Enhancing rAAV biomanufacturing: Tackling critical challenges leveraging dPCR solutions

Viki Mahendran[1], Wong Kai Zhi[1], Micheal Mateling[2], Siah An Qi[1], Sumathy Velumani[1], Yifang Lee[1], Despina Lymperopoulou[2]

1 Thermo Fisher Scientific • 35 Marsiling Industrial Estate Rd 3, #01-05 • Singapore
2 Thermo Fisher Scientific • 6055 Sunol Boulevard • Pleasanton, CA

Abstract

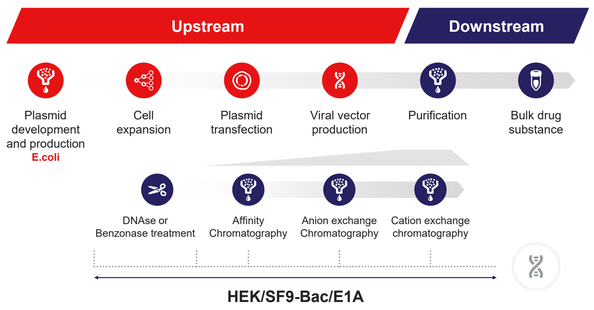
Purpose: Recombinant adeno-associated virus (AAV) vectors have emerged as promising tools for gene therapy and vaccine manufacturing. Ensuring the quality, safety, and efficacy of rAAV products is crucial, requiring rigorous testing and validation throughout the production workflow. Current regulatory guidelines for residual host cell DNA require ≤10ng per dose, with a DNA size of 200bp or lower. In viral vector production, the amount, size, and oncogenic sequences of encapsidated DNA is of additional concern. To address this need, the newly developed dPCR assays kits are designed to meet regulatory guidance. These assays detect residual contaminants such as (1) *E.coli* from plasmid production, (2) residual DNA from host cells such as HEK293, E1A and SF9/Bac. Our results demonstrate the effectiveness of dPCR assays in evaluating the quality of the vectors, providing valuable insights to help ensure product and patient safety.

Method: To assess performance, genomic DNA were diluted in a series and evaluated across a wide dynamic range for all assays. The limit of detection for each assay was determined by diluting DNA to the lowest measurable analyte, and its robustness was tested to achieve a 95% detection rate. The DNA samples were then added to a prepared Applied Biosystems™ Absolute Q™ reaction mix, loaded into MAP16 plates, and analyzed using the Applied Biosystems™ QuantStudio™ Absolute Q digital PCR (dPCR) System.

Results: Residual assays have a broad dynamic range with limit of detection (LOD) as follows: HEK293 (0.7pg/rxn), SF9-Bac (1.5fg-24fg/rxn), *E.coli* (0.08pg/rxn), E1A fragment (9 cp/rxn). The Applied Biosystems™ ViralSEQ™ dPCR AAV Titer Kit features an expansive dynamic range for accurate, extraction-free quantitation from 9 to 90,000 copies per reaction (cp/rxn). It simplifies workflows by eliminating extraction steps while maintaining quantitation accuracy between 70-130%. These assays can be utilized throughout the entire rAAV biomanufacturing process, both during in-process stages for optimization and purification, as well as for the final lot release.

Figure 1: resDNASEQ and AAV ViralSEQ Assays testing points for in-process and final lot release

Adeno-associated virus workflow



Introduction

Gene therapy and vaccine manufacturing have witnessed significant advancements with the emergence of recombinant adeno-associated virus (AAV) vectors. These vectors hold immense promise in delivering therapeutic genes and antigens to target cells, thereby revolutionizing the field of genetic medicine. However, ensuring the quality, safety, and efficacy of AAV vectors remains a critical challenge that demands robust analytical techniques.

In recent years, digital PCR (dPCR) has emerged as a powerful tool for precise and sensitive nucleic acid quantification. Its ability to partition samples into thousands of individual reactions enables absolute quantification of target sequences, overcoming the limitations of traditional quantitative PCR (qPCR) methods. Leveraging the unique advantages of dPCR, researchers have developed innovative assays to comprehensively analyze AAV vectors and assess their quality attributes.

