CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Addressing regulatory guidance for HEK293 cells & AAV-based therapeutics manufacturing

Mike Brewer

Testing and characterization in cell and gene therapy manufacturing is critical for AAV-based therapeutics. Regulatory guidance affecting those using HEK293 cells or AAV-based therapeutics is evolving over time, especially pertaining to quantitating residual host cell DNA and analyzing its size via E1A fragments, quantitating residual plasmid DNA (pDNA), and detecting the presence of the E1A oncogene. Integrated solutions leveraging real-time PCR or dPCR technologies are necessary to meet regulatory needs.

This article will cover some of the latest regulations around residual DNA amounts in the product, as well as quantitating host cell and pDNA, and the presence and size of the E1A oncogene. Integrated dPCR and qPCR assay solutions will also be introduced.

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RESIDUAL HOST CELL DNA TESTING GUIDANCE

Regulatory guidance for residual host cell DNA testing has existed since the beginning of the recombinant DNA biopharma revolution starting in the early 1980s. That guidance has evolved over time maintain pace with technological advances. For example, the most recent chemistry, manufacturing and control (CMC) guidance for human gene therapy investigational new drug (INDs) involves taking steps to minimize the biological activity of any residual DNA associated with a viral preparation. This can be accomplished by reducing the size of the DNA to below the size of a functional gene and decreasing the amount of residual DNA. The recommendation is that the amount of residual DNA is limited to 10 ng per dose and the DNA size to below ~200 base pairs (bp).

It is recommended that sponsors carefully consider the characteristics of cell lines used in the manufacture of viral vectors that may impact the safety of the final product, including the presence of tumorigenic sequences. The Human Gene Therapy for Neurodegenerative Disease FDA Guidance for Industry

advises limiting residual host cell DNA levels. Historically, guidance for industry was first developed for cell culture-based vaccine manufacturing in February 2010. This mentioned the potential risk of residual host cell DNA from certain host cell lines and provided guidance on decreasing its biological activity by size reduction.

Testing for residual DNA in AAV manufacturing occurs at the end of purification. Optimizing the manufacturing process to reduce non-vector DNA contamination in the final product is recommended.

A SOLUTION FOR MEASURING & QUANTITATING RESIDUAL HOST CELL DNA

The Applied Biosystems™ resDNASEQ™ Residual DNA Quantitation System includes optimized sample preparation and assays specific to the host cell DNA. The system is highly sensitive with an overall method limit of quantitation (LOQ) of as low as 1.5 pg/mL of test sample for mammalian DNA, and 15 pg/mL for bacterial or yeast cell culture processes. Generally, the workflow leads to a rapid result in <5 h, giving highly consistent results, especially when using the automated sample preparation options. There is typically an extremely low failure rate due to percentage coefficient of variation (CV) in the triplicate samples. This system leverages a worldwide support network, technical validation support, as well as hands-on training by expert Field Application Specialists.

The resDNASEQ Quantitative HEK293 DNA Kit is an assay specifically for HEK293 cell line-based manufacturing processes. This quantitative PCR (qPCR)-based system includes precisely quantitated, highly purified genomic DNA from an established Thermo Fisher Scientific HEK293 cell line as a standard control. The kit has a high adoption rate, with 50% of the top 100 large pharma companies worldwide relying on the reliable performance of resDNASEQ. The quantitation of the host cell DNA with this assay is independent of the DNA size. The resDNASEQ system has also been shown to give reliable results across multiple stages in the gene therapy manufacturing process.

QUANTITATING FRAGMENT LENGTHS

Guidance on specific gene testing for HEK293 cells states that in addition to controlling the host cell DNA content and size, the level of relevant transforming sequences should also be controlled. In this case, products made in 293 cells should be tested for specific genes in 293. A resDNASEQ Quantitative E1A DNA Fragment Length Kit has been developed for gene therapy and cell-based vaccine manufacturers who use HEK293 and need to quantitate the fragment lengths of residual DNA to adhere to new regulatory guidelines for the clearance of residual DNA fragments >200 bp, and to identify the oncogenic potential. It has a 3-in-1 assay design to allow the differentiation of three different fragment lengths (86 bp, 200 bp, and 478 bp). The assay has ultra-high sensitivity, being able to accurately quantitate down to 30 copies of the target in the qPCR reaction. The results shown in Figure 1 demonstrate linearity and high efficiency to enable quantitative results across a broad range of DNA concentrations. The resDNASEQ™ E1A DNA Fragment Length Kit is a comprehensive product solution with the same rapid testing and sample prep chemistry as the resDNASEQ™ Residual DNA Quantitation System.

