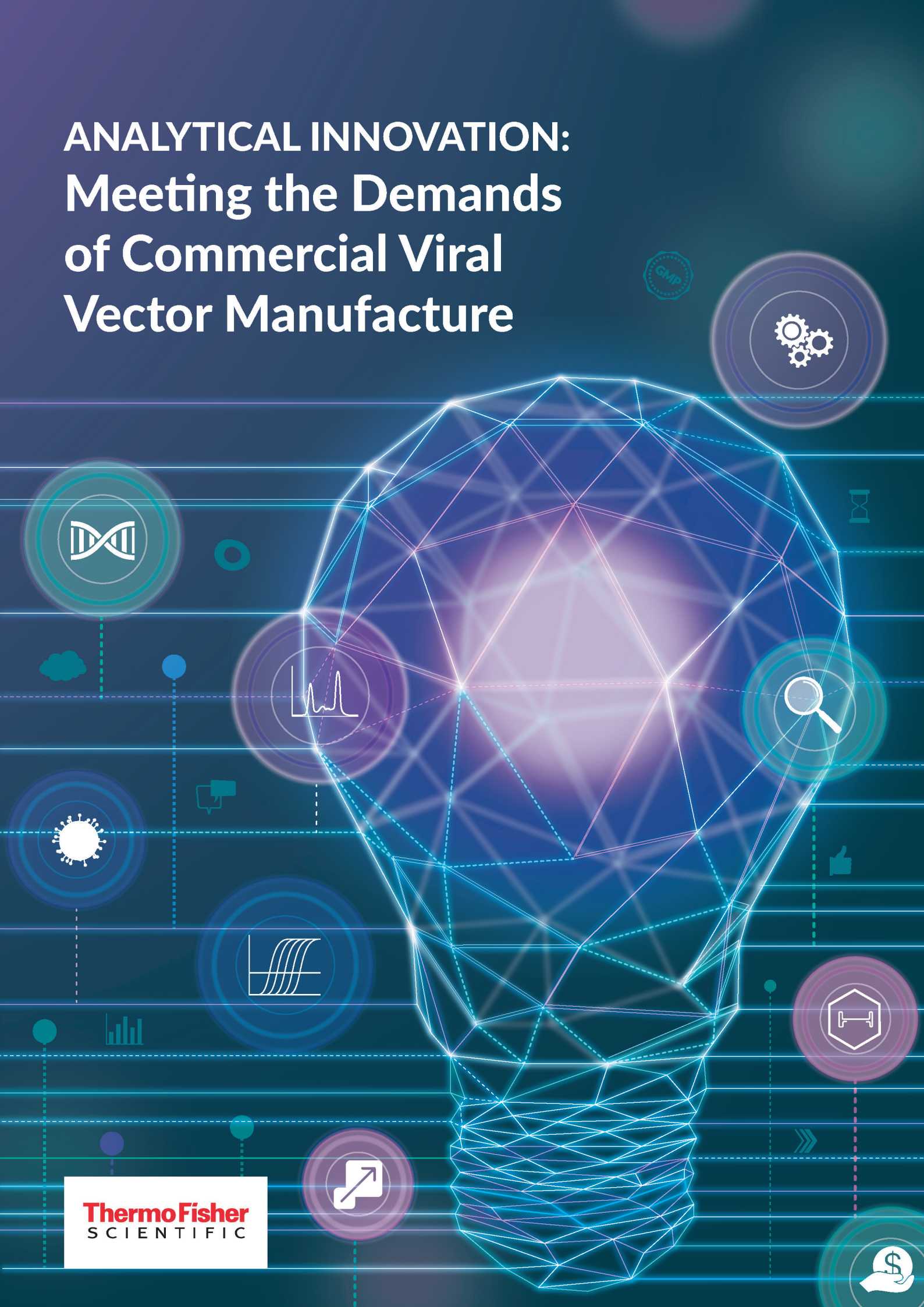
ANALYTICAL INNOVATION: Meeting the Demands of Commercial Viral Vector Manufacture



ThermoFisher SCIENTIFIC

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Meeting the Demands of Commercial Viral Vector Manufacture

David McCall, Editor, *Cell & Gene Therapy Insights*

FOREWORD

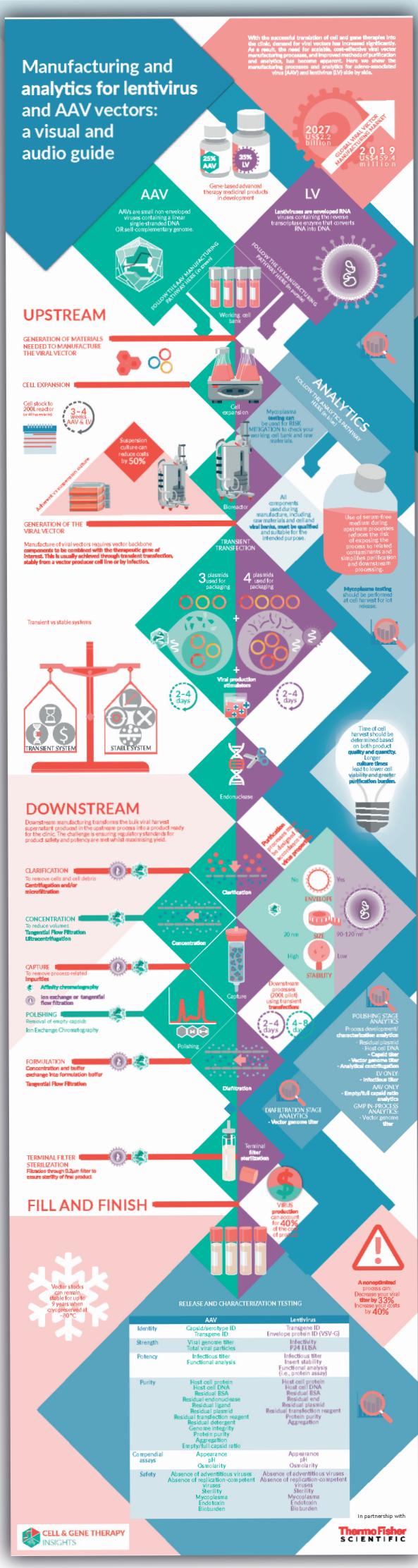
The viral vector-driven cell and gene therapy field has made its long-awaited commercial breakthrough in recent years, with a series of products achieving the landmark of marketing authorization. Six CAR-T cell immunotherapies, all of which are transduced using lentiviral (LV) vectors, have become commercial products in the hematological malignancy space. Meanwhile on the adeno-associated viral (AAV) vector side, groundbreaking *in vivo* gene therapies for rare diseases with high unmet medical need (e.g., spinal muscular atrophy, Leber congenital amaurosis) have given new hope of a cure to patients.