This scientific poster aims to showcase the application of a dPCR-based assay in the detailed analysis of AAV vectors. By employing this advanced technique, we can gain valuable insights into critical parameters such as vector genome titer, integrity, and purity, which are vital for ensuring the safety and efficacy of AAV-based therapeutics.

The dPCR-based assay utilized in this study offers several advantages over conventional methods. Firstly, it allows for accurate quantification of AAV vector genomes, enabling precise determination of vector concentrations and facilitating downstream applications. Additionally, the assay provides insights into the size distribution of encapsidated DNA, helping to ensure compliance with regulatory guidelines that mandate specific size thresholds for residual host cell DNA.

The versatility of dPCR is further demonstrated by its ability to detect and quantify potential contaminants, such as residual DNA from host cells, viral impurities, and adventitious agents. By implementing this assay, we can detect the presence of these contaminants, thus helping to ensure the safety and purity of AAV vectors throughout the manufacturing process.

Furthermore, the dPCR-based assay offers enhanced sensitivity, facilitating the detection of low-level contaminants that may have been missed by traditional methods. This increased sensitivity is particularly crucial in the context of gene therapy and vaccine manufacturing, where even minor impurities or variations in vector quality can have significant implications for downstream patient safety and product efficacy.

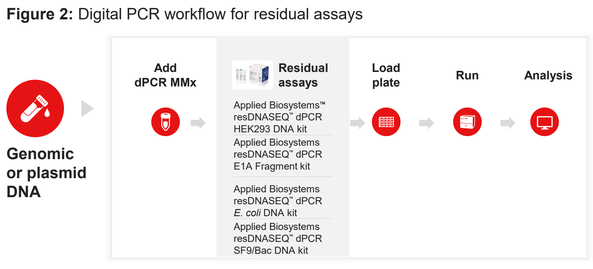
In conclusion, the utilization of a dPCR-based assay represents a significant advancement in the analysis of AAV vectors. By leveraging the power of digital PCR, we can achieve accurate quantification, assess vector integrity, monitor contaminants, and ultimately help ensure the quality and safety of AAV-based therapeutics. This scientific poster will provide a comprehensive overview of the assay's methodology, results, and potential applications.

Methods and materials

Performance was validated across multiple conditions:

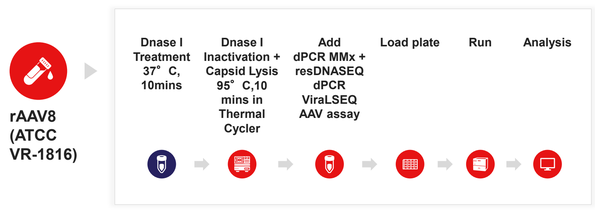
- 2 Manufactured lots
- 3-7 Days x 3 Operators x 4 Instruments
- Applied Biosystems™ QuantStudio Absolute Q™ digital PCR (dPCR) System, Software Version 6.3
- All measurements were run in triplicate

Figure 2: Digital PCR workflow for residual assays



Materials and methods (continued)

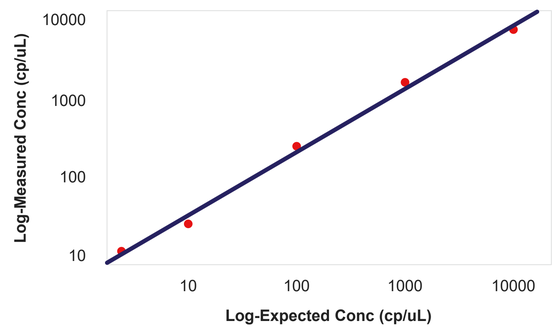
Figure 3: AAV treatment and extraction-free viral titer quantification



Results (*E.coli*)

