Digital PCR

Residual plasmid quantification in gene therapy manufacturing workflows using the QuantStudio Absolute Q Digital PCR System

Here we present:

- The novel design of the Applied Biosystems™ resDNASEQ™ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit for residual plasmid quantification in gene therapy manufacturing workflows
- The adaptation of the resDNASEQ qPCR assay to the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System
- Comparison of data between dPCR and qPCR

Introduction

Residual DNA testing is an established method that is routinely used for the assessment of product quality and safety in the development of gene therapies, vaccines, and similar biotherapeutics. The amount of residual DNA in a biologic's final dosage form must meet regulatory guidelines established by the World Health Organization (WHO), the European Pharmacopoeia, the US Food and Drug Administration (FDA), and other global regulatory agencies. The resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene (pDNA-KanR) Kit utilizes a qPCR-based system for the detection of residual plasmid DNA containing the kanamycin-resistance gene. The resDNASEQ system enables rapid, sensitive, and specific quantitation of plasmid DNA containing the kanamycin resistance gene by targeting multiple KanR variants present in commonly used plasmids. This performance helps ensure a high degree of confidence in quantitation data obtained from a broad range of sample types—from in-process samples with different sample matrices, to bulk drug substances.

Although qPCR is a robust and proven technology, interest in digital PCR (dPCR) has grown significantly. Digital PCR works by compartmentalizing a bulk PCR reaction into thousands of nanoliter-scale reactions, each containing zero, one, or just a few DNA molecules. Absolute quantification of a sample is achieved by counting positive reactions and applying Poisson statistics. This reduces variability and improves accuracy and analytical sensitivity in high-background conditions. There is no need to routinely run a quantitative control or require a standard curve for quantification.

The QuantStudio Absolute Q Digital PCR System is the newest platform in the Applied Biosystems™ portfolio. The QuantStudio Absolute Q dPCR System enables all the necessary steps for dPCR in a simple workflow identical to qPCR, improving ease of use with minimal hands-on time and maximizing consistency. Here we demonstrate how to adapt the resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit to the QuantStudio Absolute Q Digital PCR System workflow.

QuantStudio Absolute Q Digital PCR System for analytical applications in cell and gene therapies

Accuracy and consistency are critical factors in providing high confidence in the detection and quantification of residual plasmid DNA in biotherapeutic products. While dPCR is becoming a standard in precise nucleic acid quantification, many available dPCR technologies suffer from several limitations that hinder its broad adoption, such as tedious workflows, long time-to-results, and inconsistent reagent digitization resulting in high sample waste.

The QuantStudio Absolute Q Digital PCR System is a plate-based platform powered by proprietary microfluidic array plate (MAP) technology and a single instrument to enable all steps of dPCR. Reaction compartmentalization, thermal cycling, and data collection are conducted on a single instrument with a simple 90-minute workflow (Figure 1). The workflow minimizes hands-on steps to improve time-to-answer and help reduce the risk of variability and sample contamination—both of which are critical in biotherapeutic product testing.

Figure 1. QuantStudio Absolute Q Digital PCR System workflow. The standard PCR reaction is prepared with all required components for digital PCR including master mix, nucleic acid, and primers and probes, and then load into the Applied Biosystems™ QuantStudio™ MAP16 Digital PCR Plate. The dPCR experiment is run, and data are analyzed, using a single instrument.

Each QuantStudio MAP plate consists of 16 individual wells, allowing analysis of up to 16 samples in a single instrument run. Each well with the MAP technology enables a dPCR reaction to be easily and reliably compartmentalized into over 20,000 microchambers. Over 95% of the input sample is analyzed per reaction compared to 25–60% for other dPCR platforms, improving both sample utilization and data consistency. The unique architecture of the QuantStudio Absolute Q system and the QuantStudio MAP16 Plate allows flexibility, including the ability to run custom dPCR assays, which is not offered by any other dPCR platform.

