CELL & GENE THERAPY INSIGHTS

VIRAL VECTOR BIOPROCESSING & ANALYTICS:
TODAY'S KEY TOOLS AND INNOVATION
REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INNOVATOR INSIGHT

Development and validation of quantitative real-time PCR for the detection of residual HEK-293 host cell DNA

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The presence of residual DNA in therapy products may lead to an increased risk of oncogenicity, immunogenicity, and other toxicity. Current regulatory authorities (including the US FDA, EMA and WHO) limited the accepted amounts of residual DNA in biological products making it extremely important to have a sensitive method of quantifying residual host cell DNA. Among the methods of detecting residual DNA, quantitative polymerase chain reaction (qPCR) is the most widely used for residual DNA quantitation due to its sensitivity, accuracy, precision, and time-saving capability. This article examines the development and validation of a new, highly sensitive and accurate integrated solution for detection and quantitation of low level HEK-293 DNA to help meet regulatory requirements.

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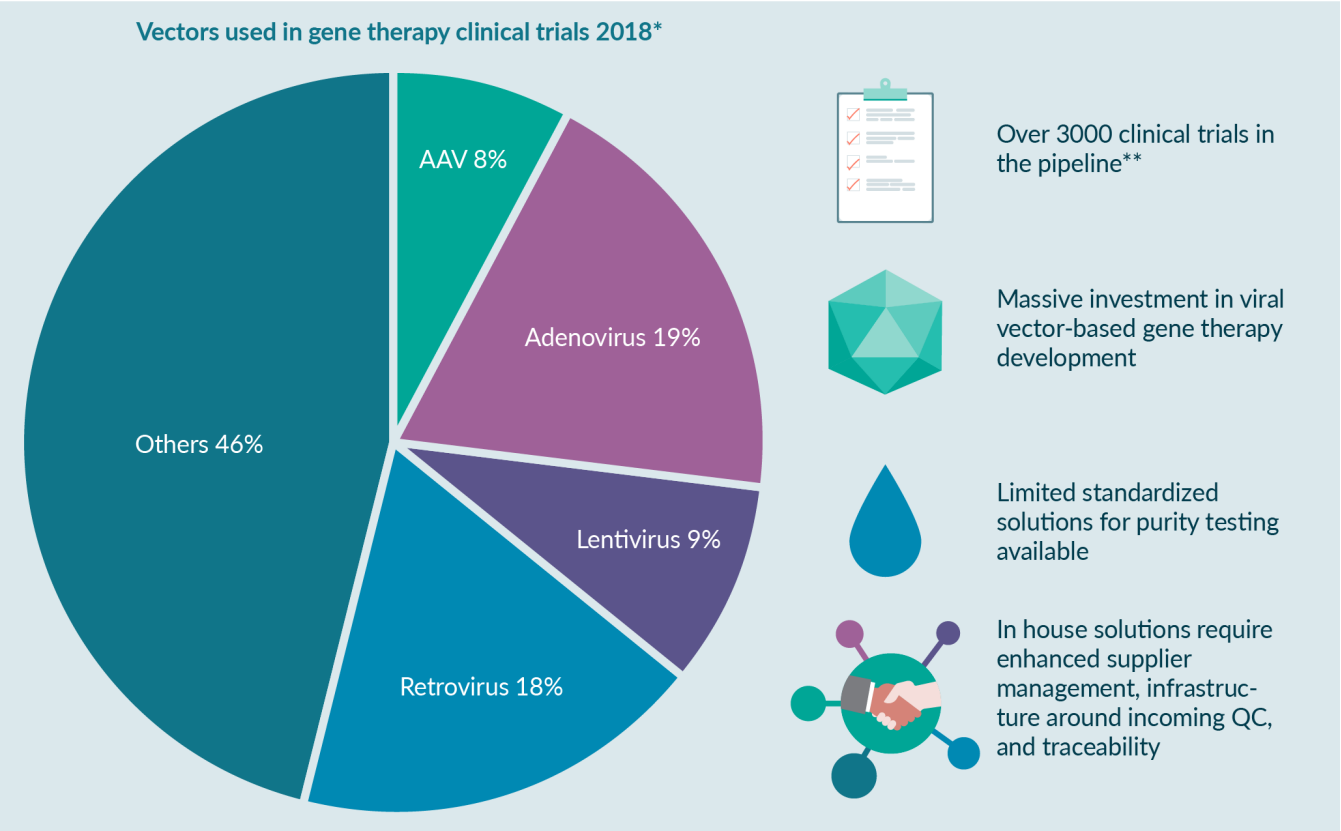
INTRODUCTION

