

#### Achieving Infectious Titer with Multi-laser Nanoparticle Tracking Analysis (NTA)

##### Introduction

The viral vector market became highly active after the launch of a number of Advanced Therapy Medicinal Products (ATMPs). Based on the number of cases in clinical trials and its success rate to date of this writing, we can likely anticipate a cascade of FDA-approved products within years.

The complexity of viral particles remains one of the biggest hurdles in the development process. Simply put, viruses have unique biological properties; they are small arrangements of protein, nucleic acid, and lipid which undergo extensive compositional and expressional changes during infection. As such, the study of viral particles is much more intricate than that of monoclonal antibodies or proteins [1]. From selecting the right viral propagation system to harvesting and purifying virus from other biological material, each step requires arduous and meticulous virus characterization in order to achieve optimized infectivity and stability.

In this note, the upstream process of analyzing viral preparations is addressed, as well as the use of multi-laser nanoparticle tracking analysis (NTA) as a cost and time efficient method to measure size, count virus particles, and correlate to infectious titer.

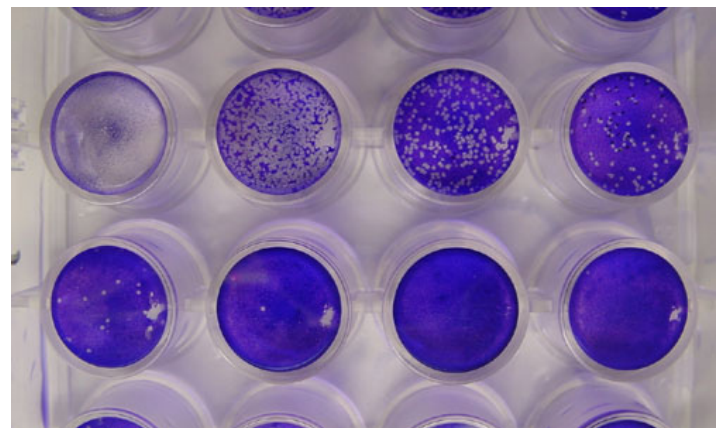
##### Typical Analysis Methods

Researchers typically follow two established analytical techniques to measure infectious titers:

1. Viral Plaque Assay
2. Quantitative Polymerase Chain Reaction (qPCR)

Both approaches quantify the amount of virus present in a solution, but cannot determine the purity of the viral sample. In a Viral Plaque Assay, serially diluted viral preparations are applied to cell culture monolayers and incubated for various durations depending on the specific virus [Figure 1]. Bacteriophages require at least 18 hours of infection while lentivirus infections can take up to two weeks, for example. During the incubation period, viruses infect the confluent cells, destroying these and

spreading to neighboring cells until regions of dead cells are large enough to appear as empty patches on the dish. These regions or “plaques” are then manually counted to determine the number of Plaque Forming Units (PFU) found in the given dilution. Multiplying the PFUs by the dilution factor determines the final infectious titer of the parent stock, expressed in PFU/mL.



**Figure 1. Serially diluted viral preparation displaying various number of plaque forming units (PFU) per mL where regions of destroyed cells visible with a dye to enhance contrast [2].**

Viral Plaque Assays are simple to perform, but require extensive preparation and processing by a technician. Plaque assays are not exact measures of viral numbers, rather, they count cell death due to viral infection. What one analyst deems as a PFU may not be in agreement with another, reducing the precision of the results. Therefore, the reproducibility of the assay dramatically decreases when multiple technicians perform the test.

Because many investigators have found the Viral Plaque Assay to be laborious and time-consuming, they select qPCR instead. One of the strengths of qPCR is its ability to amplify small amounts of viral nucleic acid and subsequently quantify viral gene expression. The technique does not discriminate between whole, broken, empty, aggregates, infectious or non-infectious viruses; it merely determines relative viral gene expression against an internal standard. The viral titer of a sample can be determined by correlating qPCR results to an experimental standard tediously derived through previous viral plaque assays performed on the same virus species to enable faster future analysis.

