Rapid and Direct Quantification of Viral Vector Particles for Gene Therapy

ViroTag Allows Biologically Relevant In-Process Monitoring of Total Particle Count

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Following notable setbacks during the previous decade, the gene therapy field is resurgent, both in the breadth and the depth of different applications being pursued using this approach. With its success, however, comes the realization of the challenges inherent with transitioning from early research and development to late-stage clinical trials and product launch. A primary issue is the consistent and compliant production of viral vectors at levels necessary to support commercialization. As the number of successful trials grows and the likelihood of multiple product approvals increases, the realization that current manufacturing technologies have not kept pace highlights this as an area in urgent need of innovation.

One of the most problematic steps is in the quantification of viral vectors during growth, harvest, purification, and release. Current methods like quantitative PCR and absorbance readings at 260 nm and 280 nm are highly variable, resulting in over- or under-estimation of particles present at any given step. The ramifications for manufacturing are lost product, delays, and cost overruns, which are serious, yet pale in comparison to the risks associated with administering too little (no therapeutic effect) or too much (adverse immune response) product to patients. Clearly, there is a critical requirement for a rapid, more precise means of quantifying viral particles used for vector-mediated gene therapy.

ViroCyt® is a Colorado-based biotechnology company focused on enabling real-time enumeration of viruses across multiple application areas. This includes viral vaccines, virus-based expression systems, and viral vector production. The primary method of detection utilizes a fluorescently labelled antibody specific to the virus of interest. ViroCyt has developed a number of these reagents under the trade name ViroTag®, and continues to launch new versions addressing the most relevant and important viruses.

In this assay, 5 µL of the ViroTag solution are added to 195 µL of the virus preparation and incubated for 30 minutes at room temperature, followed by a one-minute analysis using the Virus Counter® 3100.

In the instrument, the number of unique fluorescent events above an automatically determined threshold yields the amount of virus particles per milliliter volume (vp/mL). Due the high degree of specificity of ViroTag

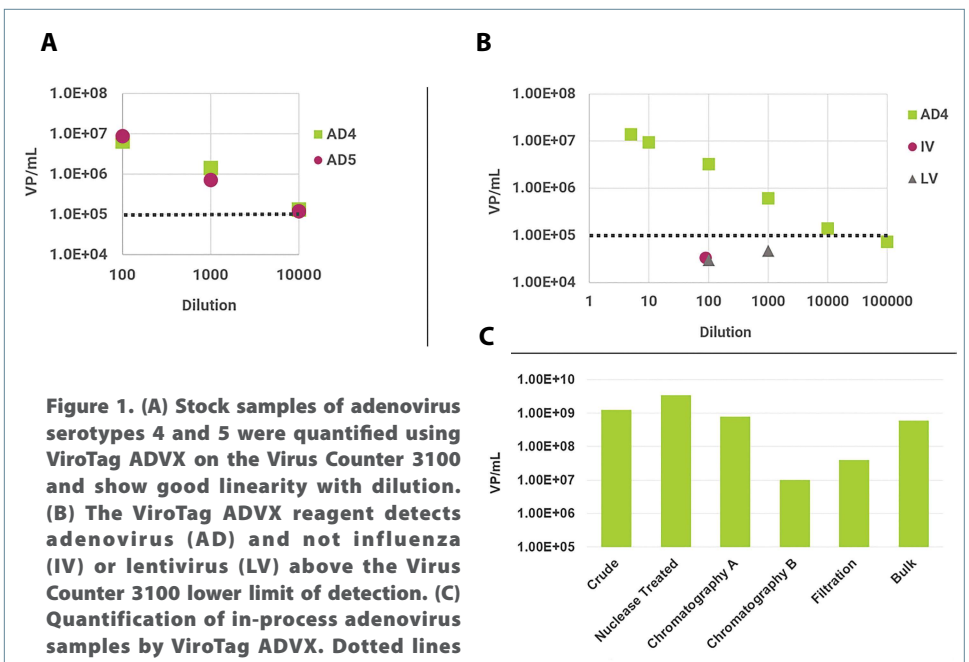

Figure 1. (A) Stock samples of adenovirus serotypes 4 and 5 were quantified using ViroTag ADVX on the Virus Counter 3100 and show good linearity with dilution. (B) The ViroTag ADVX reagent detects adenovirus (AD) and not influenza (IV) or lentivirus (LV) above the Virus Counter 3100 lower limit of detection. (C) Quantification of in-process adenovirus samples by ViroTag ADVX. Dotted lines represent the lower limit of detection of the Virus Counter.

