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Rapid, Real Time Quantification of Adeno-Associated Virus Particles Using Antibody-Based Detection on the Virus Counter[®] 3100

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Introduction

Adeno-associated virus (AAV) particles are valuable vectors for modern gene therapies. Due to initial failures of gene therapy, it is apparent that accurate enumeration of total particle count of gene therapy vectors is critical. This is important to minimize the risk of adverse immune response or other negative outcomes when dosing patients.¹ Quantification of AAV particles is difficult, often relying on costly and timedemanding methods such as electron microscopy. Rapid analysis methods are needed to monitor vector production and enumerate particles in final formulations.

The Virus Counter[®] 3100 and antibody-based Virotag[®] AAV reagents allow for the rapid quantitation of AAV particles by utilizing serotype-specific fluorescently labeled antibodies quantifying intact, fully assembled AAV particles. Other rapid viral quantification methods such as qPCR and ELISA, that quantify total genome copy (qPCR) and viral antigen (ELISA) concentration, respectively, may quantify unassociated nucleic acid and unassembled viral antigens, leading to overestimates of AAV particle concentration (Table 1).

Here we demonstrate that the Virus Counter[®] 3100 coupled with Virotag[®] AAV2-3 reagent represents a rapid, biologically-relevant method of quantification for AAV2 and AAV3 samples. Utilizing a patented, no-wash assay, AAV samples are stained in 30 minutes with data collected in 3 minutes (Figure 1). This speed allows for in-process monitoring and production optimization of AAV vector products, making the Virus Counter[®] 3100 and Virotag[®] reagents a valuable addition to bioprocessing applications utilizing AAV particles.

Results

This study was performed to contrast distinct methods for AAV enumeration with a focus on the Virus Counter[®] 3100 platform. Virus samples were diluted and assayed on the Virus Counter[®] to determine an optimal dilution for titer analysis. Samples were then prepared at the optimized dilution in replicate (n = 6) and analyzed using the Virus Counter[®] platform.

Based on measured virus count and dilution factor, a final virus count was determined for each strain tested. These results were compared with results for TCID₅₀, transducing Units, qPCR and ELISA, as available from the virus supplier.

As seen in Table 1, the results obtained using the Virus Counter[®] correlate well with TCID₅₀ for two of three strains tested. In contrast, qPCR and ELISA assays result in significantly higher calculated virion counts.

Materials and Methods

The AAV2 strain H (#VR-680, lot 62264941), recombinant AAV2 reference standard stock (#VR-1616, lot 58051221) and AAV3 strain H (#VR-681, lot 217085 reference LOT 2W) were acquired from the American Type Culture Collection (ATCC). Adeno-associated virus (AAV2) and AAV3 strains were stained using Virotag[®] AAV2-3 Reagent (#VIR-92322, Sartorius-Stedim North America).

AAV samples and sample blanks were serially diluted in Sample Dilution Buffer (Sartorius-Stedim North America) and stained with the Virotag[®] AAV2-3 reagent. Samples were incubated at room temperature for 30 minutes (protected from light) then measured using the Virus Counter® 3100.

Results below the instrument quantification limit (IQL) of 5 × 10⁵ viral particles per milliliter (vp/mL) were not used in further calculations (Figure 3). Sample results were corrected by subtracting blank results from sample results. Corrected results were log-transformed and graphed as a function of the log10 (dilution factor). The slope and R2 determined (Figure 4). The optimal dilution factor for titer analysis was determined for each AAV sample based on the linear range (Figures 5 - 7). Replicates (n = 6) were prepared for each AAV sample at the optimal dilution and quantified on the Virus Counter® 3100. Blankcorrected results were multiplied by the dilution factor to determine the titer for each sample. Individual values were averaged to determine final titer and 95% confidence intervals for each sample (Figure 8).



Mix Reagent Figure 1: Staining Diagram

Table 1: Virus Counter[®] 3100 AAV Quantification Compared to Alternate Methods

Strain	Virus Counter [®] (vp/ml)
AAV2, Strain H	1.16 × 10 ⁹ 95% C.I. 1.13 × 10 ⁹ – 1.1 ⁹ × 10 ⁹
AAV2, Reference Standard Stock	5.61×10 ⁹ 95% C.I. 5.31×10 ⁹ – 5.92×10 ⁹
AAV3	4.3 ⁹ ×10 ⁹ 95% C.I. 4.23×10 ⁹ – .55×10 ⁹