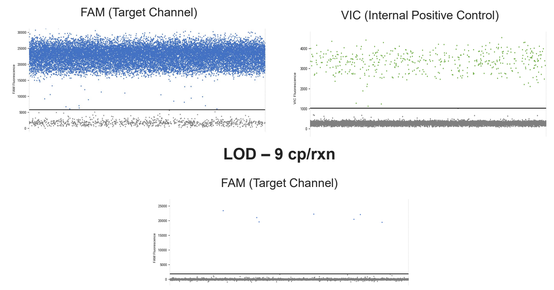
Figure 4: Assay Performance for resDNASEQ dPCR *E. coli* DNA Kit

a The standard curve for *E. coli* demonstrates a strong linear relationship between expected and measured concentrations, indicating assay accuracy across a broad dynamic range.



b The 1D plots show the distribution of fluorescence at high concentration - SC1 – 90,000 cp/rxn for FAM and VIC fluorescence plots and at the limit of detection (LOD – 9 cp/rxn). The accurate quantification at high concentration and clear detection at LOD indicates robust assay performance.

SC1—90,000 cp/rxn



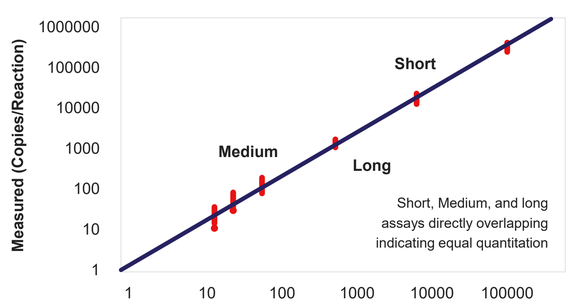
c The table summarizes LOD data for both *E. coli* assays. The mean concentration at LOD is close to the expected value of 1 cp/µL (9 cp/rxn), and the detection rate is 100%, demonstrating the high sensitivity.

LOBC/NTC	Mean Conc. of LOD (cp/uL) Expected – 1 cp/uL or 9cp/rxn	Total LOD data points	Number of LOD datapoints above LOB	Detection rate (%)
Mean				
0.01	0.88	32	32	100%

Results (E1A)

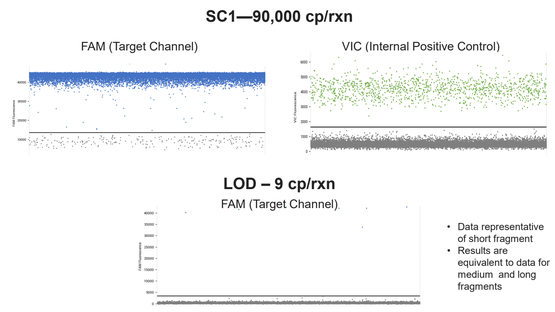
Figure 5: Assay Performance for resDNASEQ dPCR E1A DNA Kit

a The E1A standard curve for different fragment sizes demonstrates a strong linear relationship between expected and measured concentrations, the longest amplicon fragment successfully amplified is 500bp. Overlapping fragments indicate equivalent performance all fragment sizes.



b The 1D plots show the distribution of fluorescence at high concentration for short fragment - SC1 – 90,000 cp/rxn for FAM and VIC fluorescence plots and at the limit of detection (LOD – 9 cp/rxn). The accurate quantification at high concentration and clear detection at LOD indicates robust assay performance.

SC1—90,000 cp/rxn



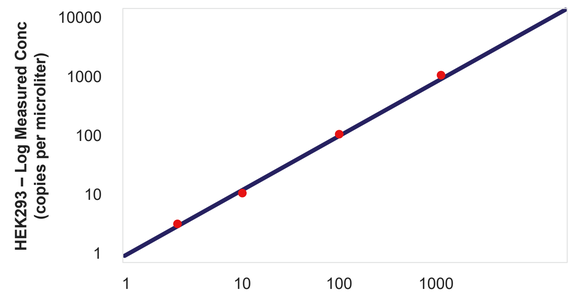
c The table summarizes LOD data for both SF9 and Bac assays. The mean concentration at LOD is close to the expected value of 1 cp/µL (9 cp/rxn), and the detection rate is 100%, demonstrating the high sensitivity.

