



Principles of Operation

Labs working with viruses need to routinely quantify the amount of virus present in their product or intermediates for a variety of purposes including method optimization and quality control. Traditional methods for determining viral titers are time and labor intensive and are often the bottleneck for many production processes. InDevR's Virus Counter instrument and assay present an alternative analytical method for rapid determination of virus concentrations to enable and support virology research and production by removing this bottleneck. This document provides a brief overview of the Virus Counter instrument and assay.

DETECTION PRINCIPLE

The Virus Counter utilizes sensitive fluorescence detection to directly quantify virus particles in solution. The assay incorporates a combination of two fluorescent dyes: one is specific for proteins and the other is specific for nucleic acids. Since virus particles contain both proteins and nucleic acids, they will be stained by both dyes. The sample staining process requires a 30 minute room-temperature incubation and is non-application specific (i.e. the same reagents are used for all viruses). All samples to be analyzed within a day can be stained at the same time. Once stained, samples are ready for analysis by the Virus Counter, which requires 5-10 minutes per sample.

While free or contaminant proteins and nucleic acids in a sample will also be stained by the respective dyes, these components will typically be dispersed molecules or small aggregates. It is rare that there will be aggregations of proteins or nucleic acids large enough to compare to the amount of protein and nucleic acids contained in a virus particle. It is even rarer that a non-viral particle (excluding prokaryotic and eukaryotic organisms, which can easily be removed from samples by filtration, centrifugation or other means) will contain significant amount of co-localized proteins and nucleic acids. Therefore, detection and quantification of particles containing appreciable amounts of co-localized proteins and nucleic acids represents an accurate measure of the virus content of a sample.



Figure 1. The Virus Counter instrument is a compact, bench top instrument with a footprint of 1.5 ft² (0.14 m²).

INSTRUMENT DESIGN

The Virus Counter instrument is similar to a basic flow cytometer in the following ways: the sample is hydrodynamically focused via a sheath fluid, a laser is used to probe the hydrodynamically-focused sample for particles of interest, and multiple fluorescence detectors are used to monitor the sample for particles of interest. Other than these similarities, the Virus Counter is not a flow cytometer and is in many ways simpler and easier to use. The Virus Counter was not designed as an open-ended research tool but is rather an application-specific tool designed to provide quick answers with minimal user input. A brief description of the key components that make up the Virus Counter, as well as a summary of the data analysis used to provide results, is given below.

Fluidics & Optics: Hydrodynamic focusing of the stained sample prior to analysis is important so that the particles within the sample can be analyzed one-at-a-time. Without hydrodynamic focusing, multiple virus particles would be in the probe region at the same time and it would be impossible to accurately gather information from or count the particles. The hydrodynamic focusing of the sample takes place as the sample and sheath fluids enter a flow cell and pass through a hydrodynamic lens. Focusing creates a very narrow sample stream within a flow channel where particles pass single file through a focused laser beam, which defines the probe region. Particles' interactions with the laser (i.e. fluorescence) are collected by two photomultiplier tube (PMT) detectors. One detector measures fluorescence from the protein-specific dye and the other detector from the nucleic acid-specific dye. Once sample particles pass through the probe region they mix with the sheath fluid and are collected in a waste container. Schematically, hydrodynamic focusing and the key components of fluorescence excitation and data collection are shown in Figure 2.

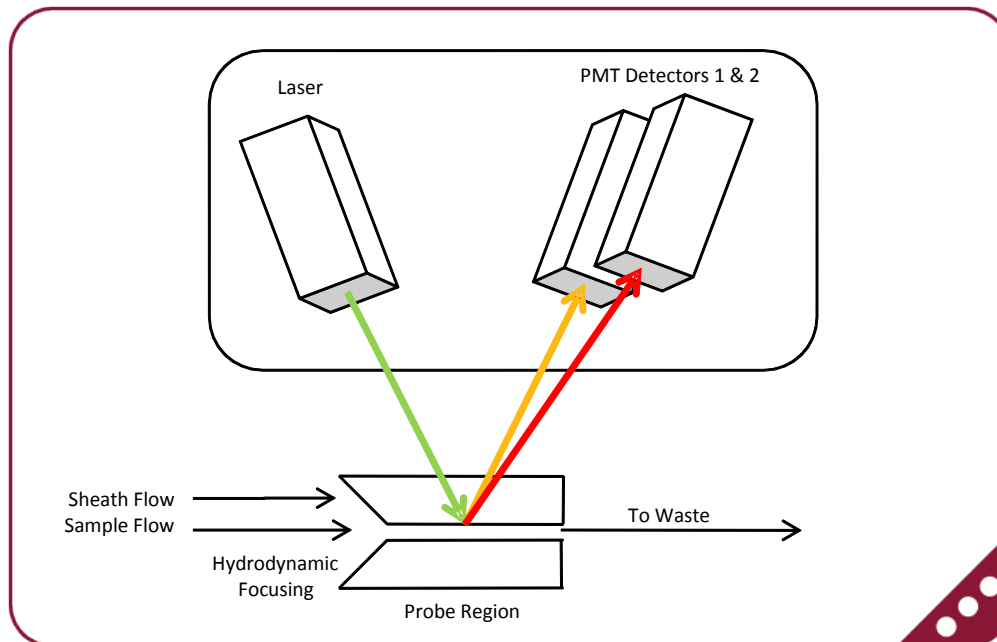


Figure 2. Schematic diagram showing the hydrodynamic focusing of the sample needed to interrogate particles individually and the components used to gather fluorescence data that help identify virus-like events.