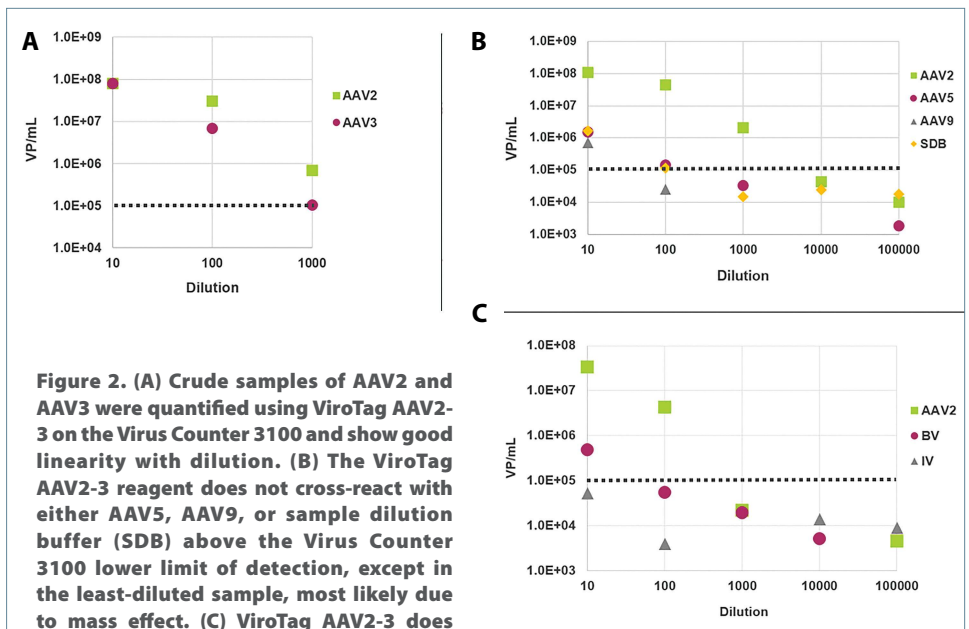

Figure 2. (A) Crude samples of AAV2 and AAV3 were quantified using ViroTag AAV2-3 on the Virus Counter 3100 and show good linearity with dilution. (B) The ViroTag AAV2-3 reagent does not cross-react with either AAV5, AAV9, or sample dilution buffer (SDB) above the Virus Counter 3100 lower limit of detection, except in the least-diluted sample, most likely due to mass effect. (C) ViroTag AAV2-3 does not detect baculovirus (BV) or influenza (IV) above the Virus Counter 3100 lower limit of detection, except in the least-diluted sample, again most likely due to mass effect. Dotted lines represent the lower limit of detection of the Virus Counter.

miRNA

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informs Dr. tenOever. During the first 24–48 hours after VP55 delivery, the elimination of cellular microRNAs impacted less than 0.35% of the over 11,000 genes expressed in the cell. After 9 days, however, almost 20% of the genes showed significant changes in expression.

"MicroRNAs are very powerful and influential in controlling the biology of the cell but they do so over the long term," declares Dr. tenOever. These findings are in agreement with knowledge that has accumulated over the years about microRNA biology, which established that microRNAs play a central role in determining how cells differentiate during development.

"While microRNAs can act on hundreds of mRNAs, their action requires several days of fine-tuning to have long-term consequences," adds Dr. tenOever. This finding suggests miRNAs are unable to significantly contribute to the acute response to virus infection.

The one exception to this observation was that, even though very few genes were affected in the first 48 hours after VP55 delivery, several genes encoding chemokines were impacted. These included chemokines responsible for recruiting antigen-presenting cells, neutrophils, and other immune cells.

An in vivo analysis of mouse lung tissue 48 hours after vector administration confirmed that several cytokines were specifically upregulated, resulting in immune cell infiltration following the degradation of all microRNAs. These results indicate that the acute viral infection is largely independent of microRNAs, and that microRNAs are primarily involved in the adaptive response to infection and other longer term processes.

MicroRNA Biomarkers Reveal Molecular Pathways of Kidney Damage

"Our approach involves looking at microRNAs from the perspective of biomarkers as a readout for kidney damage," says Vishal S. Vaidya, Ph.D., associate professor of medicine and environmental health at Brigham and Women's Hospital, Harvard Medical School, and Harvard T.H. Chan School of Public Health. "At the same time, we are exploring their utility as therapeutics."

A large number of medications and occupational toxins cause kidney damage, but many tests to assess kidney function and damage are not sufficiently sensitive or specific, opening the need for novel diagnostic strategies. MicroRNAs, which are differentially expressed between healthy and diseased states, are promising as early biomarkers for impaired renal function.