This eBook from **Thermo Fisher Scientific** and ***Cell & Gene Therapy Insights*** explores the cutting edge in analytical innovation and applications across the viral vector field.

At the end of 2017, Dr Peter Marks, Director of the US FDA's Center for Biologics Evaluation and Research (CBER), heralded the landmark approval of one such AAV-driven product, stating that it "further opens the door to the potential of gene therapies." However, meeting regulators' late-phase development and commercial-stage CMC/QC expectations has proven to be a major obstacle to further advances and product approvals in the field. CMC-related setbacks have haunted cell and gene therapy—for example, in the rejection of a number of high-profile product candidates due to the lack of sufficiently robust potency assays. Meanwhile, the rapid rise to prominence of the empty/full/partially full capsid ratio with the recognition of its importance to the safety and efficacy of AAV-based gene therapies has underlined the pressing requirement for both analytical tool innovation and new regulatory guidance in the space.

The gene therapy community is responding. Analytical technology and service providers are moving to plug the innovation gap that exists, particularly in the LV space, and progress is being made in delivering the tools and assays required to understand and characterize viral vectors. Regulators are working to deliver more specific guidance, while gene therapy developers and manufacturers themselves are recognizing the critical importance of investing in analytical development and product characterization from an early stage of R&D in order to avoid expensive delays later on.

This eBook from Thermo Fisher Scientific and Cell and Gene Therapy Insights explores the cutting edge in analytical innovation and applications across the viral vector field. Inside, you will find webinars, FastFacts videos, infographics and articles that provide invaluable insights and guidance on how to wield these tools to navigate the constantly evolving regulatory CMC landscape for viral vector-driven advanced therapies.



INTERACTIVE INFOGRAPHIC

Manufacturing and analytics for lentivirus and AAV vectors

Interactive

Highlight the key pathways:
- Analytic
- Lentiviral
- Adenoviral

With audio narration

VIEW INFOGRAPHIC

CELL & GENE THERAPY INSIGHTS

ANALYTICAL INNOVATION:
Meeting the Demands of Commercial Viral Vector Manufacture

CHAPTER 1

Simplifying analytical development of viral vector production: robust and sensitive methods for common expression systems

Srinath Kashi Ranganath

The number of viral vector-based gene therapies in clinical trials has recently grown into the thousands due to the tremendous genetic disease-curing potential they harbor. Despite this growth, the comprehensive characterization of critical quality attributes for the safety and efficacy of the material produced for these trials remains a challenge for both manufacturers and regulatory bodies alike. The demands on analytical development teams are oversized compared to legacy biopharmaceuticals and require a unique focus to address issues such as identification, characterization, and enumeration of undesired byproducts. Application of established regulatory guidance, such as limits to residual host cell DNA, requires additional scrutiny due to possible encapsidation and oncogenic potential. This article will focus on the current state of analytical methods in gene therapy workflows, and how leveraging the work Thermo Fisher Scientific has developed can help simplify the burden on analytical development teams.

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Our solutions facilitate one of two highly advanced and well-established core technologies: DNA sequencing, and real time PCR (qPCR). Our MicroSEQ™ microbial identification system utilizes gold standard genotypic DNA sequencing technology to provide accurate identification of potential fungal and bacterial contaminants. This can be used as part of an environmental monitoring plan or a robust microbial risk mitigation strategy. Our solutions for detection of advantageous agents such as mycoplasma, viruses, and quantitation of residual host cell DNA utilize qPCR to achieve fast, accurate, reliable, and actionable results.

SAFETY & EFFICACY IN THE GENE THERAPY FIELD

The gene therapy field is advancing rapidly, with seven currently approved viral-vector gene-therapy-based therapeutics worldwide, and over 3,000 clinical trials in the pipeline. Notable AAV-based therapeutics include Luxturna for treatment of Leber congenital amaurosis-2 (LCA2), and Zolgensma for treatment of spinal muscular atrophy (SMA). Investments in this area are estimated to reach $3.4 billion in 2024 and will more than double to $6.5 billion by 2030. Despite the tremendous success of many viral vector-based gene therapies, recent adverse effects in clinical trials, such as development of liver cancer, have elevated the urgency of addressing safety and efficacy. These issues need to be investigated in preclinical models and patient monitoring in absorption, distribution, metabolism, and excretion (ADME) studies. Some can likely be addressed by analytical teams via monitoring of the critical quality attributes of the drug product itself and through employing novel analytical techniques.

At the 2021 US FDA meeting, the potential vector-mediated integration of non-vector DNA and the risk of oncogenesis in viral vector-based gene therapies were addressed. Assessing the risk requires analysis and elimination of sequences in vectors that increase the risk of oncogenesis.

ANALYTICAL TESTING OPTIONS FOR GENE THERAPY CUSTOMERS

There are three options for analytical testing for gene therapy: developing home-brew methods in-house, using contract testing service labs, or using fully integrated commercial solutions. The general trend in the industry is to use commercial solutions in order to save both time and resources, and as the industry matures more solutions are being developed. This article will address the support provided by Thermo Fisher Scientific toward this goal, and the capabilities of current products to assist in characterization of nucleic acid impurities, removing the challenges of developing these analytical requirements in-house.