With over 3,000 clinical trials currently in the pipeline, the gene therapy field is moving rapidly. A variety of viral vectors are used in these therapies, including adenovirus, lentivirus and adeno-associated virus (AAV) (Figure 1). Despite massive investment in viral vector-based gene therapy development,

FIGURE 1

The field of gene therapy is advancing rapidly.



*Sourced from *The Journal of Gene Medicine*, 2018 John Wiley and Sons Ltd.
**Global Cell and Gene Therapy Market: Focus on Products, Applications, Regions and Competitive Landscape - Analysis and Forecast, 2019-202.

the speed at which the field is developing means that there are limited standardized solutions currently available for purity testing and analytics. This unmet need has led some groups to turn to the development of in-house solutions. However, these come at a considerable cost.

Residual nucleic acid is a common process impurity tested in viral vector production, and is regulated by bodies such as the FDA and EMA: they require that the DNA content in the final product is less than 10 nanograms per therapeutic dose. This can be a challenge when using AAV in particular, as these vectors can package a large amount of plasmid or cellular DNA inside the viral capsid. This article provides an overview of analytical testing in bioproduction processes and describes the development of a novel residual DNA assay.

DEVELOPMENT OF A NOVEL RESIDUAL DNA ASSAY FOR HEK-293 GENOMIC DNA

The downstream vector purification step is the point at which an analytical technique must be employed to ensure there is a minimum amount of residual DNA present in the final product.

The Applied Biosystems resDNASEQ Quantitative HEK293 DNA Kit is a qPCR-based system that is optimized for detection of host cell DNA from HEK-293 cell lines. Thermo Fisher Scientific has a long history of enabling labs in testing residual DNA, including assays for Chinese hamster ovary (CHO), *E. coli*, human, Vero, MDCK, Pichia pastoris, and NS0 DNA. Since HEK-293 cells are used in the development of viral vectors for both gene therapy

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and other biotherapeutics, the need for a specific kit has become increasingly urgent. This is not only an assay but encompasses an end-to-end workflow solution, from sample extraction to data analysis, with additional support provided for implementation and validation for the whole workflow.

Manual versus automated DNA extraction

Table 1 includes some important considerations when choosing between manual and automated sample preparation methods.

Two sample preparation methods were tested with the HEK-293 residual DNA assay. The manual sample preparation method used was the manual protocol from the PrepSEQ Nucleic Acid Extraction Kit. This is a low-throughput method allowing for 16 extractions per day. The automated method tested also uses the PrepSEQ Nucleic Acid Extraction Kit, and leverages the Kingfisher Flex System by Thermo Scientific; this is a high throughput system that extracts 192 samples per day, requiring considerably less hands-on time (less than 1 minute per extraction).

The PrepSEQ Nucleic Acid Extraction Kit is a universal solution for nucleic acid extraction. The kit works on a variety of host cell DNA types and has also been tested on mycoplasma, as well as more than 60 different viruses, including double stranded and single stranded DNA viruses, double stranded and single stranded RNA viruses, with or without envelopes. PrepSEQ has also been tested under a variety of conditions such as low pH, high salt, and high protein.

The method (Figure 2) starts with the addition of a lysis buffer to the sample, and then magnetic particles are added together with a solution that allows for optimal efficient binding of nucleic acid to the particles. The magnetic particles are collected, followed by a series of wash steps. Finally, nucleic acid is eluted off the magnetic particles. The result is a PCR-compatible nucleic acid extract significantly reduced in inhibitors.