One of the drawbacks of qPCR is that it is costly and requires an extensive prior knowledge of the viral genome sequence. Primers have to be derived against specific non-mutating regions of the viral genome and standards need to be derived from plaque assays for each virus species tested. However, as qPCR detects the presence of small, specific regions of nucleic acid, it cannot quantify invasive viral particles. Furthermore, qPCR uses isolated nucleic acid lysed from cells or precipitated from solution, meaning that the accuracy of the readout depends on the purity of the sample. As such, the development of a streamlined and efficient technology to quickly and accurately determine viral titers and sample purity will be extremely important for analyzing preparations of viruses in the expanding viral therapy market.

## Materials and Method

The underlying idea of taking an alternative multi-laser Nanoparticle Tracking Analysis (NTA) approach by the ViewSizer 3000™ technology is similar to that of qPCR: to quickly and accurately enumerate the amount of virus within a sample and then correlate the data back to PFU/mL. To achieve this, three simultaneously operating solid-state lasers with wavelengths of 445 nm, 520 nm, and 635 nm are used to visualize the displacement of each viral particle as well as their aggregates. Using multiple lasers with independently adjustable power enables complete analysis of the distribution including both single viral particles and aggregates. The detailed distribution data allows differentiation of, for example, large particle impurities such as cell wall residue from within a broad distribution of particle sizes for accurate analysis of size and concentration.

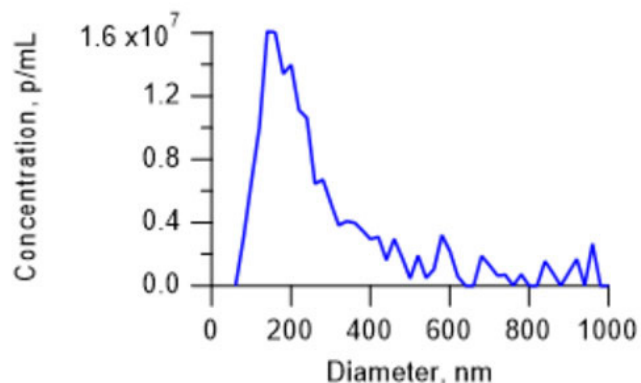


**Figure 2. The cuvette includes a black insert that houses a magnetic stir bar to keep virus aggregates particles suspended.**

In an unpublished correlation study done using a human viral vector, diluted virus preparations after a purification step were split into multiple replicates. One set of replicates were measured with plaque assay to obtain an average PFU/mL. Another set of replicates were reserved for the multi-laser NTA technique where an aliquot was

transferred to the ViewSizer 3000 cuvette [Figure 2], fitted with a magnetic stir bar, to conduct an analysis. Twenty-five videos were collected with five seconds of stirring in between each video to ensure completely independent sets of particles in each video. The measurements were recorded with the following parameters: frame rate: 30 frames/second; exposure: 15 ms; gain: 30; blue laser power: 210 mW; green laser power: 12 mW; and red laser power: 8 mW; temperature control: active, 22°C. Each run took between 15-20 minutes. Multiple users independently analyzed the samples using the same measurement and acquisition parameters described above. The data were then processed and plotted using the same settings.

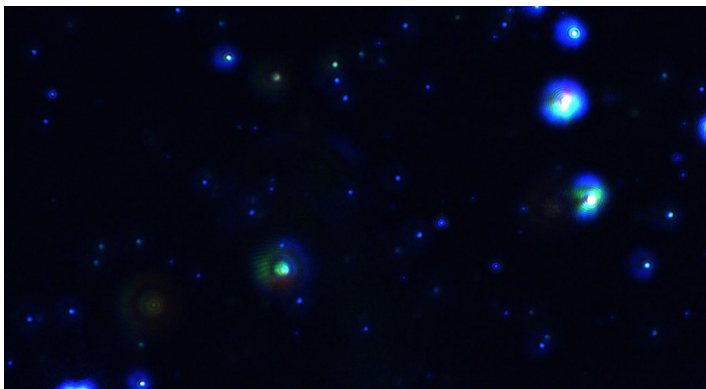
## Results and Discussion



**Figure 3. Measurement result of a human viral vector sample. Note the distribution displayed presence of host cell debris and aggregates.**

The average particle size distribution across multiple replicates measured by multi-laser NTA is shown in Figure 3. As shown, the particles within the sample exhibit a much broader size distribution beyond that expected from a perfectly purified homogenous viral preparation. Particle sizes ranged from 50 nm to 1000 nm, with a significant enrichment of particles around 200 nm. The total viral particle concentration within the sample was  $1.6 \times 10^7$  particles/mL. The distribution tailing towards 1000 nm suggests the existence of host cell debris or other background particles contained within the cell culture growth media.