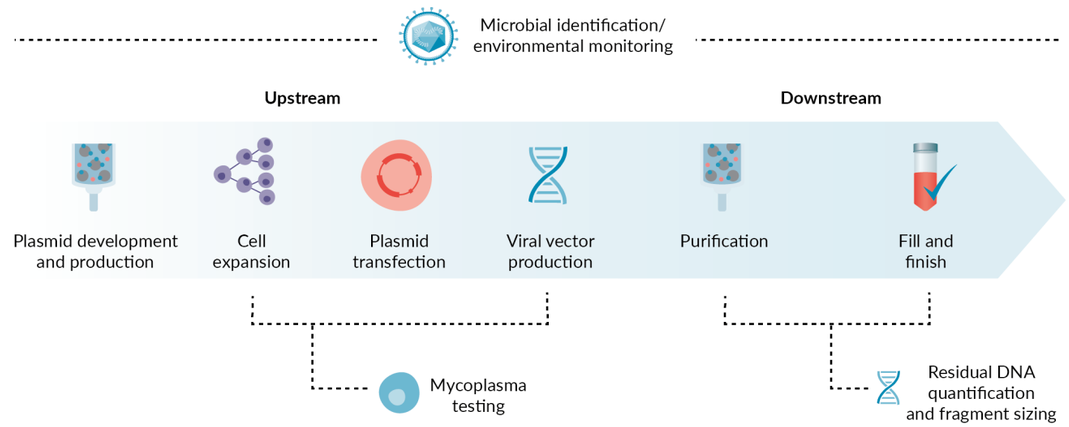
ANALYTICAL ASSAYS OFFERED BY THERMO FISHER SCIENTIFIC

In addition to the areas of increased concern and scrutiny discussed above, there are a myriad of analytical assays for characterization and release testing for viral vector products. Applied Biosystems™ resDNASEQ™ residual DNA systems were developed to rapidly and accurately quantify residual host cell DNA and plasma DNA. To address the concern of the presence of mycoplasma, the first regulatory-accepted rapid molecular method was developed: The MycoSEQ™ mycoplasma detection system. To address the requirements for the identification of potentially contaminating microbes, the MicroSEQ™ microbial identification system was developed.

Figure 1 provides a view of the analytical assays Thermo Fisher has developed to simplify in-house analytical development for viral vector manufacturers, which are designed to meet Current Good Manufacturing Practice (cGMP) regulations.

FIGURE 1

Simplifying analytical development to address established regulatory guidance.



RESIDUAL DNA QUANTITATION & FRAGMENT SIZING ANALYSIS

Residual DNA left in a product can impact quality, efficacy, and safety. Regulators worldwide therefore require limitations on the amount of residual DNA in the final dose. The WHO recommends that the amount of residual DNA per dose is kept below 10 ng. It is suggested by the FDA that a method with a sensitivity of 10 pg be used to determine DNA levels. Residual fragment length analysis is expected to demonstrate <200 base pairs (bp). There is increased concern that encapsidation is leading to viral vector products with larger amounts and longer sequences of residual DNA. Oncogenic sequences are of particular concern and must not be present in the final product.

Thermo Fisher offers end-to-end solutions consisting of all-inclusive kits with well-characterized standards and reagents. These assays have been designed to meet regulatory guidance with high sensitivity, reproducibility, and lot-to-lot consistency over several years.

The resDNASEQ™ residual DNA quantitation system (Figure 2) is the first and only fully integrated qPCR system for quantitation of residual host cell and plasmid DNA, including highly characterized DNA reference standards.

There are resDNASEQ™ solutions for both insect (Sf9) and mammalian cell culture-based viral vector manufacturing systems. This article focuses on the solutions for the mammalian expression system.

ANALYTICS FOR THE HEK293 PRODUCTION SYSTEM

Thermo Fisher Scientific offers multiple assays that apply to the HEK293 production system. These include solutions to quantify residual host cell and plasmid DNA and a residual adenovirus early region 1A (E1A) fragment sizing assay. The resDNASEQ™ quantitative E1A DNA fragment length kit is the newest assay developed specifically for HEK293 processes to address two additional aspects of regulatory guidance, in addition to quantitation of the residual DNA. This kit can simultaneously detect and quantify E1A DNA of different fragment sizes. All of these assays can be used throughout the downstream process to support the characterization and optimization of your process and for routine quality control (QC).

FIGURE 2

ResDNASEQ residual DNA quantitation system.

Ultrahigh sensitivity
0.3 pg/reaction LOQ for HEK293 DNA
30 copies/reaction LOQ for Kanamycin-Plasmid Resistant DNA
0.3 pg/reaction LOQ for Sf9 and Baculovirus DNA

Rapid testing and streamlined workflow
Time to results <5 hours with optimized sample prep

Reliable performance
Consistent performance kit to kit, lot to lot, year to year

ADENOVIRUS EARLY REGION 1A (E1A)

E1A is an oncogene integrated in chromosome 19 of HEK293 cells, providing essential genetic regulatory modulation for viral vector manufacture. This gene allows HEK293 and various related cell lines to be used to produce recombinant adenovirus, recombinant adeno-associated virus (AAV) and recombinant lentivirus. One current challenge in viral vector manufacturing is co-packaging of the host cell DNA within recombinant viral vector capsules. As E1A is both part of the HEK293 host cell genome and a known oncogene, any potential residual E1A requires detection and quantification as a harmful process-related impurity. Regulatory guidance requires the method used to demonstrate the effectiveness of the DNA reduction process to <200 bp fragments.

As shown in Figure 3, the E1A assay design involves three single-plex assays targeting known overlapping fragment sizes of short, medium, and long fragments. The assay requires three standard curves, one for each fragment size, to quantitate E1A fragments of unknown samples.

The E1A kit comes with all reagents and standards for all three single-plex assays. Each assay shows high linearity and efficiency and enables accurate qualitative results across a broad range of DNA concentrations. The kit has been validated in various matrices used in gene therapy to reflect typical application situations, including inhibitors and at varied concentrations. The kit has shown excellent performance under these conditions. The standard curve performance of the kit, as shown in Figure 4, demonstrates high linearity and efficiency to enable quantitative results across a broad range of DNA concentrations.