TaqMan Real-Time PCR Assay

Real-time PCR was improved by the introduction of TaqMan DNA polymerase that leverages the 5' nuclease activity of the enzyme, along with fluorogenic labelled probes. Applied Biosystems™ TaqMan® Assays are the industry-leading choice for 5' nuclease qPCR assays. The kit also comes with a DNA control for calibrating assay results. This control consists of precisely quantitated, highly purified genomic DNA from an established HEK-293 cell line.

The assay was validated on two systems from Thermo Fisher Scientific. The

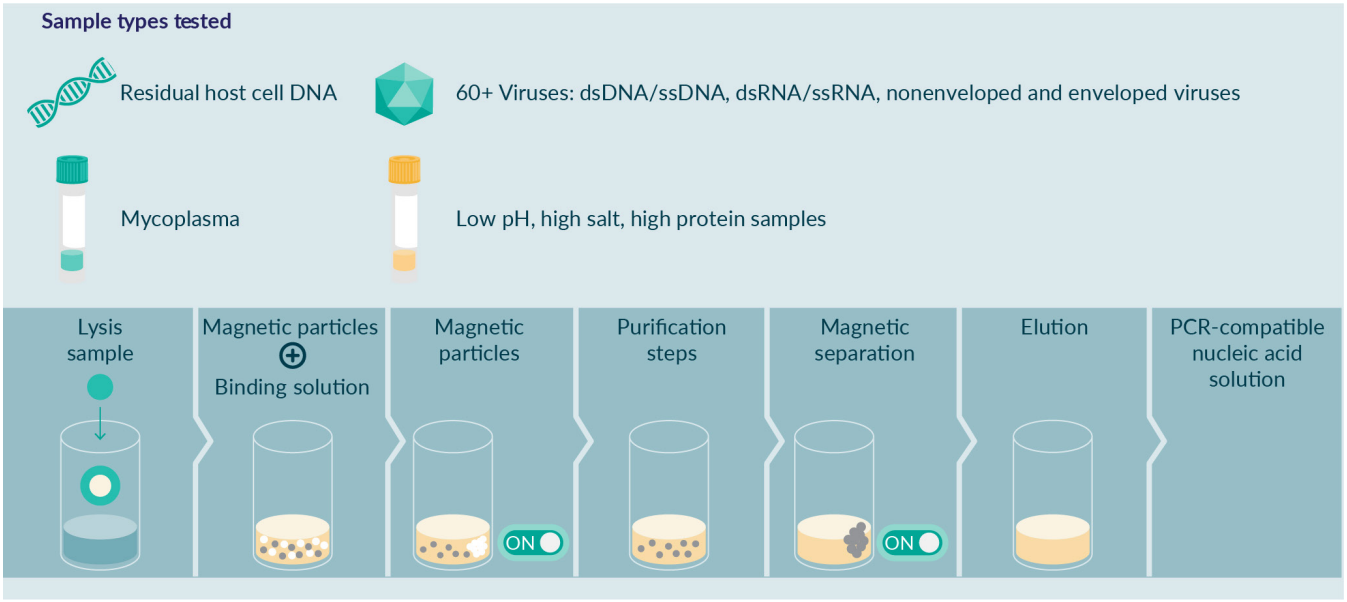
TABLE 1

Key pros and cons of manual versus automated sample preparation systems.

	Pros	Cons
Manual sample preparation system	▶ Requires minimal up-front equipment investment	▶ Additional risk and variation due to human interface - more susceptible to errors and contamination ▶ Higher labor cost ▶ Lower throughput
Automated sample preparation system	▶ Reduced hands-on time leads to minimal variation and susceptibility to errors over long-term ▶ Lower labor cost ▶ Much higher throughput	▶ Requires a large upfront equipment investment ▶ More complex, time consuming and costly implementation

FIGURE 2

Method background: PrepSEQ sample preparation kits.



workhorse system for the residual DNA portfolio is the Applied Biosystems 7500 Fast Real-Time PCR System (7500 Fast). All seven resDNA assays run on this system and are used worldwide. More recently, the QuantStudio 5 was also introduced as a validated system for the residual DNA portfolio. This addition provides precise quantification with 1.5-fold discrimination. For assays in general, the Quant Studio demonstrates excellent reproducibility, and up to a 10-log dynamic range. This improved accuracy and sensitivity enables this platform to be used across a broad range of applications in addition to residual DNA testing, including analysis of gene expression, and micro RNAs.

