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INSIGHT BRIEF

Utilizing Multiplex ddPCR to Streamline Viral Gene Therapy Workflows

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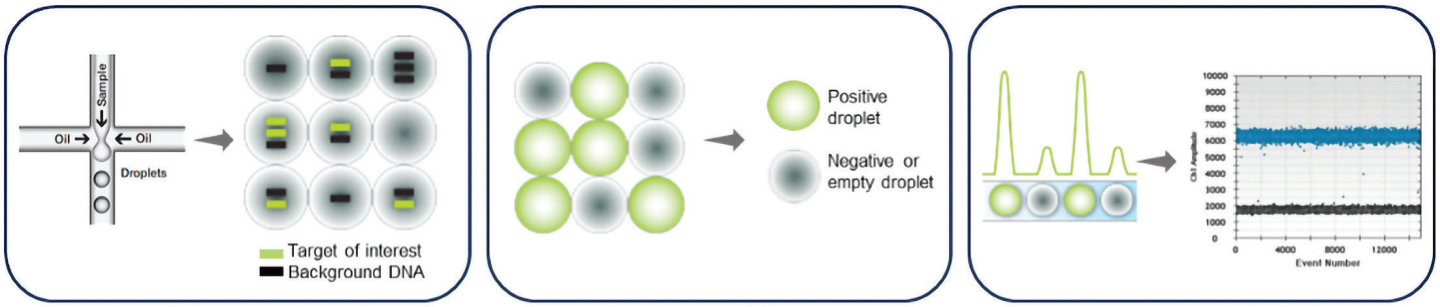
Analytical technologies play a pivotal role in the development and successful commercialization of viral gene therapies. Among these, quantitative PCR (qPCR) has long been regarded as the "gold standard" for analysis of key quality attributes for viral vector characterization and batch release. For recombinant adeno-associated vector (AAV), this includes viral vector genome (VG) titer, purity, potency, host and process-related impurities, viral capsid characterization (i.e., the ratio of empty to full capsids), and the identification of replication-competent viruses.

While qPCR is a powerful molecular tool, it is limited to relative quantification, and the precision and accuracy of the results depend on how effectively the standard curve has been optimized. In the viral gene therapy space, there is a growing need for DNA quantification techniques that can more precisely quantify viral genome titers, which is essential for dosage determination in toxicology assessments and clinical applications.[1] Novel analytical technologies like Droplet Digital PCR (ddPCR) provide a more rapid and robust approach to nucleic acid quantification for therapeutic development that better align with the industry need for better sensitivity, accuracy, and precision.

Benefits of ddPCR over qPCR

ddPCR is a relatively new form of PCR that offers distinct advantages for DNA and RNA quantification over standard qPCR methods. This technique (Figure 1) involves partitioning the sample into thousands of nanoliter-sized water-oil emulsion droplets that each serve as an individual PCR reaction for target sequence amplification. After endpoint amplification, ddPCR quantification involves the detection of positive and negative droplets using a specialized droplet reader. The fraction of positive droplets is analyzed using Poisson statistics to precisely determine the concentration of the target DNA template. In contrast to qPCR, ddPCR provides absolute quantitation without the need for standard curves and is less sensitive to PCR inhibitors and changes in amplification efficiencies. It offers enhanced sensitivity with improved signal-to-noise ratio for low-copy target detection and produces highly accurate and reproducible results with tight coefficients of variation. For example, in one direct comparison of qPCR and ddPCR technology, ddPCR was up to four times more sensitive than qPCR in the absolute quantification of single-stranded AAV vector genomes.[2]

Figure 1. Typical steps in a ddPCR workflow.



STEP 1: Sample is partitioned into thousands of droplets where the target and background DNA are distributed randomly.

STEP 2: End-point PCR amplification inside the droplets. Positive droplets contain at least one copy of the target and exhibit increased fluorescence over negative/empty droplets.

STEP 3: Fluorescence of individual droplets is measured. The number of positive droplets containing at least one copy of the target DNA gives precise quantification.

Multiplex ddPCR expands the capabilities of traditional ddPCR, allowing for simultaneous detection and quantification of multiple genomic targets in a single reaction. Each droplet contains a different combination of primers and probes specific to distinct target sequences. The reduced sample and reagent volumes also contribute to cost savings and help to conserve valuable sample material in an area of the industry that typically has low productivity and low final product volumes. While ddPCR has a more limited dynamic range than qPCR, the ability to test more samples, extract richer information, and obtain better data quality from a single experiment make it an attractive alternative for viral gene therapy workflows.

Key Considerations for Effective Implementation of ddPCR

The initial setup for a ddPCR system can involve higher upfront investment in terms of equipment, reagents, and assay development which may be a barrier for adoption for companies interested in the technology. Many factors, such as amplicon selection, primer and probe design, and sample preparation can influence the success of a ddPCR reaction.[3] Therefore, thoughtful experimental design with appropriate control strategies, optimization of primer probe sets, and a deep understanding of ddPCR's limitations are essential to getting accurate and precise results. Given the heightened sensitivity of ddPCR, careful attention must be paid to reduce the incidence of cross-contamination. Finally, after ddPCR data has been collected, it is important to have the appropriate tools and training to properly analyze the data to ensure accuracy, particularly in multiplex experiments with multiple target signals that can add complexity to analysis strategies.