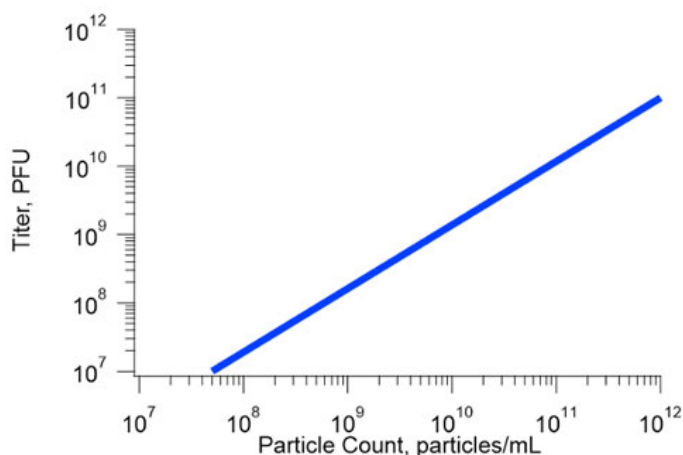
In a non-natural environment, virus particles have the tendency to form complexes with impurities or aggregations [3]; therefore, it is expected to see a profile similar to that seen in Figure 3 with a right-skewed distribution. Distinct from a conventional single laser (single wavelength) NTA device which quickly reached its technical limits when virus sample was not completely purified [4], the ViewSizer 3000 effectively captured information on viral particles and aggregates of the entire size range. The image extracted from the video recordings further validated the presence of larger viral aggregates [Figure 4].



**Figure 4. A screen shot of the measurement video of a human viral vector used in gene therapy. “Blue dots” are scattered light from individual particles. Particle motion determined from the video is used to determine particle size. Brighter spots correspond to larger, more strongly scattering particles.**

## Infectious Titer

To determine the utility of the ViewSizer 3000 approach, infectious titer data from multiple assay replicates performed by independent analysts were plotted as a function of measured particle concentration from the ViewSizer 3000 and fitted with a result shown in Figure 5. The result indicated an excellent  $R^2$  value of greater than 0.9. By using three lasers, all particles were counted and the improved correlation is presumably because infectious aggregates contribute to the titer and were quantified along with single virus particles. When a single laser is used, correlation dropped to an  $R^2$  value of 0.6 because of its inability to properly count the aggregates. This result indicates that over the course of multiple experiments, the ViewSizer 3000 accurately and reproducibly enumerated the viral titer within a heterogeneous sample.



**Figure 5.  $R^2$  value of >0.9 was achieved between titer and data collected from the ViewSizer 3000.**

## Conclusion

The enumeration of infectious viral particles within a sample is a challenging endeavor due to the complex nature of viral biology. To harness therapeutic capability of viral vectors, researchers and manufacturers alike must find a cost-effective and time-efficient method to differentiate intact virus from aggregates and other biological material, while being able to determine the concentration and infectivity of a sample during processing. This demonstrates the ViewSizer 3000’s precise ability to assess not only intact viral particles and aggregates, but also potential cell debris and background noise generated during upstream processing. Since downstream processing depends strongly on the removal of these impurities to minimize further aggregation [4], quantifying infectious viruses reliably depends on understanding both the purity of the upstream sample and the downstream analytical precision.

The conclusion is that to appropriately evaluate viral populations such as a human viral vector, a single laser is not sufficient. Using the simultaneous three laser NTA approach of the ViewSizer 3000 allows for the accurate analysis of viral preparations for concentration and sample purity, allowing for the efficient and streamlined quantification of the viral titer of a given virus sample.

## References

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2. “Virus Quantification.” Wikipedia, Wikimedia Foundation, 15 Mar. 2020, [en.wikipedia.org/wiki/Virus\\_quantification](https://en.wikipedia.org/wiki/Virus_quantification).
3. Wright JF, Le T, Prado J, Bahr-Davidson J, Smith PH, Zhen Z, Sommer JM, Pierce GF, Qu G: Identification of Factors that Contribute to Recombinant AAV2 Particle Aggregation and Methods to Prevent Its Occurrence
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