Measuring E1A fragments is an excellent approach for assessing the effectiveness of DNA size reduction steps, and also ensuring the oncogenic E1A gene has reduced to a size that is ≤200 bp E1A is an oncogene that is transformed into HEK293 cells. It is essential for the transcription of other viral genes, which are responsible for viral DNA synthesis and play roles in modulating the expression of host genes. Adeno-associated

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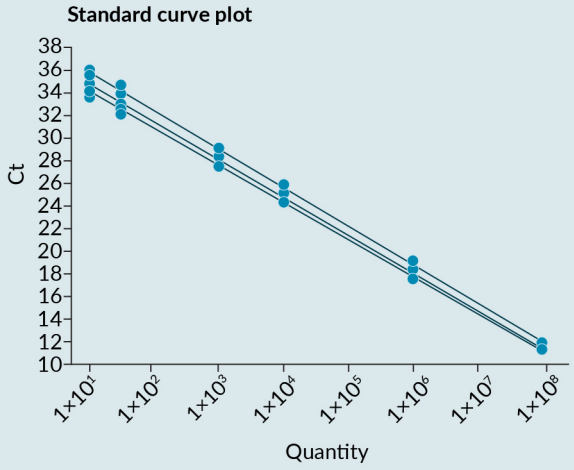
FIGURE 1

Standard curve performance of the resDNASEQ Quantitative E1A DNA Fragment Length Kit.



	Short	Medium	Long
Fragment size (bp)	86–200	200–476	>476
Slope	–3.284	–3.363	–3.433
R^2	0.999	0.999	0.999
Efficiency (%)	101	98	95

Ct: Cycle threshold: bp: Base pairs.

virus (AAV) is actively used as a gene therapy vehicle to transport modified genes into the cells. Typically, recombinant AAV is manufactured in HEK293 cells, and HEK293 is also a frequently used cell line in the production of cell-based vaccines. Manufacturers want to ensure efficiency in the DNA size reduction step (i.e., benzonase) and ensure clearance of the E1A gene in the final product.

PLASMID VECTOR QUANTITATION: KANAMYCIN RESISTANCE GENE KIT

Typically, multiple plasmids are co-transfected into the cells as part of the manufacturing process to induce the production of recombinant AAV. For gene therapy/vaccine manufacturers who are currently using plasmids in their workflow, and for plasmid manufacturers themselves, Thermo Fisher provides a robust, easy-to-use, highly sensitive multiplex qPCR assay to measure residual pDNA by targeting all common alleles of kanamycin resistance genes. This enables measurements of the presence and the removal of the pDNA during purification. It can also be used by plasmid manufacturers to quantitate pDNA through purification and in the final purified form. As a comprehensive solution, the product has high sensitivity and targets the common alleles in plasmids commonly used in the AAV workflow. The kit enables accurate quantitation as low as 30 copies in a test sample.

Amplification plots were generated using serial dilutions of pDNA standard (ranging from 15 copies to 300,000 copies provided in the kit), as shown in Figure 2. The broad linear range allows the testing of a wide range of Kanamycin-resistant pDNA samples.

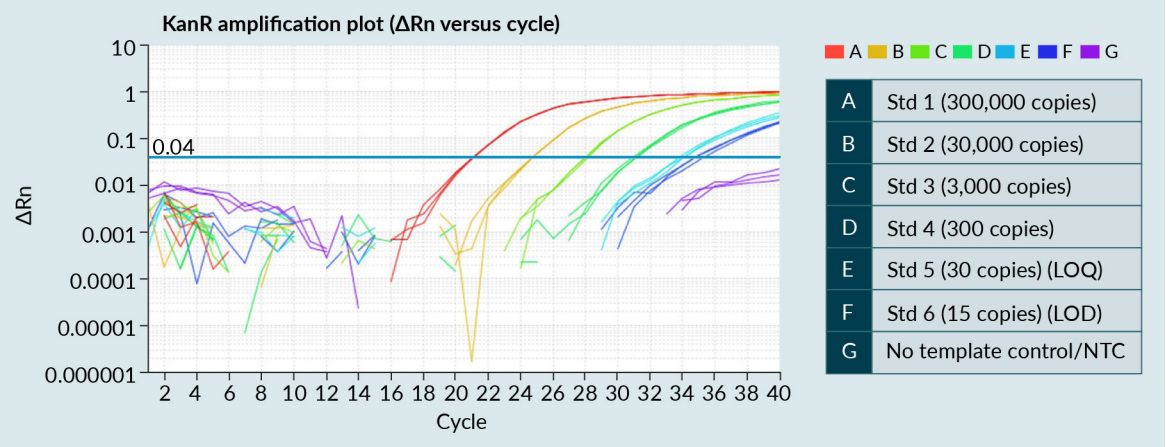
Utility is shown throughout the manufacturing process and in the manufacturing of plasmids. The standard curve performance is very linear, demonstrating that the Kanamycin Resistance Gene Kit is capable of enabling quantitation across a broad range.

