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Whitepaper

Quantification of encapsidated DNA using multiplex ddPCR

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Executive summary

The evolution and growth of the cell and gene therapy (CGT) market has seen a surge due to advances in science and technology, particularly in targeting cancer and genetic diseases. The market has a promising pipeline, with the FDA anticipating the approval of 10 to 20 CGT products annually by 2025. A cornerstone of these innovations is the production of viral vectors.

Viral vectors, especially the adeno-associated virus (AAV), are central to gene therapy. Their quantification is crucial for dosing, necessitating accurate methods like quantitative polymerase chain reaction (qPCR) and its advanced counterpart, droplet digital polymerase chain reaction (ddPCR). With its precise and absolute DNA quantification, ddPCR offers a significant leap from the relative quantification provided by qPCR. Furthermore, while qPCR has a broader dynamic range, ddPCR's efficiency and accuracy can be maintained through strategic sample dilutions.

Additional efficiencies are gained through the integration of automated technologies that optimize liquid handling. This is done by combining the throughput of robotic automation with multiplex reaction capabilities. Automated liquid handling tools significantly reduce manual errors and inefficiencies and notably quicken data production by ensuring data integrity, sample handling, and high-quality data acquisition.

This whitepaper describes the pivotal role of advanced methods and technologies in the evolution of viral vector services, focusing specifically on the following:

- The importance of ddPCR in quantifying AAV vector genome titer
- Primer and probe reaction customization for multiplex ddPCR
- The benefits of multiplex ddPCR in viral vector process development
- The value of integrating automated liquid handling

Achieving reliable quantification

Adeno-associated virus (AAV) is among the most widely used viral vectors in gene therapy. Accurately quantifying the AAV vector genome titer is vital because it determines patient dosage. Hence, a method that precisely determines the titer is essential.

The preferred method has traditionally been quantitative polymerase chain reaction (qPCR). However, the field is shifting toward droplet digital polymerase chain reaction (ddPCR). While qPCR offers relative DNA quantification based on a standard curve, ddPCR operates independently of a standard curve and delivers absolute DNA quantification.

Similar to qPCR, the ddPCR workflow involves endonuclease and proteinase K digestion of the sample. This is followed by dilutions and preparation of the sample for the PCR reaction. The plate with the diluted sample is then moved to the droplet generator. After undergoing standard PCR, the resultant fluorescence in the reaction is analyzed by the droplet reader.

The number of positive droplets is analyzed using Poisson statistics to determine the number of target DNA copies in the original sample. For each ddPCR reaction, specific parameters—such as annealing temperature, optimal primer, and probe concentration—need to be optimized at the ideal template concentration.

Reliable quantification hinges on three crucial criteria:

- A single amplification product, evidenced by two distinct populations of positive and negative droplets
- Peak resolution, which ensures a clear measure of the separation between positive and negative droplets
- Minimal 'rain' or reduced intermediate fluorescence seen between positive and negative droplets

A primary parameter to determine is the optimal concentration of the DNA template in the reaction. This parameter is vital as it dictates the optimal ratio of positive to negative droplets to fine-tune the reaction.

To ascertain the template's optimal concentration, serial dilutions are made, followed by the PCR reaction.

For each concentration, Lambda (λ) is computed using the logarithm of the quotient of negative droplets and the total droplet count.

Recent studies suggest that an optimal concentration corresponds to a λ value of 0.7, where about half of the total droplets are positive.[1]

Figure 1 depicts both positive and negative droplets: blue dots represent positive droplets, while gray and black dots stand for negative droplets. The presence of both droplet populations is essential to optimize the reaction.

