. Data from the negative control do not show this same pattern, as they are at or below the stated limit of detection of the Virus Counter of 1x10E6 vp/ml.

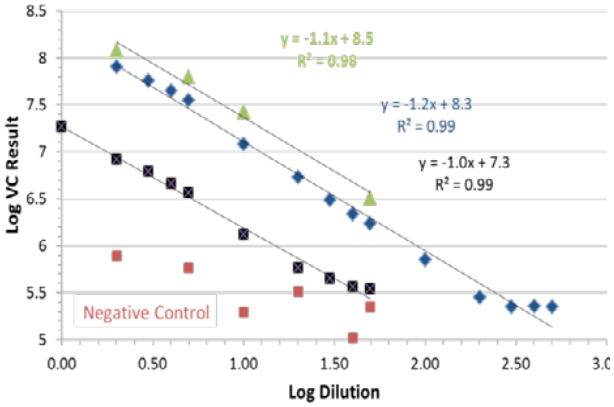

Figure 6. Virus Counter Serial Dilution Analysis of Allantoic Influenza

- Comparison of Virus Counter with TCID50. Traditional approaches to virus quantification fall into two categories: Those that determine infectivity, and those that do not. Examples of the former include plaque titer assay and tissue culture infectious dose 50 (TCID50), and transmission electron microscopy (TEM) and quantitative polymerase chain reaction (qPCR) for the later. In Figure 7, four different influenza strains were quantified using both the Virus Counter and TCID50. As is typically the case for influenza (and many other viruses), the number of intact virus particles per unit volume (vp/ml) is 1-2.5 logs higher than the number of infectious particles, due primarily to the abundance of the previously discussed Defective Interfering Particles. Since DIPs are known to play a role in immunity [3-6], these results can provide information about subsequent use of a given stock. It is worth noting that the TCID50 assay typically takes between 3-8 days to perform, and is often subject to significant variability.

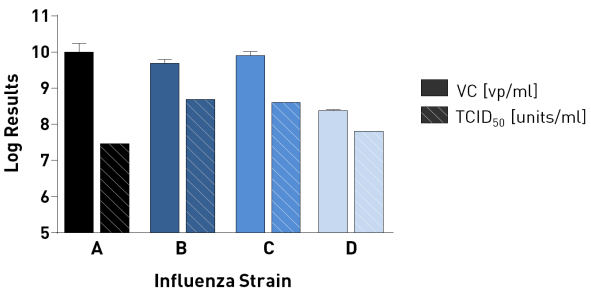

Figure 7. Comparison of Virus Counter with TCID50 Quantification of Allantoic Influenza

- **Comparison of Virus Counter with Quantitative RT-PCR.** Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) utilizes primers specific to the viral genome to amplify and simultaneously quantify a targeted RNA molecule. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. Figure 8 illustrates a close correlation between the two quantification techniques. Notice the much larger error bars for the qRT-PCR results relative to those for the Virus Counter.

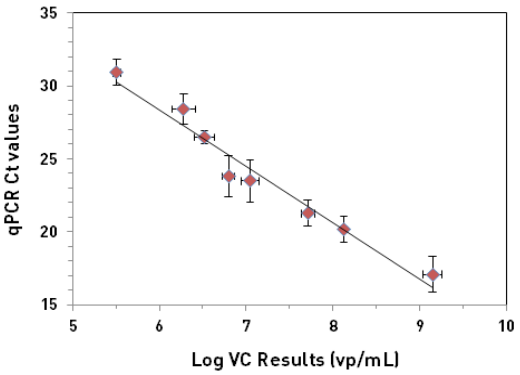

Figure 8. Comparison of Virus Counter with qRT-PCR Quantifiction of Allantoic Influenza

Conclusion: Flu Vaccine Production – Tracking and Optimizing Yield Throughout the Development and Manufacturing Process

There are many points during the process of developing, optimizing and producing vaccines that would benefit from rapid enumeration of viral particles. One of the most significant is tracking efficiency following harvest from egg- and cell-based systems. More often than not, the long and complex steps of taking crude material and transforming it into a product ready for patients results in substantial loss of material. The ability to track essentially in real time the quantity of virus at the beginning and end of each distinct stage will identify where losses are occurring and allow improvements to be made. Even small gains in efficiency at each step would lead to considerable financial benefits.

References

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