AccuSeq Analysis Software

The last part of the system, which wraps the entire qPCR workflow, is the AccuSEQ real-time PCR detection software. AccuSEQ software integrates with the QuantStudio 5 and the 7500 Fast instruments, and has been developed with security, audit and e-signature capabilities to enable 21 CFR Part 11 compliance. Figure 3 shows an example of the traceability this software can provide: every change in experimental properties is tracked and recorded in an audit trail. For example, when data are analyzed or a sample is run, the software records what was executed, when, and by whom.

ASSAY RESULTS & STUDY DESIGN

The objective of this study was to determine the performance of the resDNASEQ Quantitative HEK-293 kit. Several parameters, including linearity and PCR efficiency, were tested to ensure accurate quantification of residual DNA. Precision was also tested to ensure the data produced were consistent and reliable. Limit of Detection (LOD), Limit of Quantitation (LOQ) and assay range were also tested to ensure optimal sensitivity and to help support regulatory compliance in measurement of residual DNA. The study design included 3 operators, 2 manufactured lots of the kit, 2 sample prep methods (manual and automated) and 2 instruments: the 7500 Fast and the Quant Studio 5. All measurements were run in triplicate.

Starting with sample extraction, manual and automated sample prep methods were used to extract from a variety of matrices

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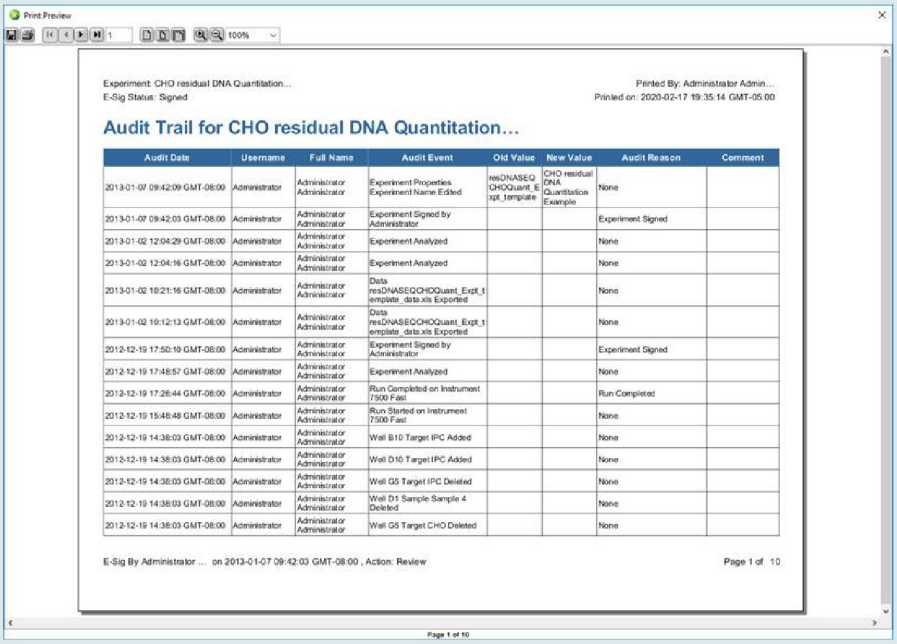
FIGURE 3

Analysis: help enable 21 CFR Pt 11 compliance.



AccuSEQ™ real-time PCR detection software

Integrates with the Applied Biosystems QuantStudio 5 and 7500 Fast Real-time PCR instruments

Developed with security, audit, and e-signature capabilities to help enable 21 CFR Pt 11 compliance

Features ensure full traceability

common to gene therapy and bioproduction workflows, outlined in Table 2. Once obtained, the extracts were spiked with an internal positive control allowing determination of whether inhibition is taking place in the sample: this serves as a measure of reliability of the assay. Internal positive controls were detected under all conditions, indicating that the PrepSeq reagent effectively removed inhibitors from these matrices.