LOBC/NTC	Mean Conc. of LOD (cp/uL) Expected – 1 cp/uL or 9cp/rxn	Total LOD data points	Number of LOD datapoints above LOB	Detection rate (%)
Mean				
0.01	1.06	56	56	100%

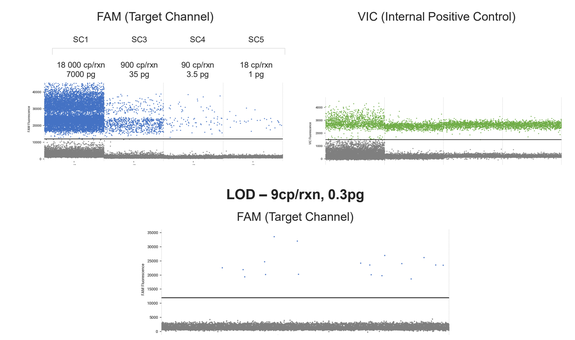
Results (HEK293)

Figure 6: Assay Performance for resDNASEQ dPCR HEK293 DNA Kit

a The standard curve for HEK293 demonstrates a strong linear relationship indicating assay accuracy across a broad dynamic range; from 7000pg to 1pg.



b The 1D plots show distribution of FAM and VIC fluorescence signals across broad range of concentration and there is clear demarcation between positive and negative microchambers. Clear detection is observed at LOD of 0.3 pg.



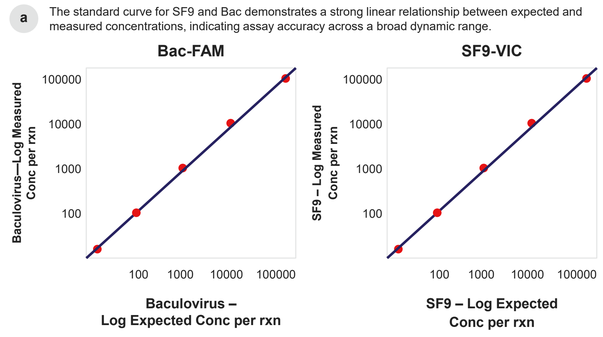
c The mean concentration at LOD is close to the expected value of 1 cp/µL (9 cp/rxn, 0.3pg), and the detection rate is 100%, demonstrating the high sensitivity

LOBC/NTC	Mean Conc. of LOD (cp/uL) Expected – 1 cp/uL or 9cp/rxn	Total LOD data points	Number of LOD datapoints above LOB	Detection rate (%)
Mean				
0.19	2	68	68	100%

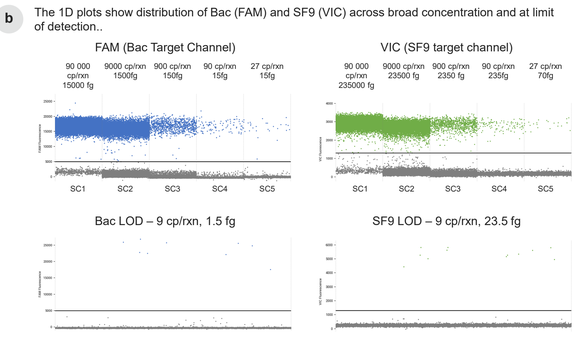
Results (SF9/Bac)

Figure 7: Assay Performance for resDNASEQ dPCR SF9-Bac DNA Kit

a The standard curve for SF9 and Bac demonstrates a strong linear relationship between expected and measured concentrations, indicating assay accuracy across a broad dynamic range.



b The 1D plots show distribution of Bac (FAM) and SF9 (VIC) across broad concentration and at limit of detection..



c The table summarizes LOD data for both SF9 and Bac assays. The mean concentration at LOD for both targets are close to the expected value of 1 cp/µL (9 cp/rxn), and the detection rate is 100%, demonstrating the high sensitivity.

