Application Note

USP solutions to confidently measure residual plasmid DNA in gene therapy products

USP Biologics™

Introduction

Many gene therapy products (e.g., those manufactured via HEK-293-based adeno-associated virus (AAV) and Lentiviral vector processes) often rely on large amounts of plasmid DNA as a critical component of the manufacturing process. Plasmids serve as templates for the therapeutic gene of interest but must be removed from the final product, as they pose a safety risk. Residual plasmid DNA can trigger unintended immune responses, transfer antibiotic resistance genes, or integrate into a patient's genome. It may also lead to false positive for critical safety assays, such as replication-competent AAV (rcAAV). A sensitive and accurate residual plasmid DNA quantification method is recommended by regulatory agencies and essential to demonstrate consistent clearance of this process-related impurity.

It is most common to use high-sensitivity PCR-based analytical tests to quantify residual plasmid DNA. Developing these methods and ensuring their consistent performance can be challenging without reference materials and standardized methods. To address these challenges in measuring residual plasmid DNA, the United States Pharmacopeia (USP) recently released an analytical reference material (ARM), Plasmid for Residual DNA Quantification ARM (catalog #1544900). This application note provides information about this reference material, as well as recommended analytical procedures.

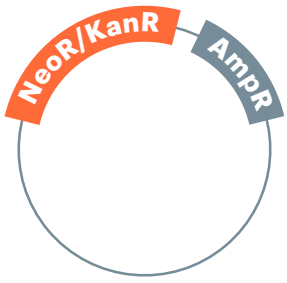


Figure 1. USP Plasmid for Residual DNA Quantification

Intended use for USP plasmid ARM

This reference material is intended to be used with digital PCR (dPCR) or quantitative (qPCR) methods, specifically designed for system suitability, assay control, and standard curve calibrant. The product is double digested and contains both the AmpR and NeoR/KanR antibiotic resistance genes, which are commonly found in plasmids used for gene therapy manufacturing (**Figure 1**). Since antibiotic resistance genes are commonly used during the selection process and present in most plasmid constructs, they are excellent PCR amplification targets.

Reference material value assignment

Along with its potential use as a system suitability or assay control, USP's Plasmid for Residual DNA Quantification ARM provides a reliable reference material that laboratories can use to validate their methods. This ARM's assigned value is based on results from multiple laboratories using a dPCR method combined with USP's quality processes to ensure greater consistency and accuracy (**Figure 2**).

Results shown in **Table 1** were collected from a multi-laboratory study (also found in the product information sheet).

Table 1. Attributes of Plasmid for Residual DNA Quantification Analytical Reference Material

Test	Result
Appearance	Colorless Liquid
Identification by Sanger Sequencing	Conforms to Sequence
Identification by Restriction Enzyme Mapping	Conforms to Sequence
DNA Concentration by Variable Pathlength Extension UV Spectroscopy using SoloVPE	29 ng/µL
Assigned Value (DNA Concentration)	3.3E+09 copies/µL (29.1 ng/µL)