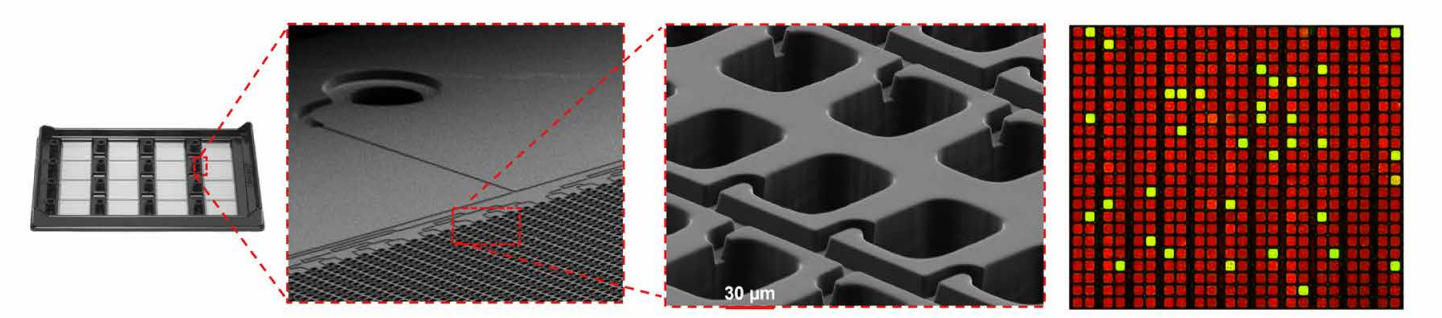

Figure 2. The QuantStudio MAP16 Digital PCR Plate.

Three different samples with known KanR variants were used to demonstrate that the resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene assay is compatible with the QuantStudio Absolute Q Digital PCR System. The resDNASEQ kit includes the Kanamycin Positive Control, 10X KanR Assay Mix, and Negative Control (Water). The kit was supplemented with the appropriate reagent and consumables to operate on the QuantStudio Absolute Q dPCR System, including Applied Biosystems™ Absolute Q™ DNA Digital PCR Master Mix (5X) and Applied Biosystems™ QuantStudio™ Absolute Q™ Isolation Buffer formulated for the QuantStudio MAP 16 Digital PCR Plates. All materials are listed in Table 1.

Table 1. Materials for resDNASEQ assay workflow.

Material	Provider
Kanamycin Positive Control	KanR kit
10X KanR Assay Mix	
Negative Control (Water)	
Absolute Q DNA Digital PCR Master Mix (5X)	Additional reagents
QuantStudio Absolute Q Isolation Buffer	
QuantStudio Absolute Q MAP16 Plate Kit	

Each variant sample was serially diluted to quantify the three KanR variants across a range of concentrations (Table 2). The serial dilutions were then used in the dPCR workflow.

Table 2. Standard curve preparation.

Standard name	DNA standard (µL)	DNA dilution buffer (µL)	Concentration (copies/µL)	Fold dilution
Std 0	10 µL from positive control	990	3×10^5	100x
Std 1	20 µL from Std 0	180	3×10^4	10x
Std 2	20 µL from Std 1	180	3×10^3	10x
Std 3	20 µL from Std 2	180	3×10^2	10x
Std 4	20 µL from Std 3	180	3×10^1	10x
Std 5	20 µL from Std 4	180	3	10x
Std 6	100 µL from Std 5	100	1.5	2x

For analysis on the QuantStudio Absolute Q Digital PCR System, a dPCR reaction mixture for each standard was prepared following the standard QuantStudio Absolute Q dPCR protocol (Table 3). Each 9 µL reaction mixture was loaded into a single well of the QuantStudio MAP16 Plate, followed by an overlay of isolation buffer (Table 3). Each standard (Std 2, 4, 5, and 6) was loaded in triplicate in the QuantStudio Absolute Q MAP16 Plate (see Table 4 for example), and subsequently loaded onto the QuantStudio Absolute Q Digital PCR System. The following thermal parameters were used for each digital PCR run: 96°C hold for 10 min, followed by 45 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 61°C for 30 sec. The Applied Biosystems™ FAM™ dye channel was used to collect the data.

Table 3. Components of dPCR reaction mix and overlay.

dPCR mix	Volume (µL)
5X Combinati MMx	1.8
10X KanR Assay Mix	0.9
H_2O	1.3
Standard/template	5
Total volume	9
Overlay	**Volume (µL)**
QuantStudio Absolute Q Isolation Buffer	15

Table 4. Example of a PCR plate setup.