"MicroRNAs can also provide information about which pathways are active and which targets can be druggable," points out Dr. Vaidya.

In a study that used microRNAs and proteins to provide a combined biomarker signature, Dr. Vaidya and colleagues examined two patient cohorts, one presenting with acetaminophen-induced kidney injury and the other one with cisplatin-induced kidney damage. "Protein biomarkers provide sensitivity, and microRNAs offer mechanistic insight," explains Dr. Vaidya.

This approach helped visualize metabolic pathways that are altered in the kidney during toxic injury. "The biggest challenge, from a therapeutic perspective, is that microRNAs regulate many mRNAs and, therefore, impact many proteins," concludes Dr. Vaidya. GEN

for the virus, both crude, partially purified, and fully purified samples are amenable to this approach, allowing evaluation throughout upstream and downstream processing.

To demonstrate proof of concept for the quantification of viral vector particles, two of the most prevalent viruses currently in development were selected for evaluation: adenovirus and adeno-associated virus.

Adenovirus

Adenovirus (AD) is nonenveloped, with a 90–100 nm icosahedral nucleocapsid containing a double-stranded DNA genome ranging from 26 to 48 kbp in size. With over 400 trials in progress, AD is one of primary vehicles for delivering genetic payloads to specific cell types, especially neoplastic cells. Because AD is known to cause respiratory disease in humans, primarily in children, it is essential that key viral genes are replaced with the required therapeutic gene or genes. To avoid this issue, as well as the likelihood of a host immune response to the vector, non-human strains of AD are also being investigated.

This point, especially as it relates to quantification, has been specifically called out by the FDA: "Given the potential toxicity of the adenoviral particles themselves, CBER recommends that patient dosing be based on particle number."[1]

ViroTag ADVX (adenovirus, cross-reactive) uses an antibody capable of detecting multiple Adenovirus serotypes and has been tested with AD 2 through 6 on the Virus Counter 3100. *Figure 1* demonstrates the results for dilution series of Adenovirus 4 and 5, as well as the specificity of ViroTag ADVX using unrelated viruses. Also included in this figure is an example of data obtained for in-process AD samples.

Adeno-Associated Virus

Adeno-associated virus or AAV, is also a nonenveloped virus, albeit much smaller than AD, with a particle size of 25 nm and a 4.7 kb single stranded DNA genome. The principal advantages of AAV as a viral vector are the absence of pathogenicity, low host immune response, and long-term expression.

A noteworthy shortcoming, however, is the limited genetic capacity of the small particle. There are currently in excess of 100 clinical studies underway using AAV-mediated gene therapy across a broad spectrum of diseases, including hemophilia, heart disease, muscular dystrophy, cystic fibrosis, and Alzheimer's disease.

While the most-often-used method for quantifying AAV is quantitative PCR, it is generally recognized in the AAV community that qPCR is at best an indirect readout of particle number, and results for the same sample can vary by as much as 3× when performed on the same instrument and even more (up to 10×) when the sample is analyzed on different hardware and/or by different laboratory personnel.

The ability to use qPCR is also often confounded by the aberrant packaging of constructs in excess of the normal 4.7 kb genome size. Because qPCR typically underreports the copy number—and by inference, particle count—concerns around injecting higher than necessary amounts of AAV particles are well-founded.

Thus far, ViroTag reagents have been developed for four different AAV serotypes (2, 3, 5, and 8), each of which was selected based on two primary criteria: importance as a viral vector and availability of well-characterized and highly specific monoclonal antibodies. All clones were chosen for their abilities to bind only intact capsids, irrespective of genome content. As is shown in *Figure 2*, ViroTag AAV2-3, which was developed to detect both AAV2 and AAV3, was evaluated for cross-reactivity and specificity both within the AAV family and across more divergent viruses.

Conclusion

The lack of robust quantification methods for viral vectors is a significant unmet need in the gene therapy arena. Direct, real-time, and specific titration of viral particles during growth, purification, final quality control, and release is essential for consistent, efficient, and scalable manufacture of safe product. ViroTag, when combined with the Virus Counter 3100, provides an immediate readout of particle count regardless of the purity of the sample, permitting the benefits of in-process monitoring such as optimizing growth conditions, maximizing yield, and identifying problems as soon as possible. GEN

REFERENCES

1. Guidance for Human Somatic Cell Therapy & Gene Therapy, FDA Centers for Biologics Evaluation and Research, 1998.

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