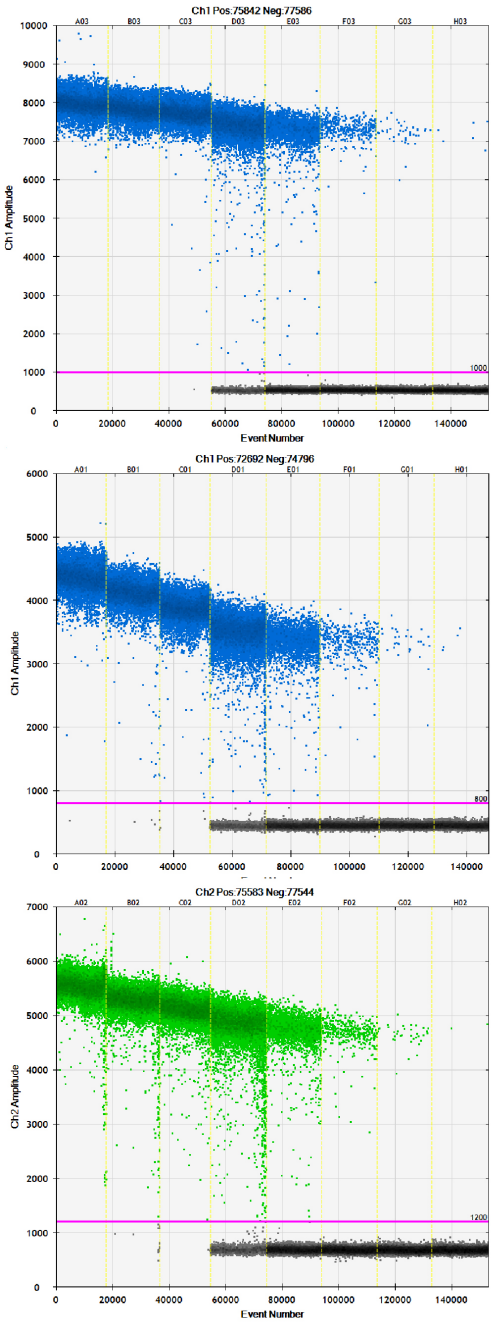


Figure 1: Optimal Concentration for three different targets (2 on channel 1 (Blue) and one on channel 2 (green)

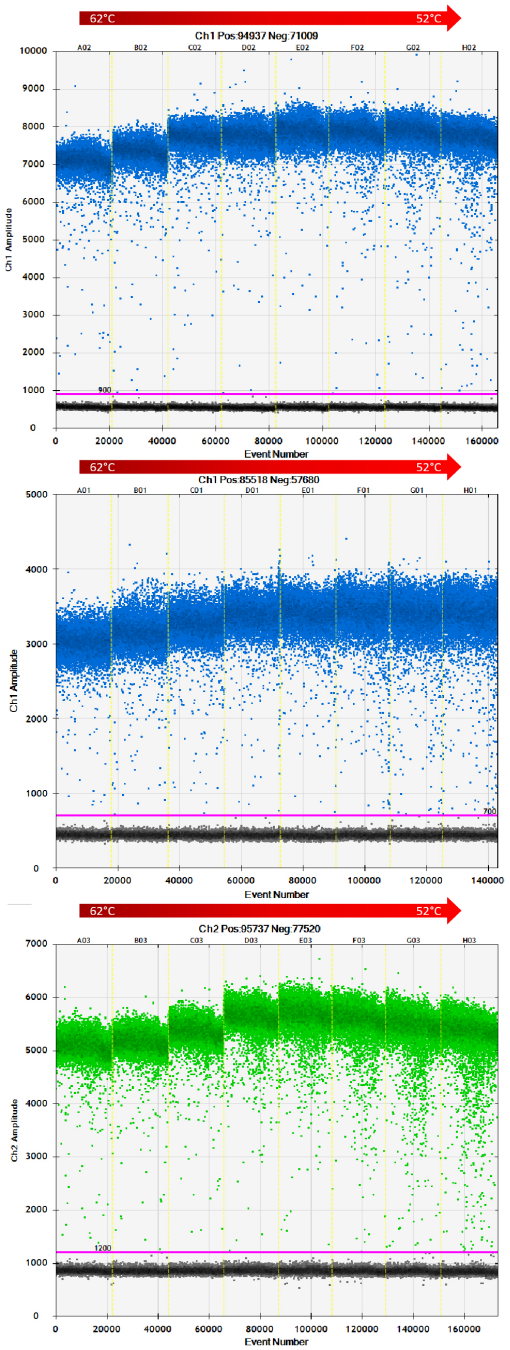


Figure 2: Temperature Gradient for three different targets (2 on channel 1 (Blue) and one on channel 2 (green)

Primer and probe reaction customization

After determining the optimal concentration, the next critical parameter to examine is the annealing temperature, pivotal in distinguishing between positive and negative populations. Different annealing temperatures undergo are evaluated during the PCR reaction. The separation between positive and negative droplets is computed by subtracting the positive droplet's amplitude from the negative droplet's amplitude, then dividing by the amplitude of the negative droplets. The objective is to achieve a value exceeding 1.5. The explored temperature gradients range between 52 and 62 degrees, as illustrated in Figure 2. Given the thermodynamic properties of primers and probes, certain annealing temperatures might prove more effective than others.