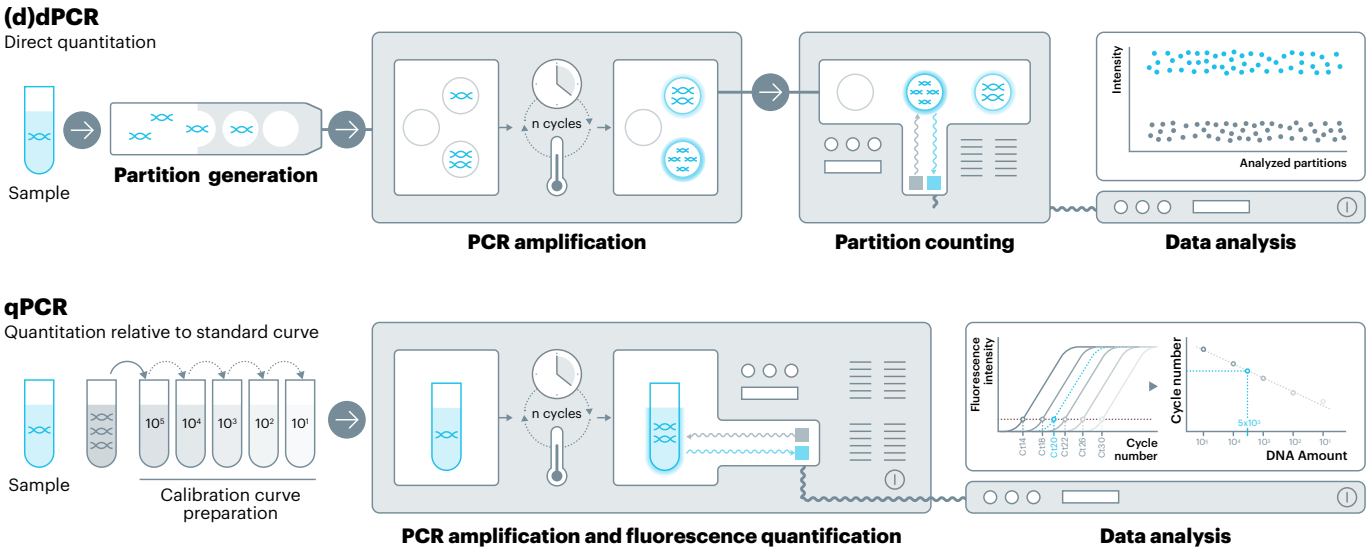


Figure 2. Use of Plasmid for Residual DNA Quantification ARM in (d)dPCR and qPCR assays.

This dPCR method was developed and assessed following ICH parameters: specificity, precision (repeatability), linearity, range, and accuracy. Method information and feasibility data are provided in the sections below.

Digital PCR method

Primers, probes and amplification conditions

The following primers and probe sequences were identified based on a literature search and *de novo* designs. Of all the screened primers and probe sets, these performed most accurately and reproducibly.

The AmpR and the NeoR/KanR antibiotic resistance gene targets can be used with equal performance and produced comparable results in all multi-laboratory study laboratories. The sequences of the primers and probe are as follows:

- **NeoR/KanR Forward Primer:**
  5′-CTGCCGAGAAAGTATCCATC
- **NeoR/KanR Reverse Primer:**
  5′-ATGTTTCGCTTGGTGGTC
- **NeoR/KanR Probe:**
  5′-ATACGCTTGATCCGGCTACCTGC
- **AmpR Forward Primer:**
  5′-CACAACATGGGGGATCATG
- **AmpR Reverse Primer:**
  5′-GTCGTTTGGTATGGCTTCATTC
- **AmpR Probe:**
  5′-ACTCGCCTTGATCGTTGG

The thermal cycling conditions are as follows:

1. PCR Initial Heat Activation: 95°C for 2 minutes
2. 2-Step Cycling (40 cycles):
   - Denaturation: 95°C for 15 seconds
   - Annealing/Extension: 60°C for 30 seconds

Multi-laboratory testing

After the method was shown to be fit for its intended use, it was employed in a multi-laboratory collaborative study to characterize the reference material. Results are provided in **Table 2**. We observe comparability between AmpR and NeoR/KanR targets, as well as a low coefficient of variance (CV) among the five collaborative laboratories.

The DNA concentration, as measured by dPCR, is 3.3E+09 copies/µL. Based on the size of the plasmid, this value is equivalent to 29.1 ng/µL.

As an orthogonal test of the value assignment by dPCR, the plasmid concentration was measured by UV-vis spectroscopy using a SoloVPE instrument. A concentration of 29 ng/µL was observed (see product information sheet), which confirms the accuracy of the dPCR-based value assignment.

Linearity and range data

To assess the linearity and range of the dPCR method, the plasmid reference material ranging from 2.8E+01 to 2.8E+05 copies/reaction (rxn) was analyzed (**Table 3**). The linearity was assessed by plotting the nominal concentration against the observed (measured) concentration of plasmid DNA from triplicate runs (**Figure 3**).