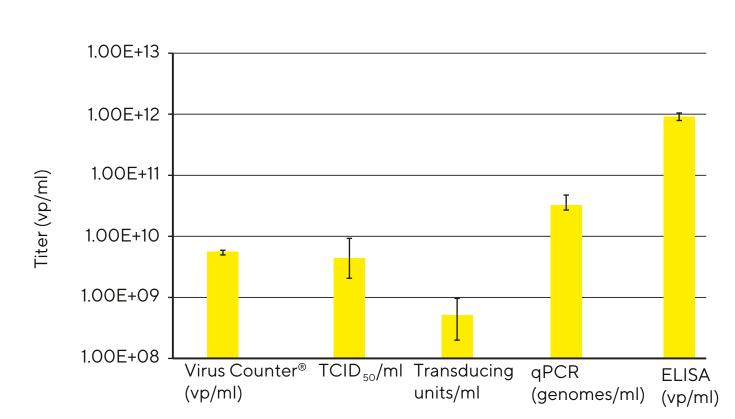


Figure 2: Reference Stock Titer Comparison

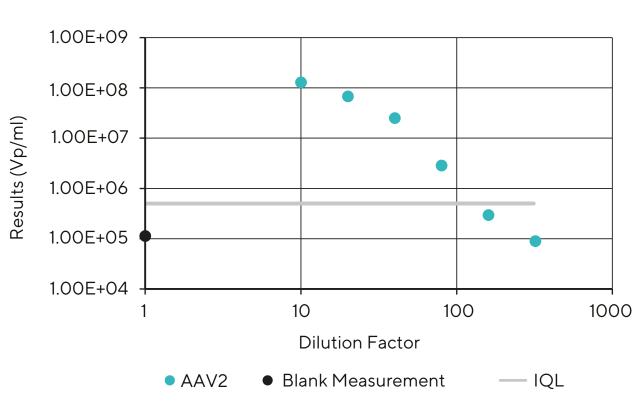


Figure 3: AAV2 Dilution Series

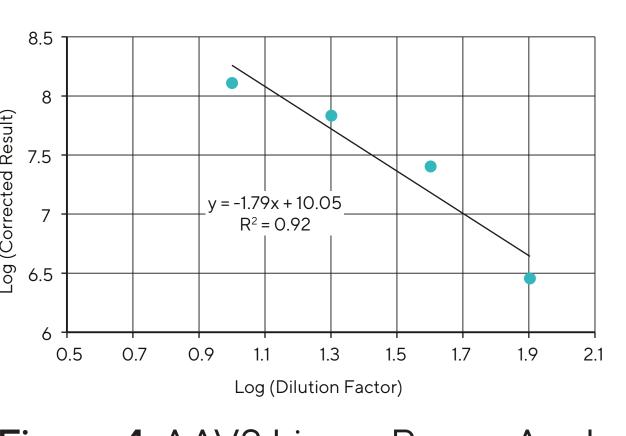


Figure 4: AAV2 Linear Range Analysis

With Virus Sample



30 min



Analyze

TCID ₅₀ /ml	Transducing Units/ml	qPCR (genomes/ml)	ELISA (p
1.10 × 10 ⁹			
4.37×10 ⁹ 95% C.I. 2.06×10 ⁹ – .26×10 ⁹	5.09×10 ⁸ 95% C.I. 2.00×10 ⁸ – .60×10 ⁸	3.28 × 10 ¹⁰ 95% C.I. 2.70 × 10 ¹⁰ – .75 × 10 ¹⁰	9.18 × 10 ¹¹ 7.89 × 10 ¹⁷
1.58×10 ⁴			