Comparing instruments

Firstly, standard curve performance was tested on the 7500 Fast. Two lots of HEK-293 residual DNA assay were tested for linearity and PCR efficiency. R-squared of the standard curve was 0.999 for both lots, and PCR efficiency was 102% for Lot1 and 101% for Lot 2. This high linearity and efficiency enable the assay to measure

TABLE 2

Results: sample extraction.

Gene therapy matrices tested		Assay performs in extracts from samples containing	
Sample derived from a bioreactor at harvest	✓	Benzonase	✓
Sample after chromatography	✓	Excess DNA of other species	✓
Sample after final purification	✓	Detergent	✓
		Cell culture media	✓

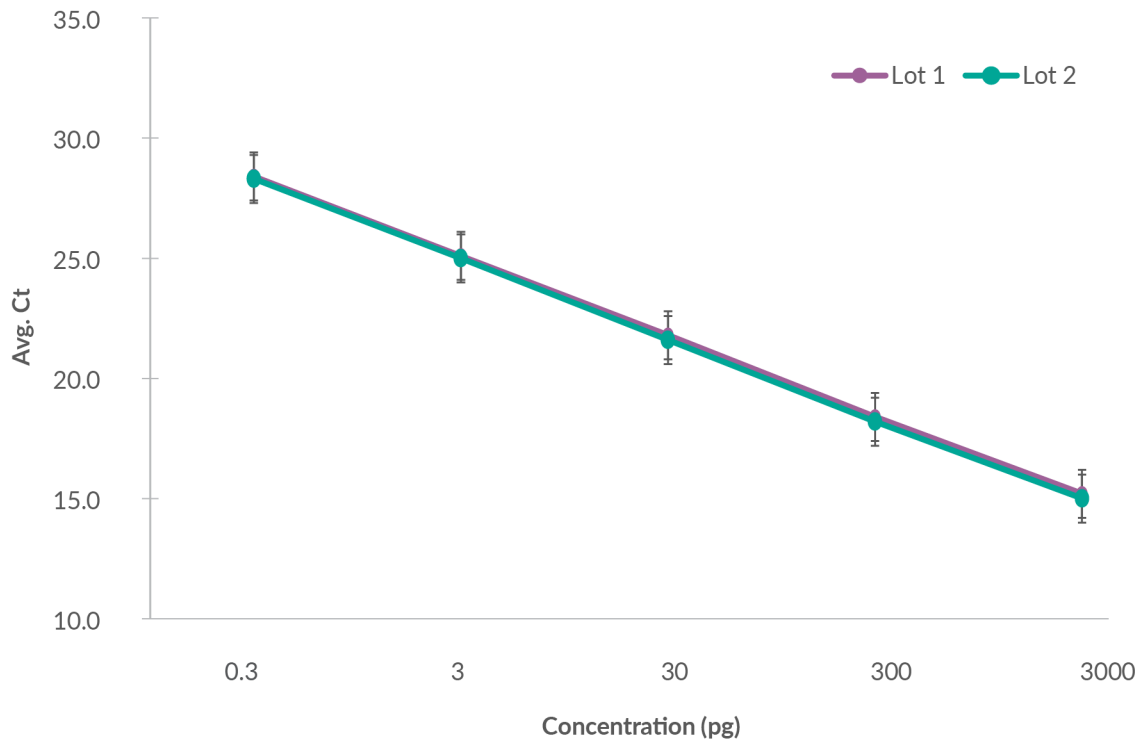
Sample matrix:

- Manual and automated sample prep methods were used to extract from matrices outlined below
- Extracts were spiked with the internal positive control (IPC)
- IPC was successfully detected across all sample types

Results show that sample prep successfully prepared samples from a variety of matrices common to gene therapy bioproduction workflows.

FIGURE 4

Standard curve performance (Applied Biosystems 7500 Fast Real-Time PCR Instrument).



Results demonstrate high linearity and efficiency to enable quantitative results across a broad range of DNA concentrations. PCR Efficiency = 102% (Lot1) and 101% (Lot 2) R^2 = 0.999 (Lot 1) and 0.999 (Lot 2), from 0.3 to 3000 pg.

TABLE 3

Precision measurement (Applied Biosystems 7500 Fast Real-Time PCR Instrument).