For the development of multiplex assays, a good understanding of the primer-probe concentration and annealing temperature is needed. As these parameters undergo optimization, observing peak resolution becomes crucial. Figure 3 provides instances of peak resolution and the percentage of rain based on the conditions applied in the PCR reaction. The left histogram reveals a broad peak, characterized by low resolution and substantial rain, resulting from the use of a non-optimal primer-probe set.

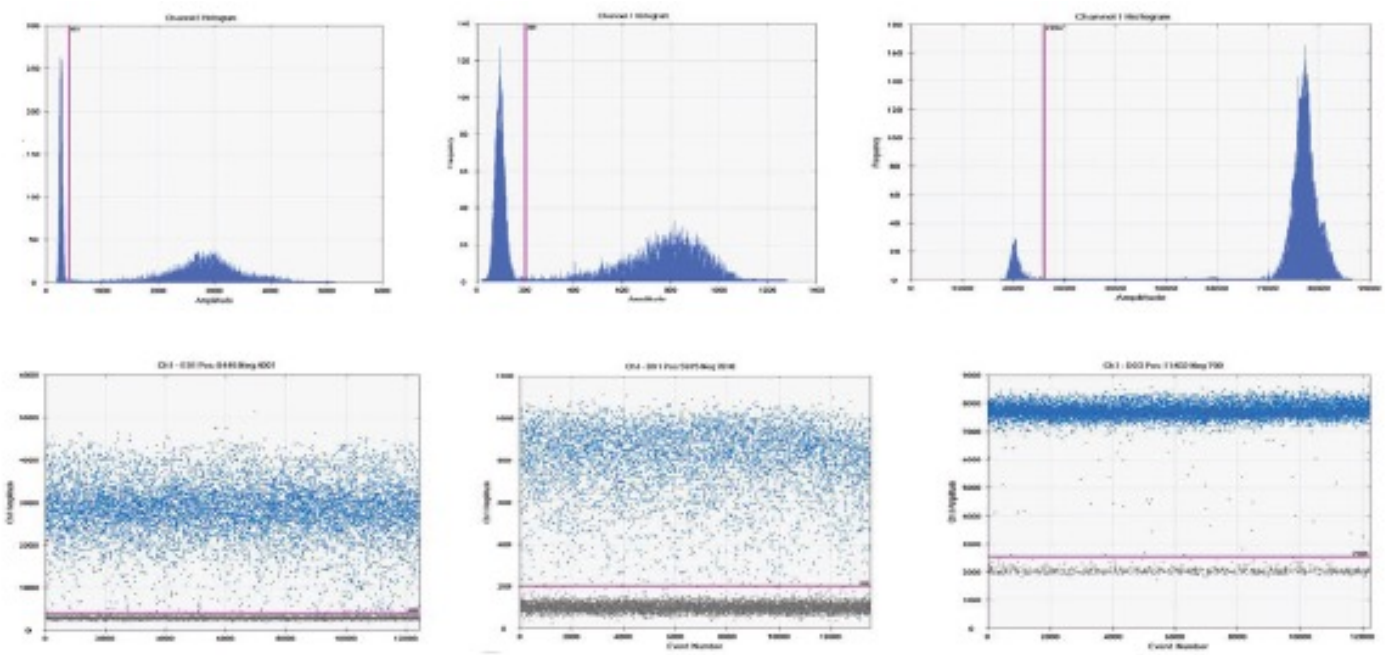


Figure 3: Peak resolution and percent rain

After optimizing the PCR conditions, peak resolution and rain improved (evident in the middle histogram of Figure 3). However, they didn't match the rightmost histogram, which embodies optimal conditions paired with an appropriate primer and probe set. It is worth noting that assays will present higher variability when accompanied by reduced peak resolution and elevated rain percentages.

It is important to ensure that each reaction has good peak resolution, low rain, and a single amplification product before attempting to go into multiplex reactions.

Multiplex ddPCR

Multiplex ddPCR allows for the quantification of multiple targets within a single reaction. A triplex ddPCR was developed using three distinct primer/probe sets to target three sequences frequently encapsidated in rAAV: the actual gene of interest, residual transfection plasmid, and residual host cell DNA.

Figure 4 illustrates that channel 1 (blue) detects two different targets, while channel 2 (green) identifies one. Whenever a droplet encompasses more than one target, its amplitude increases. This approach was also used to quantify different alleles of the Kanamycin resistance gene present in the plasmid backbone used during rAAV transfection. When taking multiple measurements from a single reaction, operational efficiency is greatly improved, resulting in improved turnaround times to support the development of viral vectors. A multiplex assay was also used to target the vector genome titer with encapsidated baculovirus DNA to support the development of viral vectors. This was achieved by designing primer/probe sets targeting both the gene of interest and the baculovirus DNA and combining them into one multiplex reaction.