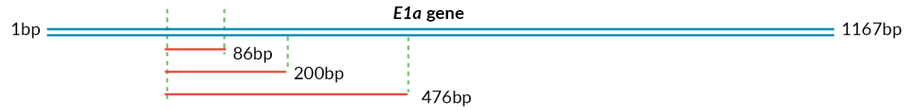
RESDNASEQ QUANTITATIVE PLASMID DNA KITS

Since its launch in late 2020, the Applied Biosystems™ resDNASEQ™ quantitative HEK293 DNA kit has seen tremendous success in the gene therapy field. The resDNASEQ™ kanamycin resistance gene kit was launched in 2021 and has been implemented in several gene therapy production processes. Both kits follow the same general workflow, including a DNA extraction procedure using PrepSEQ™ solutions and a qPCR assay for quantitation of the target DNA fragments.

The resDNASEQ™ quantitative plasmid DNA and kanamycin resistance gene kits, and the HEK293 residual DNA kit assays provide high sensitivity and broad dynamic range allowing testing of a wide range of samples. The method linearity within the dynamic range easily meets specifications, as demonstrated by R2 values of >0.99, as shown in

FIGURE 3

E1A fragment length assay concept.



Assay design

- Three single-plex assays for known fragment sizes: short, medium and long
- Three standard curves for quantitation of each fragment size
- Sample Prep with PrepSEQ Chemistry, KingFisher Flex, qPCR with 7500 Fast or QuantStudio 5
- Readout directly in AccuSEQ software in copy number for unknown fragments

Example

Quantification

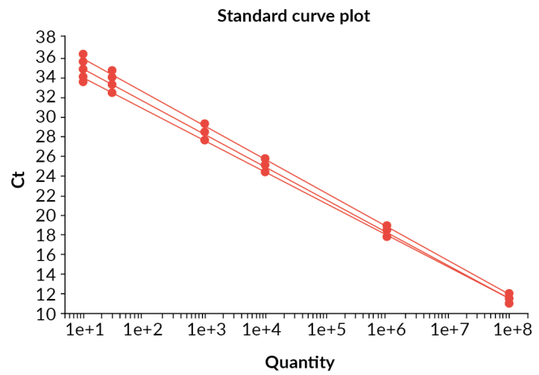
Short Frag assay (86bp)	110+100+90 = 300 copies
Med Frag assay (200bp)	100+90 = 190 copies
Long Frag assay (476bp)	90 copies

Ratio

<200bp	110/300
200–476bp	100/300
>476bp	90/300

FIGURE 4

Standard curve performance.



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

Table 1. In addition, the PCR efficiency has been calculated to 100% ± 10%.

Rigorous internal validation of the resDNASEQ™ assays has been completed. Internal validation studies are executed using our total workflow solution to verify our assays perform to specifications designed to meet regulatory guidance and validation criteria.

TOTAL WORKFLOW SOLUTION

The streamlined workflow begins with a manual or automated sample preparation. A semi-automated version utilizing the KingFisher™ Flex allows for up to 96 extractions at a time. Regardless of the level of automation chosen, the sample prep features well-established PrepSEQ™ chemistry based on magnetic particle-based suppression. The second and third steps in the workflow are setting up and running the resDNASEQ assay on one of our recommended Applied Biosciences real-time quantitative PCR (qPCR) instruments. The results are reported on the AccuSEQ™ software.

AccuSEQ™ real-time detection software supports you with the setup, running, and analysis of your qPCR experiments. The software has Security, Audit, and e-Signature (SAE) functionality that helps enable 21CFR part 11 compliance and full traceability of all actions within the software.

OPTIMIZED SAMPLE PREPARATION

Gene therapy sample matrices are typically associated with PCR-inhibiting components such as benzonase, detergents, media components, and potentially high levels of other non-target nucleic acid material. Various sample matrices were tested, and spiked DNA was successfully recovered and quantitated with expected limits across all sample types. Results show that PrepSEQ chemistry allowed to successfully prepared samples from a variety of matrices common to gene therapy bioproduction workflows.

PrepSEQ™ kits involve a combination of alcohol precipitation and magnetic bead-based extraction of nucleic acids from a plethora of sample types. This is a universal sample prep for extracting double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and RNA. The PrepSEQ kit has shown excellent performance in obtaining quantitative recovery, high precision, and consistent performance with complex matrices including low pH, high salt, and high protein.

The PrepSEQ chemistry can be used in both manual and automated workflows. Depending on the type of automation and experimental design chosen, 1–96 samples can be processed in one run. The manual method is low throughput and allows 1–16 extractions per day. The KingFisher™ Flex system is high throughput, allowing for up to 192 extractions per day. The KingFisher Flex offers many advantages such as easy setup using graphical interface, high speed purification of nucleic acids, control of cross-contamination, and high-quality nucleic acid recovery from a wide variety of sample types.

TABLE 1

Residual DNA kit specifications.

Specification	Kanamycin-resistance gene plasmid DNA kit	HEK293 DNA kit	E1a Fragment Length Kit
Linearity	$R^2 > 0.99$	$R^2 > 0.99$	$R^2 > 0.99$
PCR efficiency	100% ±10%	100% ±10%	100% ±10%
Precision	≤10% CV	≤10% CV	≤10% CV
Limit of detection (LOD)	15 copies	30fg	10 copies
Limit of quantitation (LOQ)	30 copies	300fg	30 copies
Assay range	300,000 to 30 copies	300fg to 3ng	10^8 to 30 copies
qPCR instruments tested			
Applied Biosystems™ 7500 Fast Real-Time PCR QST System			✓
QuantStudio™ 5 Real-Time PCR System, 96-well, 0.1 mL			✓
Gene Therapy Matrices tested			
Sample derived from a bioreactor at harvest			✓
Sample after chromatography			✓
Sample after final purification			✓

INSIGHT

In summary, the resDNASEQ™ system is a robust residual DNA quantitation solution for therapeutic grade AAV production. This all-inclusive system includes highly characterized DNA standards and reagents necessary for residual DNA quantitation. It provides an optimized sample preparation for quantitative DNA recovery and ultra-high sensitivity and specificity with no cross-reactivity to unrelated DNA. The streamlined workflow provides reliable data within five hours. Our quality assurance team provides consistency so that customers will receive the same high-quality performance from kit to kit, and our worldwide technical support network will assist you throughout all phases of the implementation process from early qualification and validation, all the way to lot-release and routine testing.