	Intra-run					
	HEK293 (Lot 1)			HEK293 (Lot 2)		
DNA spike amount	Avg Ct	Standard deviation	%CV	Avg Ct	Standard deviation	%CV
3,000 pg	15.2	0.08	0.51	15.0	0.04	0.30
300 pg	18.4	0.07	0.38	18.2	0.05	0.29
30 pg	21.8	0.08	0.38	21.6	0.04	0.18
3 pg	25.1	0.08	0.33	25.0	0.03	0.13
0.3 pg	28.4	0.09	0.31	28.3	0.08	0.29
	Inter-run					
	HEK293 (Lot 1)			HEK293 (Lot 2)		
DNA spike amount	Avg Ct	Standard deviation	%CV	Avg Ct	Standard deviation	%CV
3,000 pg	15.2	0.04	0.26	15.0	0.01	0.04
300 pg	18.4	0.02	0.12	18.2	0.03	0.14
30 pg	21.8	0.05	0.22	21.6	0.03	0.16
3 pg	25.1	0.09	0.36	24.9	0.03	0.11
0.3 pg	28.4	0.14	0.48	28.3	0.08	0.28

The standard curve was tested across 6 runs on the 7500 Fast.
Intra-run precision across all concentrations was less than 1% CV.
Inter-run precision across all concentrations was less than 1% CV.
High precision observed at as low as 0.3 pg per reaction.

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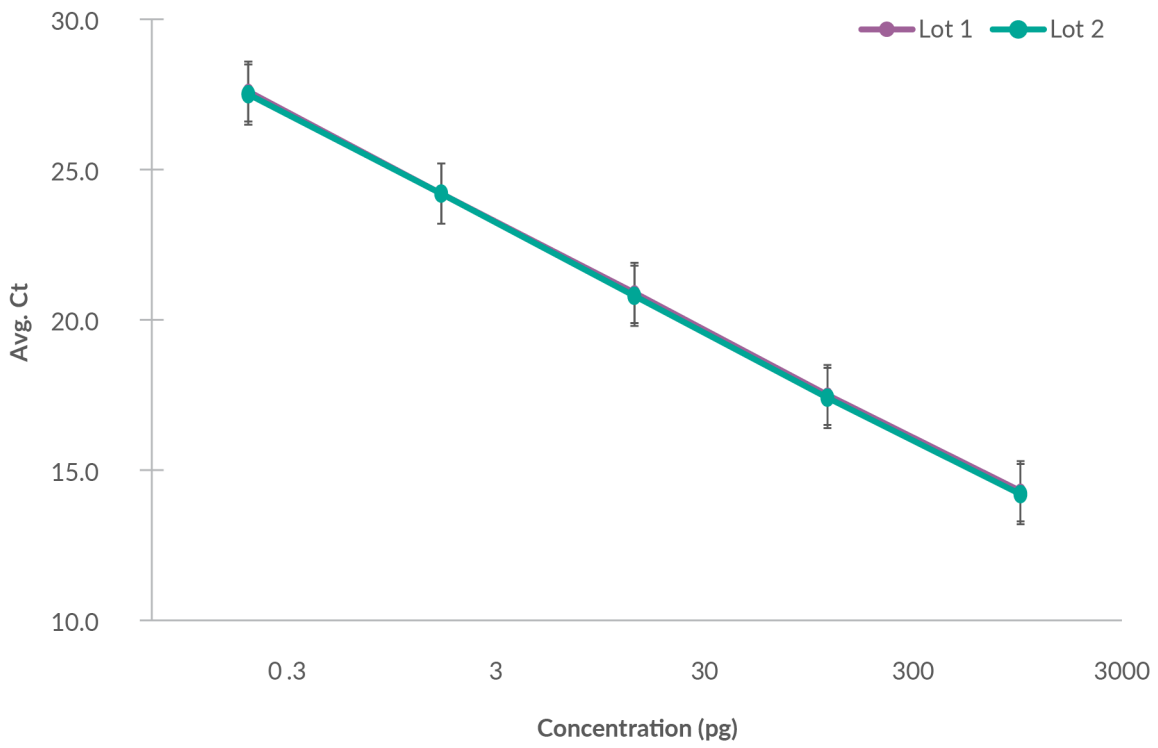
FIGURE 5

Standard curve performance (QuantStudio 5 Real-Time PCR Instrument).