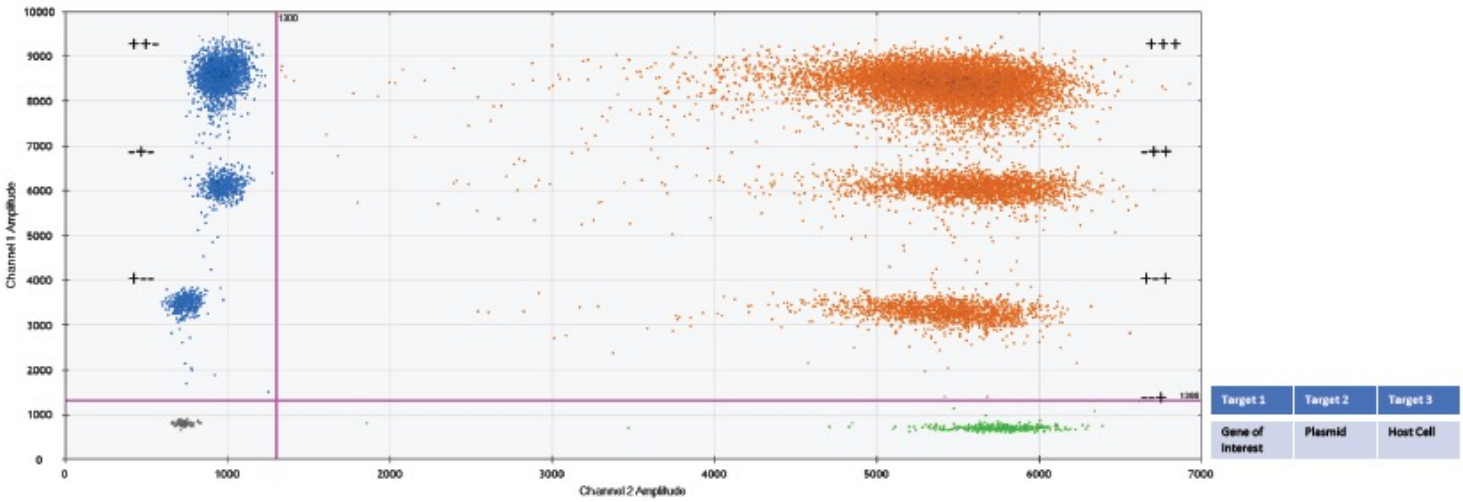


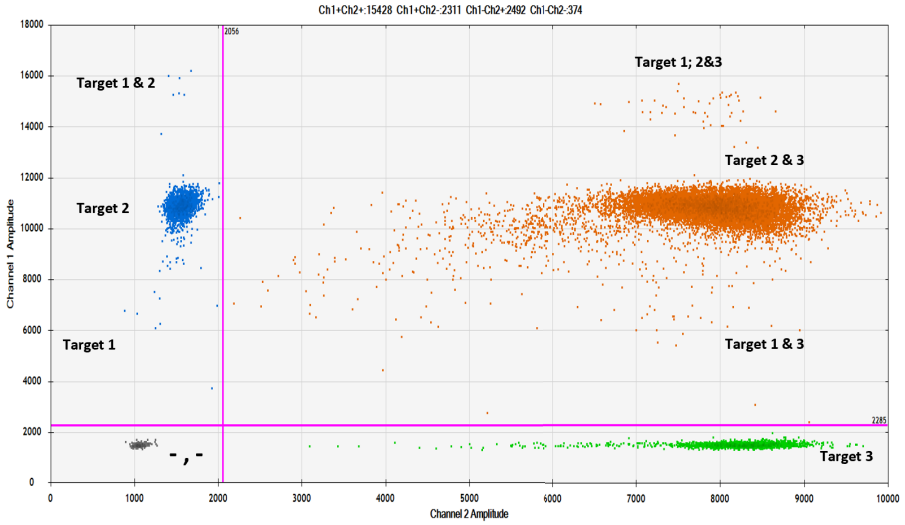
Figure 4: Triplex ddPCR reaction setup to differentiate encapsidated DNA

Finally, the limit of quantification is determined using different dilutions of the sample in several replicates to determine the accuracy and precision across the range of copy numbers. Even though it is possible to calculate a copy number down to 0.8, the precision and the accuracy decreases, which is why the limit of quantification of the reaction is set higher to ensure good repeatability. Similarly for multiplex reactions, it is important to confirm the limit of quantification of the assay for each target independently in the presence of the other targets saturated to ensure the measurements are reliable.