Q&A with Srinath Kashi Ranganath

Charlotte Barker, Editor, *BioInsights*, talks to **Srinath Kashi Ranganath**, Field Applications, Pharma Analytics Group, Thermo Fisher Scientific

SRINATH KASHI RANGANATH is a Staff Scientist – Field Applications with the Pharma Analytics group at Thermo Fisher Scientific, supporting customers in implementing, optimizing and validating the Pharma Analytics workflows for biomanufacturing processes across various therapeutic modalities. Prior, Srinath served as a Bioassay Scientist and an SME for the development and optimization of assays for residual DNA and other process impurities for 6 years. Srinath has an MS in Pharmaceutical Sciences from Campbell University, NC. His thesis is focused on understanding the expression profile of certain intracellular signaling molecules and how altering their function will affect the downstream cell signaling.

Q Can I use the resDNASEQ quantitative DNA fragment length kit instead of the resDNASEQ HEK293 DNA kit?

SKR: The resDNASEQ™ HEK293 kit is specifically designed to yield highly sensitive quantitation of residual HEK293 DNA samples. The E1A DNA fragment length

kit will provide additional information about the fragment sizes in the sample. There are significant differences in the kit design; the HEK293 kit targets a conserved repeat region of the genome giving the confidence in sensitivity requirements designed to meet regulatory guidance, while the E1A kit targets just one gene. There is preliminary data to show that there is some correlation between the E1A quantitation and the total residual host cell DNA for HEK293. It is not linear, but when you look at clearance studies in the downstream purification process, similar orders of clearance are observed between the two types of DNA. At this point, I do not think you will be able to use the E1A kit to replace HEK293 testing.

Q What is the regulatory track record for the HEK293 residual DNA kit?

SKR: That information comes from feedback from the field, but because many of customers purchase via the website and do not require a lot of support during implementation, qualification, and validation, it can be hard to collect. It has been purchased by more than 100 different customers and, to date there are no known issues with the acceptance of this method by regulatory agencies.

Q Do you have any available solutions for a droplet digital PCR (dPCR) platform?

SKR: These kits are designed to work very well on qPCR, but customer feed-back indicates that the E1A, kanamycin, and Sf9 baculovirus strips also perform well on dPCR platforms. A droplet dPCR platform will probably not work well with an assay design that targets a highly repetitive element.

Q There are two genes encoding kanamycin resistance that are commonly used in plasma. Does your kit detect both?

SKR: The kanamycin resistance gene kit was developed to pick up the vast majority of antibiotic-resistant plasmids used in biopharmaceutical manufacturing by targeting sequences common to three gene families of kanamycin resistance. So it is very likely that it will work with most plasmids containing the kanamycin gene. The address specific questions about whether or not the kanamycin resistance gene kit is able to quantitate a specific plasmid, please contact a Thermo Fisher Scientific representative, and bioinformatic information may be available.

Q Is there a cross-reactivity between this kit and other non-HEK293 cells?

SKR: It is important for a residual DNA kit to be specific. Extensive exclusion primer testing is performed so that these kits meet specificity requirements. Additionally, resDNASEQ kits are species-specific, but not specific to a certain cell line. For instance, the HEK293 kit will pick up and actively quantify human DNA. In addition, the only low level off-target reactivity is that with Vero (African Green Monkey) DNA, which is unsurprising given the close relationship between humans and monkeys. For unrelated species, no cross-reactivity was observed.

Q Does the residual human DNA kit over or underestimate the DNA concentration of HEK293 DNA? If so, what is the magnitude of the difference?

SKR: There is data to show that the HEK293 and human DNA standard curves line up on top of each other. There should not be any major differences between measures with both kits. The HEK293 kit is specifically designed for gene therapy customers who use HEK293 cell lines, and includes a HEK293 DNA standard, although both kits are shown to quantitate the HEK293 or human DNA the same way.

Q Do you see any interference from the presence of digested envelopes in DNA quantitation assays?

SKR: In development, Thermo Fisher Scientific tested a representative sample that might not correlate with the results that you see in your sample because your process might be unique. There are a few known customers who are working to answer the same question: is detection of any incorporated residual DNA in the capsules possible without protein digestion and without lysis

Q Are the qPCR assays compatible with the QuantStudio 6 and 7?

SKR: It is highly recommended that the kits be used on the complete system that have tested and validated during in-house testing: the 7500 Fast and the QuantStudio 5 instrument with the AccuSEQ software. This is particularly because 21 Code of Federal Regulation (CRF) Part 11 compliance is only possible with AccuSEQ. It is not possible with the software on the QuantStudio instruments such as the design and analysis such as the QuantStudio rtPCR software. If this is used simply for research and development testing and 21 CRF Part 11 compliance is not needed, then the kit works just as well as any qPCR instrument that fulfils the filter and sample block requirements.

Q Where in my workflow do I need to test for residual fragment length?

SKR: Given the rapid turnaround, fragment size testing can be performed at any point during the process. However, the clear utility of this kit is to measure the efficiency of the DNA size-reduction steps such as benzonase, further upstream, or right at the harvest stage when you are performing the DNA size-reduction step and the lot-release testing stage. That being said, the kit may also be used in process development to test different elusions for the presence of the E1A gene to demonstrate clearance.

Q Are there any plans to add other types of cell line to the resDNASEQ family?