AAV VIRAL PARTICLE QUANTITATION

At the 70th Cellular Tissue and Gene Therapy Advisory Committee meeting in September 2021, additional guidance surrounding the risk of hepatoxicity observed in clinical trials with high doses of AAV vectors was discussed. The fact that many AAV products contain

FIGURE 2

Amplification plots showing the sensitivity and dynamic range of the Kanamycin Resistance Gene Kit.



Std: Standard.

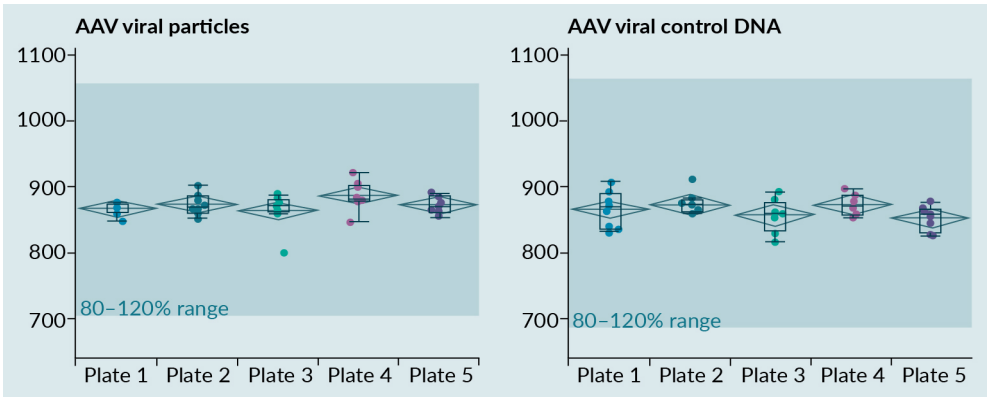
significant amounts of empty capsids was noted. It was concluded that assays for empty capsids need better standardization, and more effort is needed to comprehensively characterize empty capsids and other byproducts of AAV manufacturing.

However, before specific guidance on acceptable levels of empty capsids can be provided, there needs to be a better understanding of the manufacturing process. To help address this issue, Thermo Fisher has a dPCR assay that provides a sensitive and accurate quantitation of AAV genomes. dPCR is a method of quantifying nucleic acid targets without standard curves by dividing the bulk reaction into thousands of smaller, independent reactions. Individual molecules are amplified in each microchamber, and positive and negative microchambers are simply counted.

Results of the readout of the dPCR assay for counting viral genomes of AAV are shown in Figure 3. This assay targets the ITR2 sequence of AAV viral particles, as well as a DNA control. The Absolute Q dPCR instrument demonstrates consistent quantification

FIGURE 3

Demonstrating consistent AAV quantification across plates.



AAV: Adeno-associated virus.

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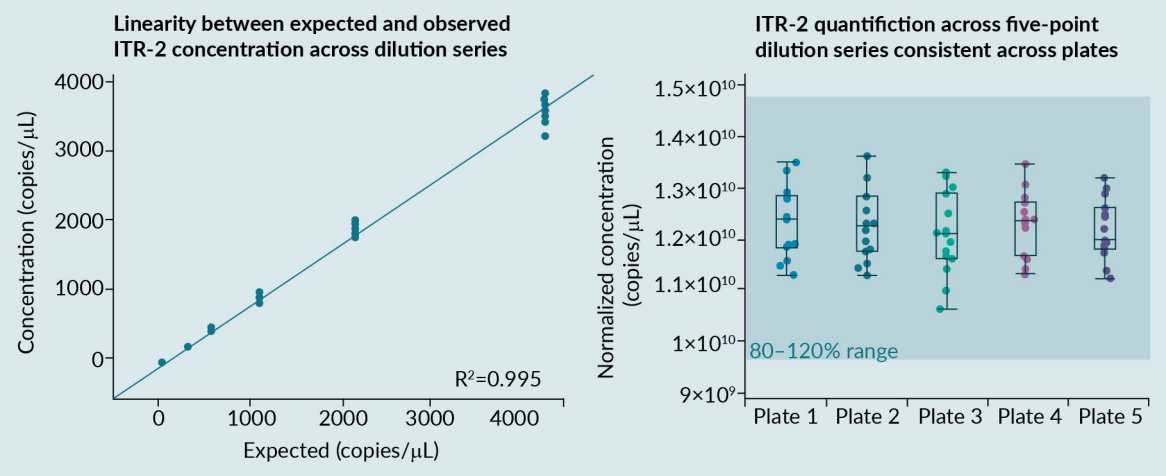
FIGURE 4

Linearity between expected and observed ITR-2 concentration across dilution series.