While adopting ddPCR for viral gene therapies may involve initial challenges, the benefits it offers in terms of precision, sensitivity, and accuracy can contribute to better data for making well-informed decisions that accelerate the development and approval of safe and effective gene therapy treatments. Partnering with a contract development and manufacturing organization (CDMO) can offset these challenges, offering strategic benefits for implementing ddPCR technology — access to expert knowledge and advanced equipment, faster implementation, cost efficiency, flexibility, and risk mitigation — that all contribute to reducing time to market. By outsourcing specialized activities like ddPCR, companies can channel internal resources toward advancing their own development goals.

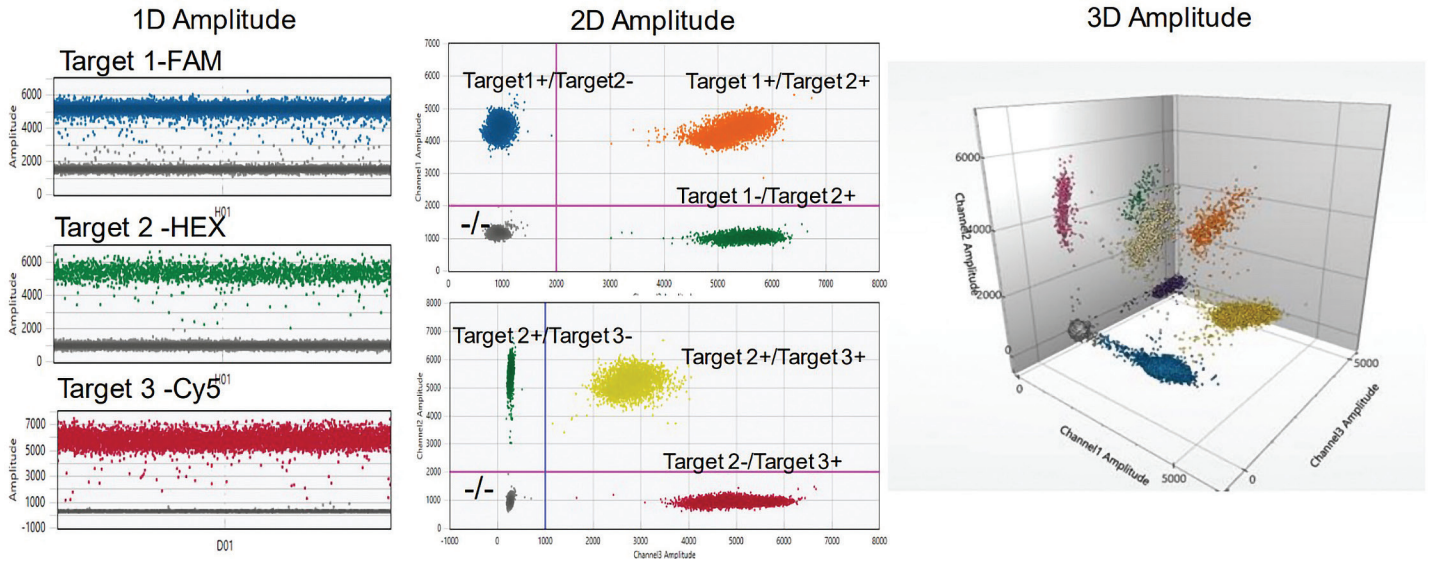
Leveraging CDMO Expertise for ddPCR

FUJIFILM Biotechnologies has established itself as **a key CDMO partner in the field of viral vector manufacturing** and analytics for viral gene therapy programs and strives to stay at the forefront of new analytical technologies that can enhance our analytical capabilities, improve product characterization, and provide clients with more accurate, precise, and actionable data.

Within our analytical laboratories, we have validated the Bio-Rad QX ONE ddPCR System for use in our programs. This innovative platform integrates droplet generation, thermal cycling, droplet reading, and analysis to streamline and automate the ddPCR workflow. Our team has developed and optimized methods for in-process characterization and product release using this platform for vector genome titer assays for AAV vectors, product (i.e., Helper and RepCap plasmids) and host cell-related (i.e., E1A gene, Large T antigen) impurities analyses and the detection of replication-competent vectors. This includes primer/probe design, annealing temperature, limits of detection (LOD) and limits of quantification (LOQ) and other factors such as the resolution of the digital assay (clear separation between positive and negative droplets), threshold determination, and reduction of rain during analysis to ensure data accuracy.