SKR: The resDNASEQ™ portfolio has evolved over time. The Chinese hamster ovary (CHO) assay came first, but now there are many more targets. As emerging needs become clear, additional targets will be added. If a particular residual DNA assay is needed for your process, please get reach out to Thermo Fisher Scientific. More feedback received by Thermo Fisher Scientific helps enable quicker development of solutions to customer problems.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

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CHAPTER 2

Expression systems for viral vector production: advantages of the Sf9 baculovirus system and simple solutions to address its specific analytical challenges

Yi Fang Lee & Srinath Kashi Ranganath

The gene therapy landscape has exploded in the last few years, bringing a multitude of viral vectors to the clinic. At the forefront of this evolution is the application of lentivirus, adeno and adeno-associated virus (AAV), and plasmid-based therapies to genetic diseases of all types. With each program that progresses through the clinic, the body of manufacturing and clinical knowledge grows – and so does the availability of regulatory guidance. Adeno-associated virus (AAV) is increasingly popular, and the baculovirus-Sf9 platform has been established as a promising alternative to mammalian cell-based methods. However, the Sf9 baculovirus production system poses some unique analytical challenges. Here, we will discuss two of these issues – quantifying residual DNA and detecting the adventitious agent Sf-rhabdovirus – and solutions developed by Thermo Fisher Scientific designed to meet regulatory guidelines and ensure product quality and safety when utilizing the baculovirus-Sf9 platform for AAV production.

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AN ESTABLISHED ALTERNATIVE TO RAAV PRODUCTION IN MAMMALIAN CELLS

As an alternative to recombinant AAV (rAAV) production in mammalian cells, the baculovirus Sf9 platform has been notably established as a GMP-compatible and scalable system generating as many vector genomes per cell as mammalian cell-based methods – i.e., up to 2 x 10^5 vector genomes per cell in crude harvests.

The baculovirus Sf9 production system offers many advantages over other production platforms when considering various safety issues. The system uses serum-free media, and despite the discovery of adventitious virus transcripts in Sf cell lines, most of the viruses infecting insects do not actively replicate in mammalian cell lines. There is also no need for a helper virus or plasmid to produce recombinant AAV in insect cells, besides the baculovirus.

Although this system offers many advantages, the Sf9 baculovirus production system poses some analytical challenges relating to process impurities from the system itself. The manufacturing process for Glybera™ drug product (uniQure), the first gene therapy product produced using the Sf9 baculovirus system, is an illustrative example. A comparability assessment of the product from different process stages was performed; of critical importance was the comparability between the process used for the clinical studies, and the commercial process. The results indicated that the product quality has improved throughout the development of this manufacturing process. In most analyses, the commercial process quality was comparable if not better than the clinically used process, except for a significantly higher carry-over of baculovirus DNA.

In this relevant case, the residual baculovirus genetic sequences unintentionally encapsulated in AAV1 capsids, and co-purified with the product, which was one of six major product quality objections raised during the licensure assessment of the Glybera drug product by the European Medicines Agency.

Another important analytical challenge is detection of Sf9 rhabdovirus, which is an adventitious virus infecting insect cells, and is present in most Sf9 and Sf21 cell lines used in bioproduction.

Solutions to these two important challenges are discussed below.

QUANTITATION OF RESIDUAL SF9 HOST CELL & BACULOVIRUS DNA

To address the issue of residual DNA in the Sf9 baculovirus system, there is a need for an analytical method to accurately quantitate residual Sf9 and baculovirus DNA. The FDA describes the residual DNA limits for non-tumorigenic cells to be less than 10 nanograms per dose, and the DNA size to be below approximately 200 base pairs in the recent CMC guidance for industry document revised in 2020.

Similar guidance was provided by the FDA in a briefing document from 2012 for vaccines produced in cell lines derived from human tumors. The most recent WHO recommendation also sets the upper limit for residual cellular DNA at 10 nanograms per dose.

In a typical AAV production process, process impurities such as residual DNA and host cell protein are expected to be present. Therefore, they should be characterized and substantially reduced and controlled to an acceptable range by appropriate purification steps. Residual host cell DNA is present in two forms – as a nuclease-sensitive process-related impurity that is non-specifically co-purified with the desired AAV vector product, and as a nuclease-resistant product-related impurity that is encapsulated within AAV particles. Minimizing these distinct forms of residual host cell DNA requires different manufacturing process optimization strategies, and a robust residual DNA testing method is therefore necessary to determine the residual DNA levels in the process.

The design and control of an in-house residual DNA assay poses a number of challenges. Designing a custom residual DNA assay requires extensive development and validation to ensure that the primers are specific to the target sequences, and are not amplifying non-specific targets. For a multi-target system such as the Sf9 baculovirus platform, multiple assays are needed for both Sf9 and baculovirus.

Establishment and maintenance of a robust standard or DNA control requires significant expertise and is highly time consuming. These assays require multiple components, and strong inventory management is required for the individual components to ensure lot-to-lot reproducibility. Additional infrastructure may also be required for each component around incoming quality control.

The resDNASEQ™ Quantitative Sf9 Baculovirus DNA Kit

The resDNASEQ Quantitative Sf9 Baculovirus DNA Kit is a duplex quantitative PCR-based system for the simultaneous detection of residual DNA from the Sf9 baculovirus platform used in the development of gene therapy, cell-based vaccines, and similar biotherapeutics. The assay is reliable, rapid, and enables sensitive and specific quantitation of Sf9 host cell DNA and baculovirus DNA.

All resDNASEQ kits are provided in a comprehensive product solution format, with all-inclusive reagents and genomic DNA standards. The rapid testing and streamlined workflow allow for a fast time to result of under 5 hours with optimized sample preparation.

The reliable performance of the kit also allows its use in multiple stages of the gene therapy manufacturing process. The kit is designed to meet the specifications listed in the USP 509, the residual DNA testing guidance document published by the United States Pharmacopeia.

Validation was conducted by eight operators spanning three continents, using multiple lots, and over four days. Two sample preparation methods were used; manual sample preparation and automated sample preparation with KingFisher Flex using the PrepSEQ™ Sample Preparation kit. Real-time PCR was evaluated on the Applied Biosystems™ 7500 Fast™ and the QuantStudio™ 5 instruments.