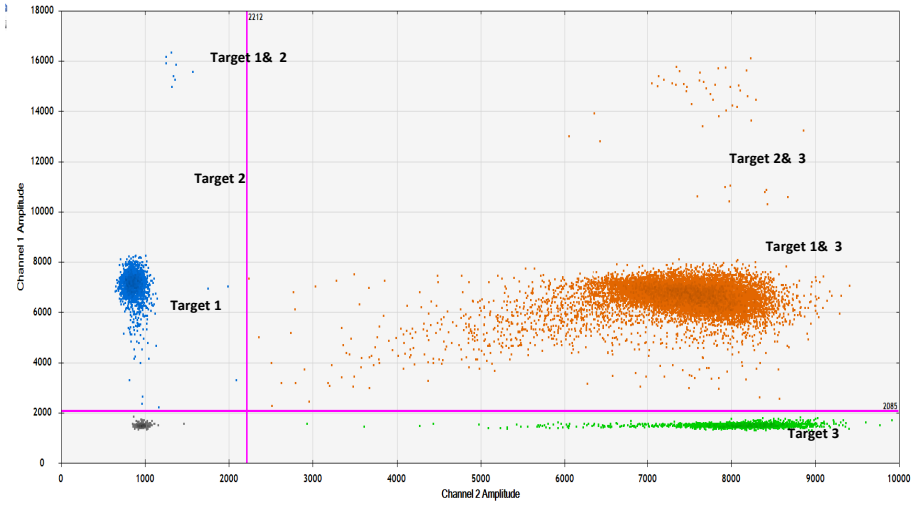
The ability to detect each target at the limit of quantification was also tested. An experiment was performed where two out of three targets were saturated while one target remained constant at the limit of quantification.

In conclusion, ddPCR can be used to determine vector genome titer as well as to quantify residual host cell and plasmid DNA within the same reaction. Although the dynamic range is not as large as qPCR, dilutions can be designed so that at least one dilution falls within the range of the assay. As opposed to qPCR, ddPCR is used. It is crucial that the conditions/parameters are optimized as described for successful ddPCR.

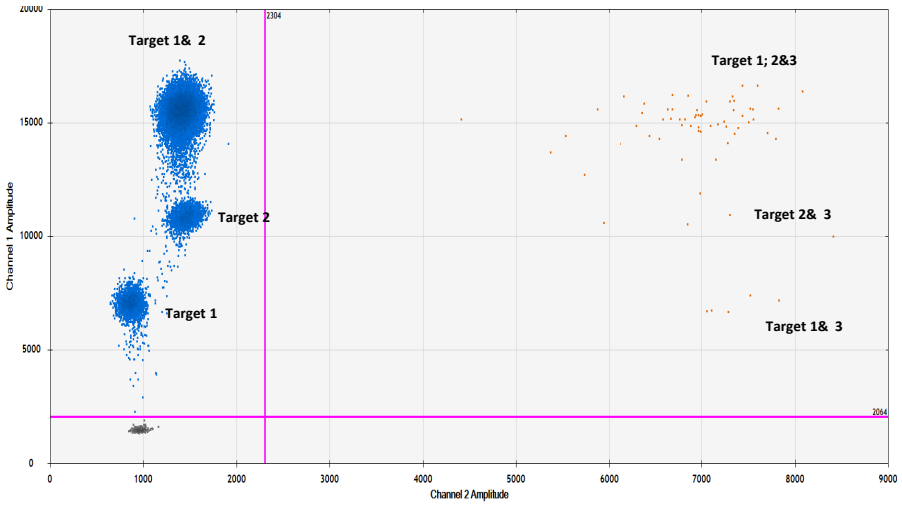
By performing triplex ddPCR, it is possible to quantify several targets without introducing inter assay variability.



2D graph showing Target 1 at 4 copies/µL



2D graphs howing Target 2a t4 copies/µL



2D graphs howing Target 3a t4 copies/µL

Figure 5: Duplex ddPCR used to determine vector genome titer and residual baculovirus DNA

Integrated automated liquid handling.