The expanded multiplex capabilities and higher throughput capacity of the instrument over traditional ddPCR platforms allows us to simultaneously analyze multiple targets within a single reaction, while also providing higher throughput to process a larger number of samples within a shorter timeframe. Our optimized protocols, coupled with the multiplex capabilities, enable us to efficiently detect both high titer and low residual targets simultaneously. Utilizing all four fluorophore channels, we have the capability to detect as many as four distinct targets within the AAV vector genome. Alternatively, with a lo/hi amplitude multiplex strategy, we can extend this detection to encompass up to eight unique targets. Taken together, these capabilities translate into streamlined process development and laboratory process characterization. Figure 2 shows an example of the 1D, 2D, and 3D amplitude views from a multiplex experiment looking at three target sequences. The 2D and 3D views provide more detailed and nuanced information about complex molecular interactions between different targets compared to traditional qPCR data.

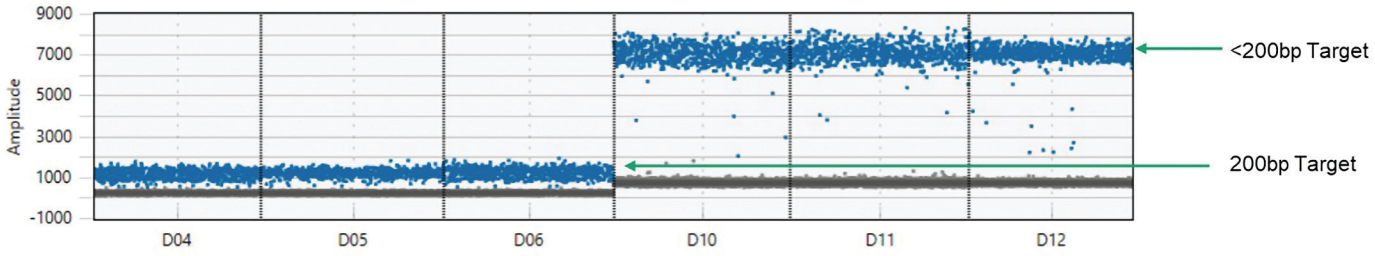
Figure 2. Example of data in 1D, 2D, and 3D views on QX ONE Instrument with three targets.



While AAV production processes are carefully designed to minimize host- and process-related impurities, there remains a possibility that they could be encapsulated in the AAV particles. The predominant cell line used to manufacture AAV products are HEK293 cells, known to carry the oncogene E1A, which has the potential to trigger tumorigenic events in recipients if it is present. Therefore, utilizing analytical technologies like dPPCR for E1A detection is an important safety measure to monitor and control the quantity of residual host DNA present in in-process and final AAV products. Figure 3a shows an example of amplitude multiplexing to detect separate E1A target fragments. Using primers designed to detect two separate E1A targets — one that is <200bp and second target that is ≥200 bp — we can effectively differentiate between the two based on their difference in amplitude using a single fluorescence probe/channel (FAM). In comparison to a commercial E1A qPCR kit, the copy numbers generated using FUJIFILM Biotechnologies' E1A method show good data correlation (Figure 3b).

Figure 3. a) E1A detection using amplitude multiplexing for two separate E1A targets (<200bp and ≥200 bp) in the FAM channel. B) ddPCR data generated using FUJIFILM Biotechnologies methods compared to a commercial E1A qPCR kit shows good correlation.

a)



b)

Sample	qPCR Result (copies/µL)	ddPCR200bp Target (copies/µL)	% Difference ddPCR vs. qPCR (200bp target)	ddPCR <200bp Target (copies/µL)	% Difference ddPCR vs. qPCR (<200bp target)
E1A DNA Control	3333.3	2592.6	–25.0%	2723.3	–20.6%
	333.3	261.9	–24.0%	271.9	–20.6%
	10.0	7.96	–22.7%	8.44	–17.4%
	3.3	2.44	–30.9%	2.29	–36.0%
AAVSample	15562.0	22082.0	34.6%	24627.0	45.1%

At FUJIFILM Biotechnologies, we take a forward-thinking approach to analytical development that goes beyond meeting existing viral vector characterization requirements. It is important to us as an organization to stay at the leading edge of analytical technologies to anticipate the potential future characterization demands and proactively explore innovative methods, such as linkage analysis to assess reverse packaging for AAV using ddPCR. This ensures that we not only keep pace with the evolving landscape of advanced therapies but help to shape its trajectory. With combined experience spanning more than 100 gene therapy projects, our dedicated Analytical and Quality Control teams have the expertise and leading-edge technologies to help you navigate the complex regulatory landscape of viral gene therapies.

Learn more about our capabilities here: **https://fujifilmbiotechnologies.fujifilm.com/capabilities/advanced-therapies/**

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About the Author

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Michael is responsible for overseeing FUJIFILM Biotechnologies' Viral Gene Therapy operations in the UK. Prior to this he spent 4 years at Fujifilm's College Station location in Texas USA where he was Head of Process Development, and was responsible for leading a rapidly expanding process development team. He has previously served in a number of technical roles at FUJIFILM Biotechnologies' sites in Texas and the UK and before joining Fujifilm, worked for MSD Biologics and Avecia Biotechnology. Michael gained a BSc in Medical Microbiology and Immunology and an MPhil in Developmental Biology from Newcastle University.

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