The key performance criteria evaluated were:

- PCR efficiency
- Linearity
- Range of the standard curve
- Precision
- Reproducibility
- LOQ/LOD
- Specificity
- Sample preparation spike recovery
- Singleplex versus duplex performance

These criteria were evaluated based on the ICH Q2 R1 guidelines, and the criteria for precision and accuracy were taken from the USP 509 document.

The assay is able to achieve an R2 of greater than 0.99 and a PCR efficiency of 100 +/- 10%, (Figure 1). Regarding the precision, as outlined in our USP, the requirement is to meet less than 30% CV for the quantity values. The kit is able to achieve this comfortably, with less than 30% of the back-calculated quantity percent CV. The LOD is 30 femtograms, the LOQ is 300 femtograms, and the assay range is 300 femtograms to 3 nanograms. The assay range was evaluated in multiple gene therapy matrices derived from bioreactor at harvest, samples from after chromatography, and samples after final purification.

FIGURE 1

Specifications of the quantitative Sf9 and baculovirus DNA kit.

01 Comprehensive product solution
All-inclusive kits with genomic DNA standards and all reagents

02 Rapid testing and streamlined workflow
Time-to-results < 5 hours with optimized sample prep

03 Ultrahigh sensitivity
0.3 pg LOQ for Sf9 and Baculovirus DNA

04 Reliable performance
Reliable results across multiple stages in the gene therapy manufacturing process

Specification	
Linearity	R^2>0.99
PCR efficiency	100% +/-10%
Precision	⊠30% back-calculated quantity CV
Limit of Detection (LOD)	30fg
Limit of Quantitation (LOQ)	300fg
Assay range	300fg to 3ng

Gene Therapy Matrices tested	
Sample derived from a bioreactor at harvest	✓
Sample after chromatography	✓
Sample after final purification	✓

High sensitivity & broad dynamic range

The broad linear range provided by the assay allows for testing of a wide range of Sf9 and baculovirus DNA samples (Figure 2). This is particularly important for rigorous process characterization studies used to assess the impact of the small process changes on the residual DNA content.

Workflow integration

All Thermo Fisher resDNASEQ assays use the same workflow as illustrated in Figure 3, which involves extraction of the nucleic acid material from the test articles using the PrepSEQ sample preparation kit, followed by the detection and quantitation using the redDNASEQ kits on a QPCR instrument. The sample preparation can be performed manually or in a semi-automated manner using the Pharma Kingfisher™ Flex instrument, allowing flexible throughput of ~10–500 samples per week.

The qPCR instruments we recommend for this type of testing are the 7500 Fast, or the QuantStudio™ 5 PCR instruments, because they are compatible with the multiplex nature of the assays, and provide the ability to integrate the AccuSEQ™ software. This allows for fully automated data analysis on a single software in a 21 CFR part 11 compliant environment, and prevents the need to migrate the data into excel or perform other manual calculations.

The PrepSEQ Sample Preparation Kit is a universal kit for all our QPCR-based applications and can be used to extract residual host cell DNA, plasmid DNA, and mycoplasma DNA. The kit was also evaluated for extraction of over 60+ enveloped or non-enveloped viruses that contain single or double-stranded DNA or RNA. The kit has demonstrated robust extraction efficiency from samples containing simple to complex matrices, including ones with low pH, high salt, and high protein concentration.

It is important to note that while this assay may be able to run on other real-time PCR instruments, we have performed the validation using AccuSEQ software that is only compatible with the 7500 Fast and QuantStudio 5 PCR instrument. The AccuSEQ software is designed specifically for these PCR instruments and assays to enable compliance with 21 CFR part 11 features. These features are security, audit, and e-signature capabilities. Any adjustments tools or changes to the data are recorded and audited so that everything is fully traceable.

DETECTION & QUANTITATION OF SF-RHABDOVIRUS

Rhabdovirus was first identified in Sf9 in 2014 by the FDA Center for Biologics Research and Evaluation [1]. It was then independently reported by other institutes, suggesting that it is a universal contaminant of Sf9 cells.

The virus is a 13 kb negative sense strand RNA virus, belonging to the rhabdoviridae family. Variants have been reported within Sf9 cell lines, and the Sf21 parental cell line, but they are largely conserved.

Studies have suggested that the virus is non-infectious to mammalian cells, and thus does not replicate in human cell lines. However, the safety data is quite recent, and the long-term effects of the virus are still unknown. Therefore, there is a compelling reason for this virus to be monitored for its absence in bioproduction using Sf cells. In the publication on discovery of the rhabdovirus by the FDA, the authors emphasize the need to demonstrate the absence of Sf-rhabdovirus in manufactured biological products at different stages of manufacturing.

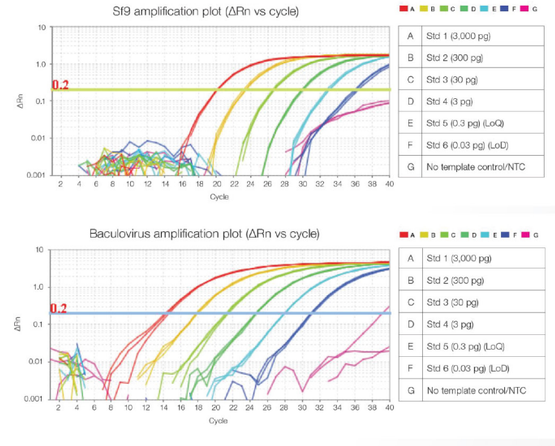
Based on FDA Q5A viral safety guidance, an appropriate virus testing program in assessment of virus removal and inactivation is required for biological products. Similarly, adventitious viral agent testing is required by the FDA under human genome therapy INDS guidelines.

Virus safety is a critical aspect of biopharmaceutical production, and relies on the well-established principles of prevent, detect, and remove to assure drug safety for patients (Figure 4). These principles are the foundation of every viral safety strategy.