Table 2. Multi-Laboratory testing dPCR data

Plasmid DNA Quantification by digital PCR for the Analytical Reference Material (copies/µL)									
Target Region	Lab A	Lab B	Lab C	Lab D	Lab E	Mean	CV (%)	Total Mean	Total CV (%)
AmpR	3.41E+09	2.74E+09	3.46E+09	3.36E+09	3.37E+09	3.3E+09	9.11	3.3E+09	8.4
NeoR/KanR	3.40E+09	2.83E+09	3.55E+09	3.28E+09	3.39E+09	3.3E+09	8.34		

The linear regression analysis showed a slope and R^2 close to 1.00, and an intercept near 0. Additionally, the recovery at each point ranged from 95 – 103%. These results support the suitability of the method in a range of 2.8E+01 to 2.8E+05 copies per reaction.

Precision (Repeatability)

To assess the method's repeatability, eight (N = 8) samples of the reference material were prepared (**Table 4**). A 1.9% CV was observed, confirming that the method is precise and repeatable.

Accuracy in the presence of AAV sample matrices

To assess method performance in different AAV product-like sample matrices, we performed spike-in recovery experiments. First, to establish a baseline DNA concentration, the amount of residual DNA was determined in four separate recombinant AAV samples (**Table 5** (A)). The AAV samples averaged ~500 copies/µL, illustrating the challenges of removing plasmid DNA. To each sample, 200 copies/µL of material was added as spike-in and the recovery was assessed by calculating the concentration of plasmid DNA in the spike-in samples. Accuracy was assessed by calculating the measured amount of spike-in DNA and comparing it to the nominal spike in amount. The results demonstrate accuracy and robustness in the presence of different AAV product matrices.

Specificity with host cell sample matrices

To assess method specificity, the presence of plasmid DNA was analyzed in different host cell genomic DNA samples by performing 0.4% (by mass) spike-in experiments. In these experiments, plasmid DNA was not detected in genomic samples that were not spiked with plasmid DNA and demonstrated good recovery when plasmid DNA was spiked into the gDNA samples (**Table 6**). These data demonstrated robustness and specificity from host cell DNA sources. These results also show that the primers/probe combinations are specific to the target regions.

Table 3. dPCR linearity/range data

Nominal (copies/µL)	Observed (copies/ µL)	Recovery (%)	% CV
2.8E+05	2.8E+05	100%	4.4%
2.8E+04	2.7E+04	95%	4.1%
2.8E+03	2.7E+03	95%	4.7%
2.8E+02	2.8E+02	96%	4.0%
2.8E+01	2.9E+01	103%	12.3%

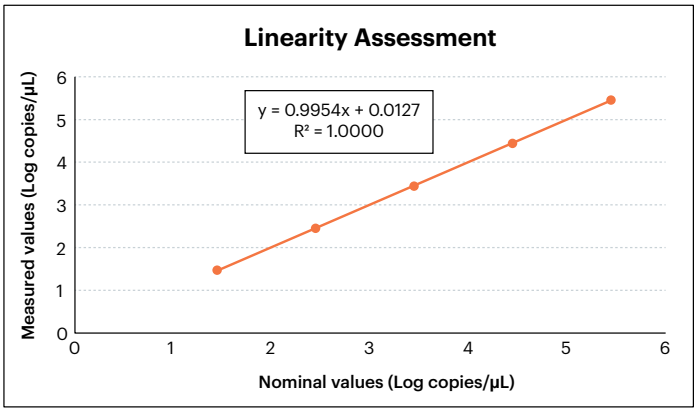


Figure 3. Linearity assessment of dPCR method

Table 4. dPCR precision (repeatability) data

Determination	Titer (copies/µL)	Average Titer (copies/µL)	CV
#1	3.11E+09	3.09E+09	1.9%
#2	3.09E+09		
#3	3.10E+09		
#4	3.13E+09		
#5	2.97E+09		
#6	3.16E+09		
#7	3.10E+09		
#8	3.03E+09		