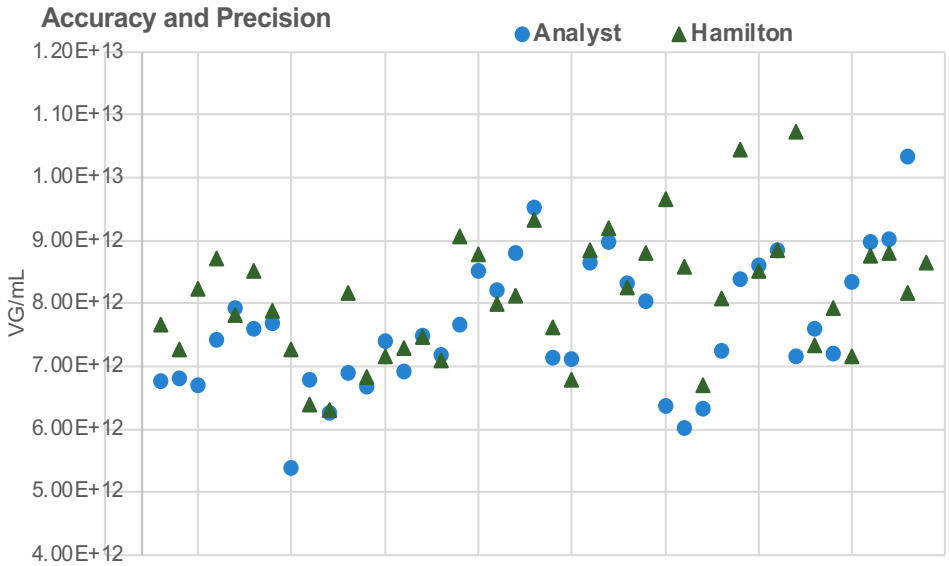
Automated liquid handling technology is becoming an integral part of many high-throughput molecular biology and biochemical assays. Ranging from simple, single-channel pipettors to more complex multi-channel devices that can handle multiple samples simultaneously, automated liquid handling systems use robotic devices or systems to transfer liquids precisely and accurately in the lab setting. The benefits of integrating these capabilities with ddPCR include:

- Accuracy and precision
- High throughput
- Consistency
- Viral titer estimation
- Reduced risk of contamination
- Resource optimization
- Data integration
- Standardization

In this regard, the integration of automated liquid handling technology with ddPCR allows for more efficient, accurate, and high-throughput analyses—a critical combination for optimizing production processes and ensuring viral vector product quality and safety.

Automating the VG titer method, which is traditionally performed using manual pipettors, resulted in data with higher precision being produced. The automated workflow was optimized for robustness and consistency.

Data produced by the instrument showed lower variability after several executions than the data produced by the five analysts.



Operator	Result
5 Analysts (N=48)	0.96 ± 15%
Hamilton (N=42)	1.04 ± 12%
Overall (N=90)	**1.00 ± 14%**

Conclusion

In summary, ddPCR is a robust tool for determining vector genome titer and quantifying residual host cell and transfection plasmid DNA, delivering accurate and precise results. While its dynamic range doesn't rival that of qPCR, appropriate dilution strategies ensure at least one dilution aligns with the assay's range. Unlike qPCR, ddPCR offers advantages, such as diminished matrix effects on titer. However, for effective ddPCR execution, it's crucial to optimize conditions and parameters as highlighted.

By utilizing triplex ddPCR, it's possible to quantify multiple targets without introducing inter-assay variability. The precision of triplex ddPCR enables laboratories to optimize their analytical workflows. To ensure that measurements remain reliable, it is essential to validate the limit of quantification for each target individually while other targets are saturated. Integrating automated liquid handling technologies adds further efficiencies by expediting turnaround times and enhancing the overall quality of results.

These advancements empower analysts to shift their focus away from labor-intensive processes and toward high-priority tasks like data analysis and presentation, thus ushering in a new era of productivity and accuracy in the laboratory.

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With more than a decade in the biopharmaceuticals industry, Diego Matayoshi has held roles in both analytical development and quality control. At Thermo Fisher, he has applied his expertise to the research, design, optimization, and qualification of methods for viral vector analytics, essential for the release of clinical vector products. Mr. Matayoshi earned his BS in biology from the University of Florida.

[1]Lievens, A., Jacchia, S., Kagkli, D., Savini, C., & Querci, M. 2016. "Measuring Digital PCR Quality: Performance Parameters and Their Optimization." PLoS ONE 11 (5): e0153317. https://doi.org/10.1371/journal.pone.0153317.

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