ITR: Inverted terminal repeats.

of ITR2 sequence using both AAV particles and AAV DNA control.

Figure 4 shows the linearity of the dPCR solution across a five-point AAV dilution series. The accuracy and consistency of the data are high, even at lower concentrations of the AAV targets (<1,000 copies/test sample).

CELL CULTURE IMPURITIES: qPCR TESTING FOR MYCOPLASMA

Mycoplasma testing guidance from FDA states that vector safety testing should include microbiological testing, such as sterility, mycoplasma, endotoxin, and adventitious agent testing, in order to ensure that the chimeric antigen receptor(CAR-T) cell drug product is not compromised. Mycoplasma testing is required at cell culture harvest for virus used in gene therapy and for transduction of T cells in cell therapy. In the manufacturing process of recombinant AAV, mycoplasma testing is typically done at the stage of bulk harvest from the bioreactor producing the recombinant virus. Downstream, residual DNA impurities such as plasmid host cell DNA and E1A are measured.

The MycoSEQ Mycoplasma Detection System is an integrated sample preparation and qPCR assay for the detection of mycoplasmas that is accepted by regulators across multiple therapeutic modalities, including recombinant proteins, monoclonal antibodies, vaccines, and viral vectors. It can also be used in cell-based therapy manufacturing as a release test.

This assay was designed to exceed the guidelines as a nucleic acid amplification technique (NAT)-based alternative method for lot-release testing. It includes an optimized sample preparation developed for a protocol starting with 10+ mL of the starting test sample to enable an analysis of 1 mL of test sample equivalent in a qPCR reaction. The system and instrument have an integrated software platform that enables full compliance with 21 CFR Part 11 electronic records expectations. Instrument Installation Qualification and Operational Qualification services are offered, with an extensive network of field application scientists offering instrument training and providing guidance on method qualification, implementation, and validation.

The method provides highly confident results, uses objective multi-parameter analysis, and incorporates a proprietary and patented discriminatory positive control. It enables the differentiation of real mycoplasma targets and accidental cross-contamination

with a positive extraction control. It is also highly sensitive and consistent across the various mycoplasma species: the assay is designed to support the detection of over 140 species and is sensitive down to 1–3 genome copies per qPCR reaction. It has also been demonstrated to be specific to mycoplasma species and does not detect microorganisms or non-mycoplasma microorganisms that are related genetically to mycoplasma.

Following validation, regulatory filing, and review, end users have received regulatory acceptance to use MycoSEQ across a variety of therapeutic modalities including cell culture, cell therapy, and tissue therapies. This complete sample-to-answer solution typically uses the cartridge-based magnetic bead processing system the AutoMate Express. Following extraction of the DNA from the samples, the MycoSEQ qPCR assay is employed, typically running on the Applied Biosystems™ QuantStudio 5 or 7500 Fast Real-Time PCR instruments, with analysis on the fit-for-purpose, application-specific AccuSEQ software.

The Ct value obtained with this assay is a measure of the amount of DNA present at the beginning of the qPCR reaction. Ct data can be compared from experiment to experiment and lab to lab, and is shown to be consistent across studies.

SUMMARY

The constantly evolving regulatory environment for HEK293 cells and AAV-based therapeutics manufacturing necessitates integrated solutions to quantitate residual host cell DNA, fragment lengths, residual pDNA, AAV genomes, and cell culture impurities. Thermo Fisher Scientific has established a multitude of real-time qPCR and dPCR assays in order to enable the manufacture of HEK293 and AAV-based therapeutics whilst meeting all regulatory requirements. Each of these assays has been validated by multiple end users globally.

Q&A

Mike Brewer

Q I've heard that sample preparation is not needed for analysis with dPCR. Can you provide more details of that?