Table 5. dPCR accuracy assessment using spiked-in AAV samples

Sample	(A) Non-Spiked PCR Measurement (copies/µL)	(B) Spiked PCR Measurement (copies/µL)	(C) Measured Spike Amount (C = B – A) (copies/µL)	Nominal Spike (copies/µL)	Recovery (%)
rAAV5	786	989	203	200	101%
rAAV5	391	579	188		94%
rAAV9	556	754	198		99%
rAAV9	266	453	187		94%

These findings demonstrate the method's feasibility, making it suitable for routine quality testing.

Use in qPCR assay

In addition to its use in a dPCR method, USP has demonstrated that the Plasmid for Residual DNA Quantification ARM is also suitable for qPCR-based measurements. When testing by qPCR, for both the AmpR and NeoR/KanR targets, the same primer and probe sets were used. The following thermocycler conditions were used for qPCR measurements:

1. Initial Heat Activation: 95°C for 10 minutes
2. 2-Step Cycling (40 cycles):
   - Denaturation: 95°C for 15 seconds
   - Annealing/Extension: 60°C for 60 seconds

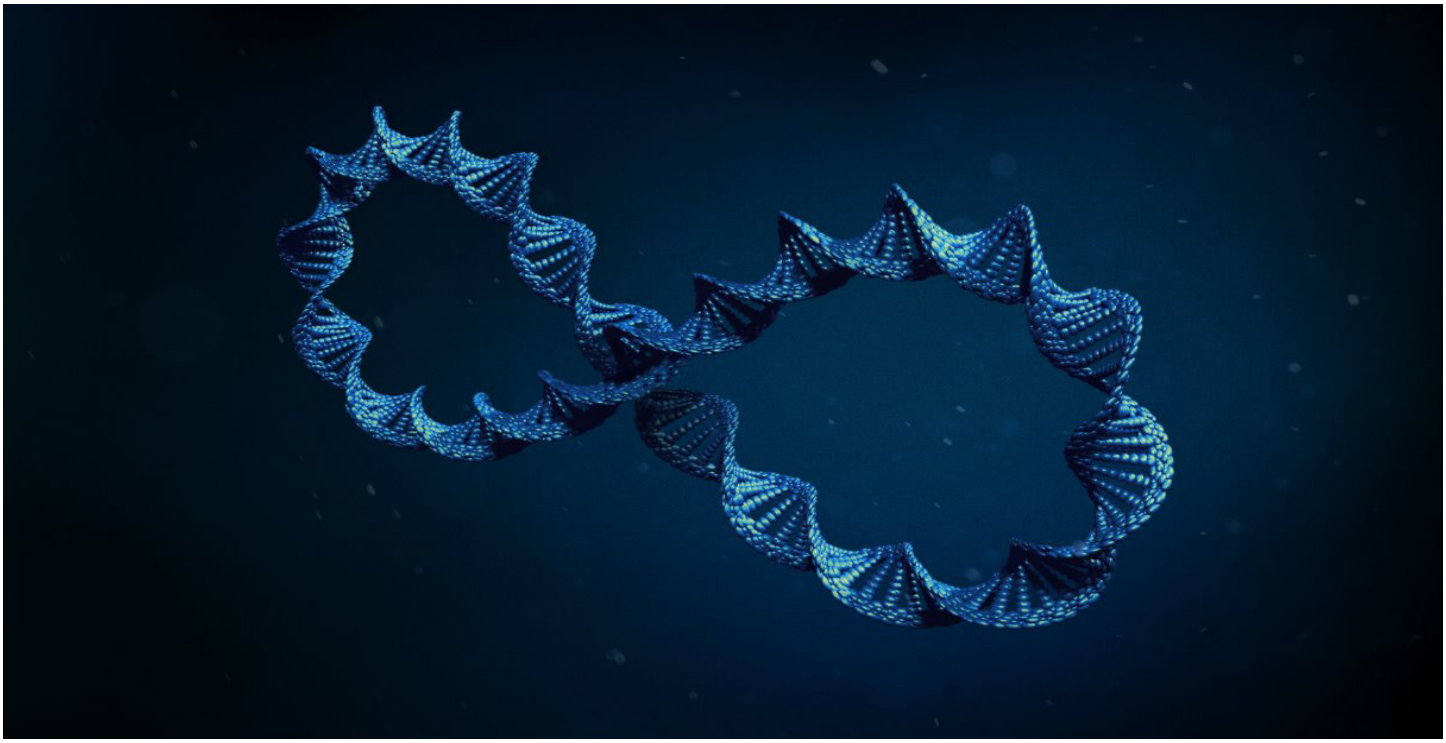
When assessing the utility of the product as a qPCR control, linearity (R^2 >0.99) was achieved from 0.0001 – 10 pg/µL for both the AmpR and NeoR/KanR targets. The accuracy was also analyzed by assessing the recovery of a spiked-in sample using the qPCR method with USP's Residual Plasmid DNA ARM as the calibration standard. In this study, a plasmid sample, which was measured using an orthogonal method, was added at three concentrations ranging from 0.01 – 1.0 pg/µL to assess the accuracy at various points on the curve. The data, as shown in **Table 7**, showed a recovery ranging from 95 – 108%. This recovery meets the system suitability requirements of the USP *<509> Residual DNA Tests for Genomic DNA*, which is relative standard deviation ≤30% for three replicates of sample solutions and positive control solutions.