Add 24 µL of QuantStudio Absolute Q Isolation Buffer to each unused well in the QuantStudio Absolute Q MAP16 Plate.				
	1	**2**	**3**	**4**
A	Std 2	Std 2	Std 2	NTC
B	Std 4	Std 4	Std 4	NTC
C	Std 5	Std 5	Std 5	NTC
D	Std 6	Std 6	Std 6	Empty

Results

Data output from the Applied Biosystems™ QuantStudio™ Absolute Q Digital PCR Software is reported in copies/µL, and therefore was subsequently adjusted based on sample input volume (5 µL) in order to compare with the qPCR results. In contrast, the KanR qPCR protocol designates a 10 µL sample input volume and generates data in terms of copy number. As such, to compare dPCR results to qPCR data, the dPCR results were adjusted based on the formula below.

Expected qPCR copies = ((dPCR copies/µL) x 2) x 10

Three different KanR variants were tested using the new dPCR workflow. For all three variants, the dilution conditions representing the qPCR LOQ (Std 5) and LOD (Std 6) were detected with precision using the dPCR workflow (Figure 3).

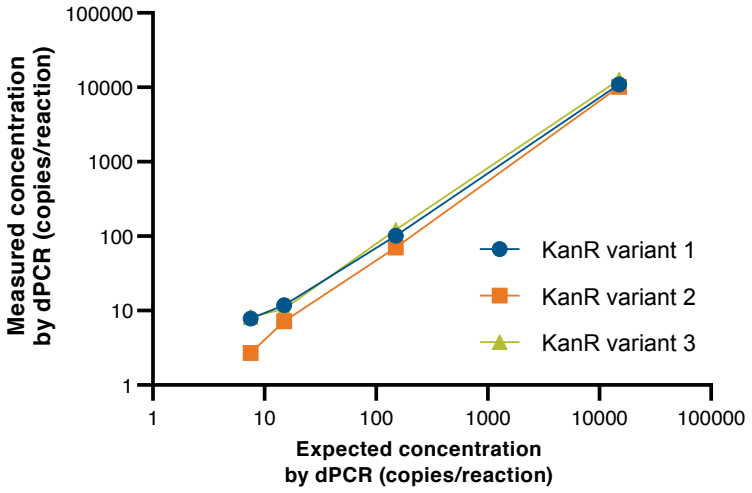

Figure 3. dPCR results for the 3 different KanR variants tested. The concentrations of the dilution samples representing the qPCR LOQ (Std 5) and LOD (Std 6) were measured with precision for all three KanR variants.

Conclusions

Gene therapy products are synthesized using various biological processes. The regulatory authorities responsible to control the safety measures of such products must be attentive to the amount of potential contamination carried over in the manufacturing process. The resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene (pDNA-KanR) Kit is a critical assay to ensure that residual plasmids are not carried over to the final drug product. Here we demonstrated the feasibility of transitioning this assay to the QuantStudio Absolute Q Digital PCR System. With the KanR assay, absolute quantification of samples is possible using the QuantStudio Absolute Q dPCR System's protocol. Quantification of low standards with low copies per reaction (LOQ and LOD) was possible without the need to generate or use a standard curve. The simplicity and flexibility of the QuantStudio Absolute Q dPCR System, including its compatibility with custom assays, means samples are analyzed in as little as 90 minutes with a streamlined workflow that complements current qPCR analytical processes.

Ordering information

Product	Cat. No.
resDNASEQ Kits	
resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit	A50337
resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit with PrepSEQ Residual DNA Sample Preparation Kit	A50460
QuantStudio Absolute Q assays, reagents, and consumables	
Absolute Q DNA Digital PCR Master Mix (5X)	A52490
QuantStudio Absolute Q Isolation Buffer	A52730
QuantStudio MAP16 Plate Kit	A52865
Absolute Q Custom Assay	Please inquire
QuantStudio Absolute Q dPCR System	
QuantStudio Absolute Q Digital PCR System	Please inquire

Note: This application note is a demonstration of a workflow. The resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit has not been fully validated on the QuantStudio Absolute Q Digital PCR System.

Learn more about the QuantStudio Absolute Q Digital PCR System at **thermofisher.com/absoluteq**

Learn more about resDNASEQ kits at **thermofisher.com/resdnaseq-plasmid**