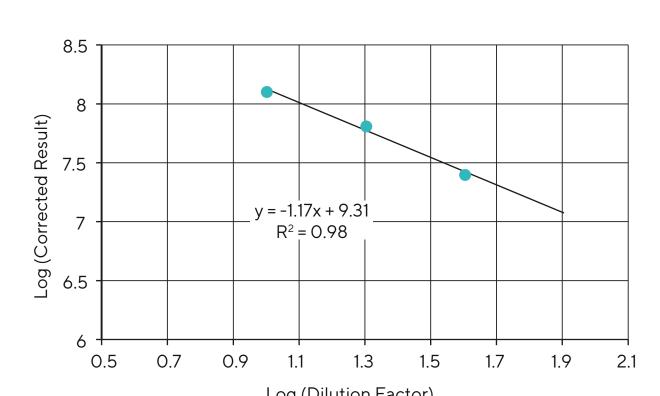


Figure 5: AAV2 Optimized Linear Range

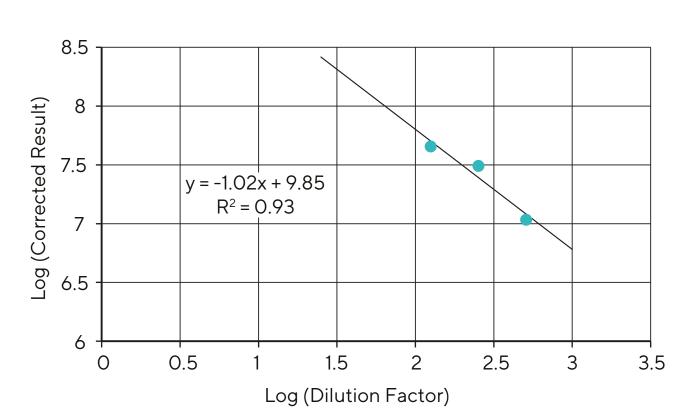


Figure 6: AAV2 Reference Standard Optimized Linear Range

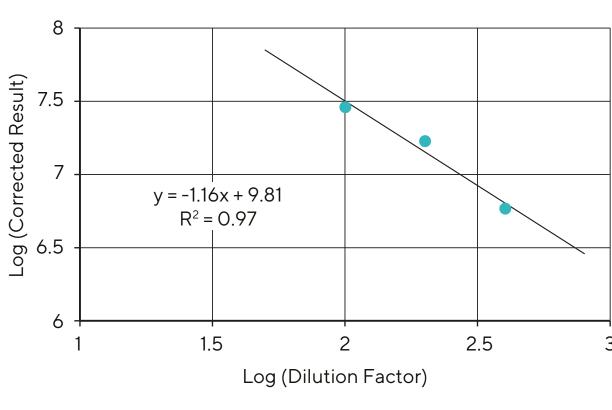


Figure 7: AAV3 Optimized Linear Range

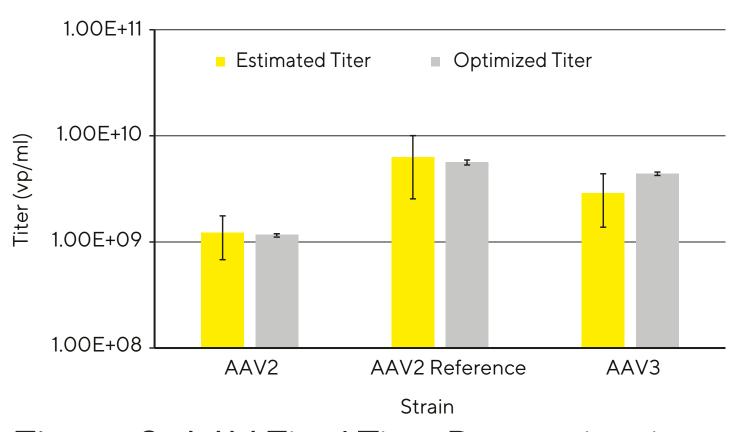


Figure 8: AAV Final Titer Determination

Conclusion

- Differences in Virus Counter[®] 3100, TCID₅₀, transducing units/mL, qPCR, and ELISA demonstrate the need for well-qualified standards for reference and highlight the importance of biologically-relevant and specific measurements of particle count. Alternative methods such as ELISA and gPCR have become more commonplace but may be biological limited as they tend to over-estimate particle count when compared to methods that count particles directly or those directly tied to the biological activity such as $TCID_{50}$ and measures of transducing units (Table 1; Figure 2).
- The Virotag[®] AAV2 3 reagent, based upon a fluorescently-labeled monoclonal antibody, allows for direct enumeration of free viral particles in solution, providing a biologically-specific platform of AAV vector quantification.
- Combined with the Virus Counter[®] 3100, the AAV2-3 reagent provides a direct, rapid, real-time method for the quantification of unassociated AAV2 and AAV3 particles in solution, allowing for improved process monitoring and optimization.

References:

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

Simplifying Progress

Discussion

(particles/ml)

¹¹ | 95% C.I. $1^{11} - .05 \times 10^{12}$

AAV particle quantification has traditionally relied upon time- and labor-intensive methods such as electron microscopy, TCID₅₀, or transducing units per milliliter analysis. These methods require extensive training, resources, and most critically, valuable time in the production process. Alternative methods such as ELISA and qPCR have become more commonplace but may be biological limited as they tend to over-estimate particle count when compared to methods that count particles directly or those directly tied to the biological activity such as $TCID_{50}$ and measures of transducing units (Table 1; Figure 2).

- The rapid particle quantification of the Virus Counter[®] 3100 coupled with the biological relevance and specificity of the AAV2 – 3 reagent allows for accurate, real-time process optimization and control, rather than the multiple day waiting period associated with TCID₅₀ and transducing units/mL analysis.
- The AAV3 results highlight the importance of total particle quantification in relation to $TCID_{50}$; products that only take TCID₅₀ into account in final product formulations may be inadvertently including an excess (here, >100,000 – fold higher) of non-transducing particles. This may lead to adverse patient reactions due to high viral antigen load in compared to a more modest amount of the target therapeutic gene.
- The significant (1,000 fold) difference between the different methods of measurement of the AAV2 Reference Standard Stock (Table 1) highlights the importance of direct enumeration of free, unassociated viral particles. These results show that qPCR and ELISA overestimate viral particle count when compared to biologicallyspecific measurements such as the Virus
- Counter[®] 3100, TCID₅₀ and transducing units/mL. ELISA and qPCR are not the most biologically relevant metrics for understanding total AAV particle count in a sample. For example, incorrectly assembled viral capsids and capsid fragments likely contribute to the differences in TCID₅₀ and Virus Counter[®] 3100 results compared to ELISA. Optimization of production processes for high ELISA titers may result in a large amount of AAV antigen that is incorrectly assembled and unable to deliver therapeutic genes to target cells. This may not be ideal for process optimization.

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