Assay	LOB/NTC	Mean Conc. of LOD (cp/uL) Expected – 1 cp/uL or 9cp/rxn	Total LOD data points	Number of LOD datapoints above LOB	Detection rate (%)
SF9 (FAM)	0.02	1.06	32	32	100
BAC (VIC)	0	1.19	32	32	100

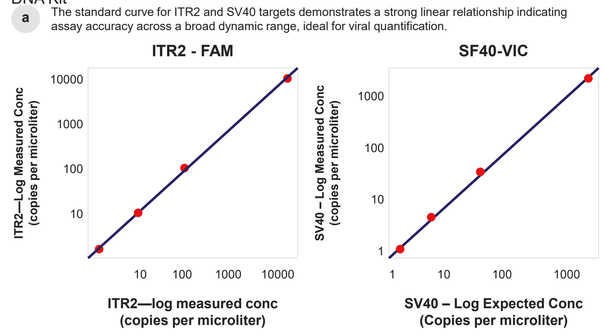
Results

- **resDNASEQ host cell DNA residual assays:** To test for accurate quantitation and broad dynamic range for residual assays, a standard curve was generated. All standard curves generated using serial dilutions to generate 5 standards were run in triplicate using dPCR and is shown in Figures 4a-7a.
- **All assays have a broad dynamic range:** Standard curve fit parameters demonstrated strong linearity with an $R^2 \geq 0.99$. The coefficient of variation (CV%) for the data points were less than 10%, and the relative accuracy ranged from 70-130% for all quantitation points up to the Limit of Quantitation. These results show that the assays deliver accurate quantification, eliminating the need for a standard curve. The broad dynamic range of the assay allows for reliable quantification of samples with high to low abundance analyte concentrations.
- **Limit of Detection:** All residual assays—E. coli, E1A, HEK293, and SF9/Bac—demonstrate high sensitivity, as illustrated in Figures 4c-7c. The assays exhibit a high signal-to-noise ratio between negative and positive microchambers, enabling precise threshold determination. The limit of detection (LOD) is consistently achieved at 95% across all assays, indicating their robustness.
- **HEK293 cell line:** Given the extensive use of the HEK293 cell line in cell and gene therapy, several key factors were incorporated into the assay design: (1) targeting high-copy genes, (2) selecting genes distributed across chromosomes, and (3) helping to ensure accurate quantitation with targets of minimal variability. Additionally, quantification of host cell genomic HEK293 can be paired with assays to identify various fragment sizes of the E1A oncogene. These strategic considerations help ensure reliable detection and quantification, which are crucial for maintaining the quality and efficacy of cell and gene therapy products.

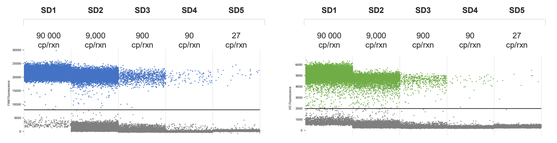
Results (AAV extraction- free viral quantitation)

Figure 8: Assay Performance for the Applied Biosystems™ ViralSEQ™ dPCR AAV DNA Kit

a The standard curve for ITR2 and SV40 targets demonstrates a strong linear relationship indicating assay accuracy across a broad dynamic range, ideal for viral quantification.



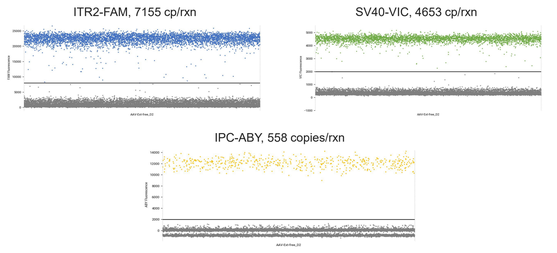
b The 1D plots show distribution of FAM and VIC fluorescence signals across broad range of concentration and there is clear demarcation between positive and negative microchambers.



c The mean concentration at LOD is close to the expected value of 1 cp/µL (9 cp/rxn) and the detection rate is 100%, demonstrating the high sensitivity.