PCR Efficiency = 103% (Lot1) and 101% (Lot 2) R^2 = 0.999 (Lot 1) and 1.000 (Lot 2), from 0.3 to 3000 pg. Results demonstrate high linearity and efficiency to enable quantitative results across a broad range of DNA concentrations.

TABLE 4

Precision (QuantStudio 5 Real-Time PCR Instrument).

	Intra-run					
	HEK293 (Lot 1)			HEK293 (Lot 2)		
DNA spike amount	Avg Ct	Standard deviation	%CV	Avg Ct	Standard deviation	%CV
3,000 pg	14.3	0.06	0.39	14.2	0.02	0.13
300 pg	17.5	0.04	0.22	17.4	0.02	0.10
30 pg	20.9	0.05	0.23	20.8	0.02	0.10
3 pg	24.2	0.06	0.24	24.2	0.03	0.12
0.3 pg	27.6	0.06	0.20	27.5	0.02	0.08
	Inter-run					
	HEK293 (Lot 1)			HEK293 (Lot 2)		
DNA spike amount	Avg Ct	Standard deviation	%CV	Avg Ct	Standard deviation	%CV
3,000 pg	14.3	0.01	0.06	14.2	0.04	0.27
300 pg	17.5	0.08	0.43	17.4	0.07	0.39
30 pg	20.9	0.05	0.23	20.8	0.05	0.24
3 pg	24.2	0.06	0.25	24.2	0.04	0.16
0.3 pg	27.6	0.05	0.19	27.5	0.08	0.30

The standard curve was tested across 4 runs on the QuantStudio 5. Intra-run precision across all concentrations was less than 1% CV. Inter-run precision across all concentrations was less than 1% CV. High precision observed as low as 0.3 pg per reaction. Results demonstrate high precision, indicating that data are consistent and reliable within runs and between runs, even when quantitating very low levels of DNA.

DNA quantitatively across a broad range of concentrations.

Figure 4 demonstrates the type of standard curve obtained from both lots. From lot-to-lot, the data are extremely consistent between 0.3 and 3,000 picograms (pg). Standard curve performance was then compared across six runs on the 7500 Fast. Intra-run precision across all concentrations was less than 1% coefficient of variation (CV), and run-to-run precision was also very tight at less than 1% CV. These results demonstrate extremely high precision, indicating that data will be consistent and reliable both within runs and between runs. (Table 3).

Finally, detection at very low levels of DNA was tested. For this example, a no template control (NTC) was run in parallel with DNA samples at 30 pg. The NTC values were consistently higher than control template values, demonstrating the capability of detecting 30 pg in an extract.

For the QuantStudio 5, standard curve performance was again investigated on two lots of the HEK293 residual DNA kit. Linearity was once again very high with an R-squared value of 0.999 for Lot 1, and 1.0 for Lot 2 (Figure 5). PCR efficiency was 103% for Lot 1 and 101% for Lot 2. Together, these data show that quantitative results may be achieved across a broad range of DNA concentrations on the QuantStudio 5. Results also demonstrated extremely consistent standard curves from Lot to Lot. Intra-run precision was less than 1% CV across four runs of the standard curve. Intra-run precision across four runs was also less than 1% CV in both assay lots. Finally, a 30 pg sample run in parallel with an NTC demonstrated a distinguishable difference, indicating reliable detection at this low DNA concentration. (Table 4).

CONCLUSION

These data show that HEK293 resDNASEQ is a comprehensive system that can provide consistent and reliable data even when quantitating very low levels of DNA. It also provides a rapid workflow, with a time to results of less than 5 hours, including optimized sample preparation. The development of solutions such as resDNASEQ is needed in order to ensure that gene therapy manufacturers are able to meet the strict limitations on residual DNA required by regulators.

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VIEW RESOURCES

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AUTHORSHIP & CONFLICT OF INTEREST

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