Table 6. dPCR specificity using genomic DNA matrices

Test Sample	Test Results
Positive Control	Tight cluster of positive partitions
No Template Control	No detectable signal
No Amplification Control	No detectable signal
5 ng HEK293 gDNA	No detectable signal
5 ng Sf9 gDNA	No detectable signal
5 ng CHO gDNA	No detectable signal
5 ng E. coli gDNA	No detectable signal
5 ng HEK293 gDNA spiked with 20 pg	98% Recovery
5 ng Sf9 gDNA spiked with 20 pg	98% Recovery
5 ng CHO gDNA spiked with 20 pg	98% Recovery
5 ng E. coli gDNA spiked with 20 pg	103% Recovery

Table 7. qPCR recovery data

Test Number	Nominal Concentration (pg/µL)	Measured Concentration (pg/µL)	Recovery (%)
1	1.00	1.08	108
	0.10	0.10	97
	0.01	0.01	95
2	1.00	0.98	98
	0.10	0.10	100
	0.01	0.01	105



Conclusion

The USP Plasmid for Residual DNA Quantification ARM and PCR methods described here can help gene therapy manufacturers ensure that their processes consistently remove residual plasmid DNA impurities. These reference materials and methods build on USP's long history supporting residual DNA testing with both USP General Chapters and physical reference materials. These materials can be used to monitor trends and control method performance, assist with method transfers and staff training, and ensure that manufacturing processes are consistent. The Plasmid for Residual DNA Quantification ARM can serve as a standard for quantitation, system suitability or spike-in control. Using materials and methods for residual DNA testing that have been developed by USP can give gene therapy manufacturers greater confidence in their results and in the quality of their products.

Additional USP resources

<1130> *Nucleic Acid-Based Techniques – Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)*

<509> *Residual DNA Testing*

CHO Genomic DNA (Catalog #: 1130710)

E. Coli Genomic DNA (Catalog #: 1231557)

Quantitative HEK-293 Genomic DNA (Catalog #: 1592106)

Quantitative MRC-5 Genomic DNA (Catalog #: 1592112)

Quantitative BHK-21 Genomic DNA (Catalog #: 1592100)

Quantitative Sf9 Genomic DNA (Catalog #: 1592170)

Quantitative MDCK Genomic DNA (Catalog #: 1592111)

Quantitative Vero Genomic DNA (Catalog #: 1292190)

More information

AAV analytical guide: https://genetherapyanalyticalguide.usp.org/

Questions: uspbiologics@usp.org

Ordering information: store.usp.org