MB: This is a somewhat nuanced issue. In cases where the concentration of analyte is significantly high, where the sample can be diluted prior to analysis by dPCR, there is no sample prep needed. That is also the case for qPCR. As you move deeper into a purification process where the amounts of target DNA are reduced and the amount of protein or virus and excipients in the sample are much higher, it is often necessary to use a sample prep, whether using qPCR or dPCR.

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Q I noticed that the regulatory specifications on host cell DNA levels are in nanograms of DNA per dose. Can dPCR, which reports in copies of DNA in a reaction, be used for host cell DNA testing?

MB: It is often the case that when you are looking to find the right tool for a job, there is a good tool and a best tool. dPCR counts copies of the target, whilst qPCR measures the amount of DNA in a test sample through the comparison of qPCR of the DNA in the test sample to a standard curve generated by qPCR analysis of known amounts of the standard DNA. Typically, qPCR reports in nanograms, picograms, or femtograms of DNA, aligned with the regulatory guidance. Regulatory guidance and the expectations on limits that are acceptable per therapeutic dose are in nanograms.

When using dPCR, a well-designed study must be performed to be able to accurately correlate the number of copies in the digital readout to the mass of the DNA in the test sample. That can be a challenging experiment to carry out, especially because the size of the DNA can make a big difference.

BIOGRAPHY

MICHAEL BREWER is the Director, Global Principal Consultant, Regulatory for the BioProduction Group (BPG) at Thermo Fisher Scientific. In this role, Michael is responsible for providing global support to BioProduction customers and serving as the regulatory thought leader and expert across all technology areas within BPD. Prior to moving to this role, he led the Pharma Analytics business, a team responsible for development and commercialization of testing applications for Microbiology, Analytical Sciences and Quality control. The products are fully integrated solutions for Glycan profiling, Bacterial and Fungal identification, Mycoplasma and Viral detection and host cell DNA and protein quantitation. Michael has over 30 years of experience in the Biopharma industry, including, Scios, Synergen and Amgen in a variety of roles including Discovery Research, Analytical Sciences and Quality Control. Prior to joining Thermo Fisher Scientific, he led a group at Amgen that developed qualified, validated and implemented molecular methods for host cell DNA quantitation, contaminant (mycoplasma, virus and bacteria) detection, contaminant identification, strain typing and genotypic verification of production cell lines. Additionally, his group supported regulatory submissions including IND, NDA, and CMC updates, Regulatory inspections, NC/CAPA investigations, contamination investigations and remediation and developed regulatory strategy for implementation of new methods.

AFFILIATION

Mike Brewer
Director and Global Principal Consultant, Regulatory,
Thermo Fisher Scientific

ThermoFisher SCIENTIFIC

AUTHORSHIP & CONFLICT OF INTEREST

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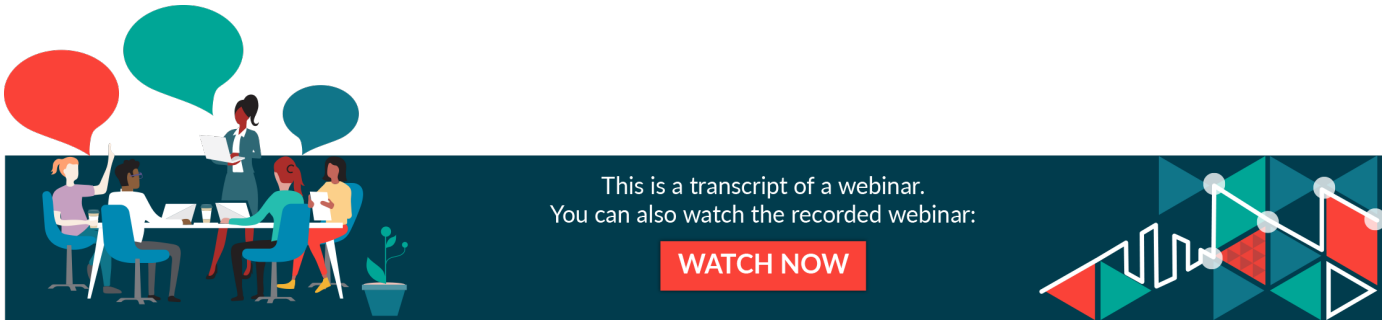

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A fully integrated, easy-to-use solution to help you meet regulatory guidance

Applied Biosystems™ resDNASEQ™ kits are quantitative PCR-based assays designed to enable accurate quantitation of residual host cell DNA and residual plasmid DNA. This is crucial in demonstrating the removal of host cell and process-based plasmid impurities during the purification of biopharmaceutical products—a global regulatory requirement.

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