Assay	LOB/NTC	Mean Conc. of LOD (cp/µL) Expected – 1 cp/µL or 9cp/rxn	Total LOD data points	Number of LOD datapoints above LOB	Detection rate (%)
ITR2	0.04	1.08	64	64	100
SV40	0.00	1.03	64	64	100

d rAAV8 (ATCC VR-1816) was quantified using direct digital PCR (extraction free method). AAV8 virus concentration was determined using ITR2 target. SV40 target together with ITR2 maybe used as an indicator of packaging efficiency.



For extraction-free samples,

- If ITR2 concentration from dilution 2 is 795 copies/µL, sample input is 3uL into 9uL reaction volume per well, Dnase dilution is 20, accumulated sample dilution is 10000, then;
- 795cp/uL x (9/3) x 20 x 10000 x 1000 = **4.77 x 10^{11} copies/mL**
- **100-fold dilutions were carried out twice from Dnase treated samples*
  ^ AAV8 virus concentration extracted using extraction kit was detected at 3.52 x 10^{11} copies/mL

Results

- **ViralSEQ dPCR AAV Titer assay:** A standard curve was generated to assess the accuracy of quantitation and the broad dynamic range of ITR2 and SV40 targets using a multiplex assay. Serial dilutions produced five standards, and all standard curves were run in triplicate using dPCR, as depicted in Figure 8 .
- **Multiplex assay with a broad dynamic range:** The standard curve fit parameters showed strong linearity with an $R^2 \geq 0.99$. The coefficient of variation (CV%) for the data points was less than 10%, and the relative accuracy ranged from 70% to 130% for all quantitation points up to the Limit of Quantitation. The broad dynamic range enables accurate quantitation of AAV8 particles, which is crucial for assessing both low and high viral loads at various stages of AAV manufacturing, quality control and therapeutic application. Additionally, the use of a multiplex assay allows for simultaneous detection and quantification of multiple targets, increasing throughput and efficiency.
- **Extraction free AAV8 quantification:** Two separate experiments were conducted to compare quantification methods for AAV8. In the first experiment, a direct approach was used where AAV8 was treated with DNase to remove free-floating DNA and then lysed using a thermal cycler. In the second, simultaneous experiment using the same viral stock, AAV8 particles were extracted with the Applied Biosystems™ PrepSEQ™ Residual DNA Sample Preparation Kit (data not shown). Results showed that direct dPCR detected higher levels of AAV8 compared to extracted samples, suggesting potential losses during the purification process (Figure 8). This indicates that direct dPCR may offer greater efficiency for AAV8 quantification when sample integrity is crucial. However, for matrices with components that could inhibit dPCR, sample extraction before analysis is recommended to ensure accuracy and reliability.

Conclusions

The optimization of the recombinant AAV (rAAV) manufacturing workflow and adherence to regulatory guidelines require the development and integration of residual and AAV assays. To support gene therapeutics, we have developed robust, highly sensitive, and reliable digital PCR assays for quantitation in HEK293 cell lines. These assays have successfully met the following important criteria:

- **Robustness:** The standard curves for all assays had a broad dynamic range and exhibited R-squared (R2) values greater than 0.99, indicating a strong correlation between the measured values and the expected values.
- **Sensitivity:** All residual assays demonstrated high sensitivity, with the limit of detection (LOD) detected at 95% without any dropouts. This facilitates reliable quantification of even trace amounts of residual contaminants.
- **AAV Multiplex Assays**: Our AAV multiplex assays have the capability to quantify virus concentrations across a wide range. The use of direct digital PCR in the AAV workflow has shown improved efficiency in virus quantification. This suggests that direct digital PCR is a more efficient method for accurately measuring virus concentrations.

Acknowledgements

The authors would like to thank Adeline Tee, Jonas Buege, Cuilan Chen, Samuel G. Booth and Bryce Coundley for their assistance in the project.

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