Data Analysis: Data collected from the fluidic and optical system described above is processed in real time to convert the observed fluorescence information into a quantitative volumetric measure of virus-like particles per mL (vlp/mL). As noted above, the majority of events normally observed by either PMT detector will be viruses stained with both fluorescent dyes. However, real-world samples will often contain some protein aggregates and/or large segments of nucleic acids. The source of such contaminants may be host cell debris, viral degradation products, or defective virus particles. These contaminants will be stained by the fluorescent dyes used to identify virus particles and may even contain enough protein or nucleic acids to rival the virus particles in fluorescence intensity observed by the PMT detectors. However, such events will not contribute to the vlp/mL result reported to users as they can be differentiated from virus-like particle events by the data processing system. To illustrate how this happens, Figure 3 shows simulated PMT data as a function of time. In this example, each detector observes three fluorescence events that all exceed a predetermined amplitude threshold used to identify events that have virus-like characteristics. However, only one event occurs simultaneously on both channels. It is these simultaneous events that indicate virus-like particles containing co-localized protein and nucleic acids. The hardware and software tally all events on both channels, but the reported vlp/mL results accounts for only the simultaneous events. In this way the data processing system can discriminate virus-like particles from other particles in the sample and report a value that has been shown to correlate well with titer values obtained from standard infectivity assays such as plaque assays or antibody-based ELISA assays. Additionally, the information obtained by the individual PMT detectors can be compared to the number of simultaneous events to provide the user with a rough measure of sample purity and the relative amount of excess free protein and nucleic acid.

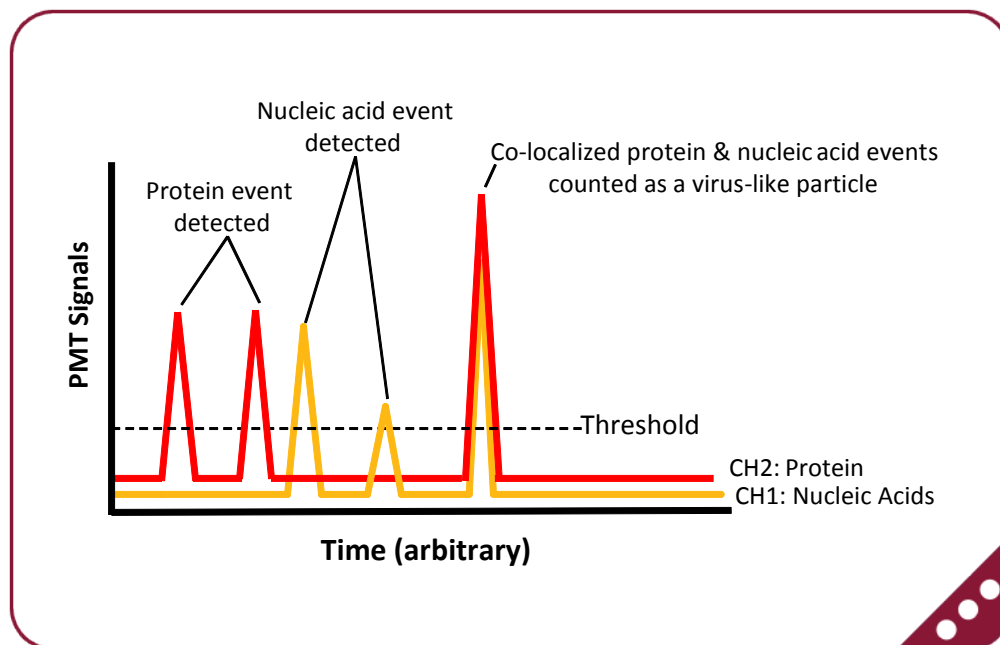


Figure 3. Simulated fluorescence data showing how fluorescence events detected by the instrument's PMTs are analyzed to provide a vlp/mL result. Only events that occur simultaneously on both channels contribute to the vlp/mL result. However, non-simultaneous events can also provide useful sample information as the increasing occurrence of such events is an indicator of sample contamination by non-viral proteins and/or nucleic acids.

CONSIDERATIONS

The Virus Counter instrument and assay have been shown to work well for a variety of viruses and a variety of sample matrices. For example, influenza, adenovirus, baculovirus, respiratory syncytial virus, rubella and cytomegalovirus samples have been successfully analyzed. In most cases multiple viral strains and sources have been explored, including many real-world samples provided by collaborators from the antigen and vaccine production fields. However, the following factors should be considered in evaluating if the Virus Counter assay fits well with your specific needs:

The Virus Counter assay is ideal for virus concentrations $>10^6$ particles/mL. This means that samples which contain at least 10^6 vlp/mL should yield statistically meaningful results. In some instances the lower end of the linear dynamic range can be extended down to $\sim 10^5$ vlp/mL by the use of systematic washing and cleanup methods. These methods are simple but require a few additional minutes per sample run.

As a general rule, the more purified the virus sample and the simpler the suspension medium, the more successful the analysis will be. For example, gradient purified virus samples are ideal. These purified samples have very few non-simultaneous events while crude virus preparations containing large amounts of host cell debris may show event rates on individual channels that are up to 3-4 times in excess over the number of simultaneous events.

It should be noted that the Virus Counter is not a diagnostic tool. It is intended to provide users with virus titer information for samples that contain a known virus. If a mixture of viruses is present, the resulting vlp/mL value will be reflective of the total virus particle count for the sample.

The Virus Counter has a minimum amount of fluorescence required to accurately trigger counting events. The lower limit is ~ 200 fluors per particle. This means that viruses with very little nucleic acid or protein content may not be detectable. For example, adeno-associated virus samples contain insufficient proteins and nucleic acids for successful Virus Counter analysis.

It should be noted that the Virus Counter assay is not a biological infectivity assay. Results have shown Virus Counter vlp/mL results to correlate well with titer values from infectivity assays. However, each application should be evaluated independently to confirm the relationship between user's current assays and the Virus Counter's results.