The testing strategy for Sf-rhabdovirus also follows these principles. Given the wide variety of samples that may require testing, it is important to create a rapid and robust assay that is rapid, and most importantly suitable

FIGURE 2

High sensitivity and broad dynamic range.

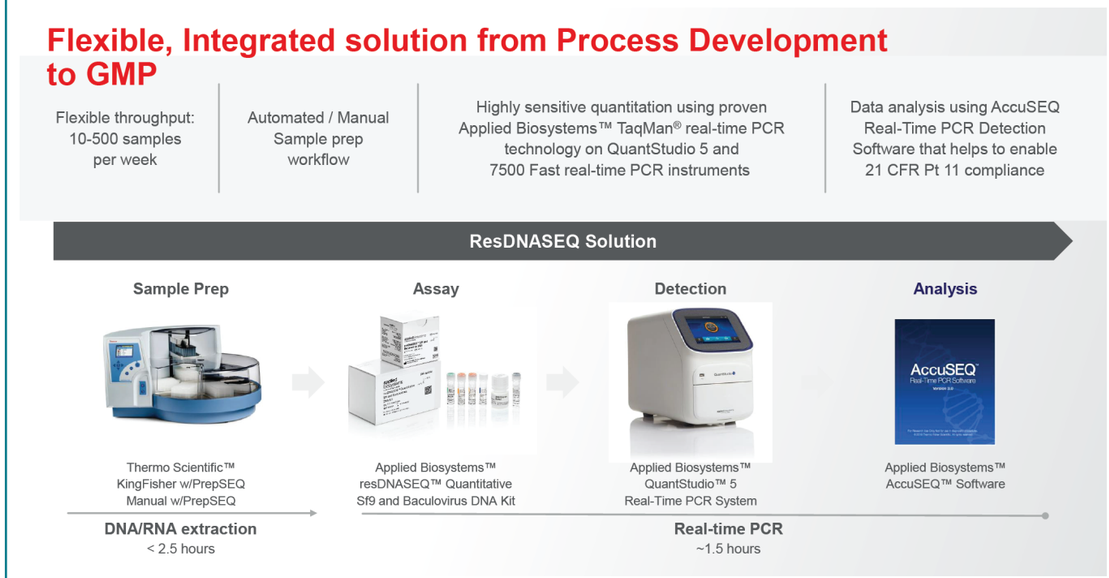


- The amplification plots were generated using 10-fold serial dilutions (ranging from 3 ng to 30 fg) of Sf9 and Baculovirus mixed DNA control, provided in the kit.
- The broad linear range allows testing of a wide range of Sf9 and baculovirus DNA samples

Figures A and B show amplification plots for both Sf9 and baculovirus using ten-fold serial dilutions ranging from 3 nanograms to 30 femtograms of the mixed DNA control provided in the kit.

FIGURE 3

Representative workflow of a Thermo Fisher resDNASEQ assay.



for various stages of the biopharmaceutical production process.

The ViralSEQ™ Quantitative Sf-Rhabdovirus Kit intends to address the requirement for fast and sensitive quantitation of Sf-rhabdovirus genome RNA. Coupled with the PrepSEQ nucleic acid sample preparation kit, the ViralSEQ Quantitative Sf-Rhabdovirus Kit is compatible with samples from various stages in the manufacturing process.

Similarly to the quantitative Sf-baculovirus resDNASEQ kit, the Sf-rhabdovirus kit follows a workflow from sample extraction to real-time PCR readout, with report generation and regulatory compliance compatibility. The main difference is that a reverse transcription step is required prior to real-time PCR (RT-PCR) detection. We recommend the ABI Veriti™ 96-well Fast Thermal Cycler in our validated workflow.

The RT-qPCR workflow is a two-step process. In the first step of reverse transcription, genome strand-specific primer will reverse transcribe the RNA. The primer incorporates a non-viral tag sequence to the cDNA. In the qPCR step, the primer specifically amplifies the products from the tagged cDNA test line for specific detection of the genome RNA (Figure 5).

Validation summary

Validation was conducted by six operators over two locations in Singapore and the USA, using three validation lots over 5 days. Two separate preparation methods were used; manual sample preparation and automated sample preparation with KingFisher Flex using the PrepSEQ kit. The real-time PCR was evaluated on the ABI 7500 Fast Real-Time PCR, and the QuantStudio 5 Real-Time PCR Instrument.

Together, 79 runs were analyzed for the validation study. The main performance criteria assessed were:

- PCR efficiency
- Linearity
- Range of the standards
- Precision
- Reproducibility
- Limitation of quantitation (LOQ) / limit of detection (LOD)
- Specificity
- PrepSeq spike-recovery

We observed that the standard curve has a good PCR efficiency of close to 100%, and a good linearity of $R^2 > 0.99$ More importantly, we found that the result is reproducible at both the US and Singapore test sites. In terms of sensitivity, we found that the assay is robust, from 10 copies to 107 copies, with an LOQ of 30 copies per reaction, and an LOD of 10 copies per reaction. The LOD is cut off at above 95%.

We evaluated for inter-variable precision across plates, including inter-operators, inter-lot, inter-instrument, and inter-day analyses. In general, the average back-calculated percentage coefficient of variation was less than 30% across all standard dilutions down to 30 copies.

The detection of viral mRNA may undermine the accuracy of the tests in detection of truly infectious viral copies. Therefore, we designed the assay to only detect the genome RNA specifically. To test for genome RNA specificity we tested 107 copies of positive-strand RNA, which are representative of the mRNA strands. We found that despite the high copy numbers, the cycle threshold (CT) is either undetected, or above the LOD, suggesting that the assay is highly specific towards the genome RNA strand, which has a much lower CT value.

We tested for exclusivity of the assay with a panel of genomes from different species or cell lines, and observed non-detection of amplification with the exclusion panel, suggesting that the assay is specific to rhabdovirus.

The recovery of spike controls with the PrepSEQ nucleic acid kit, manually and on

FIGURE 4

Viral safety assurance strategy for assuring the absence of Sf-rhabdovirus in manufactured biological products.

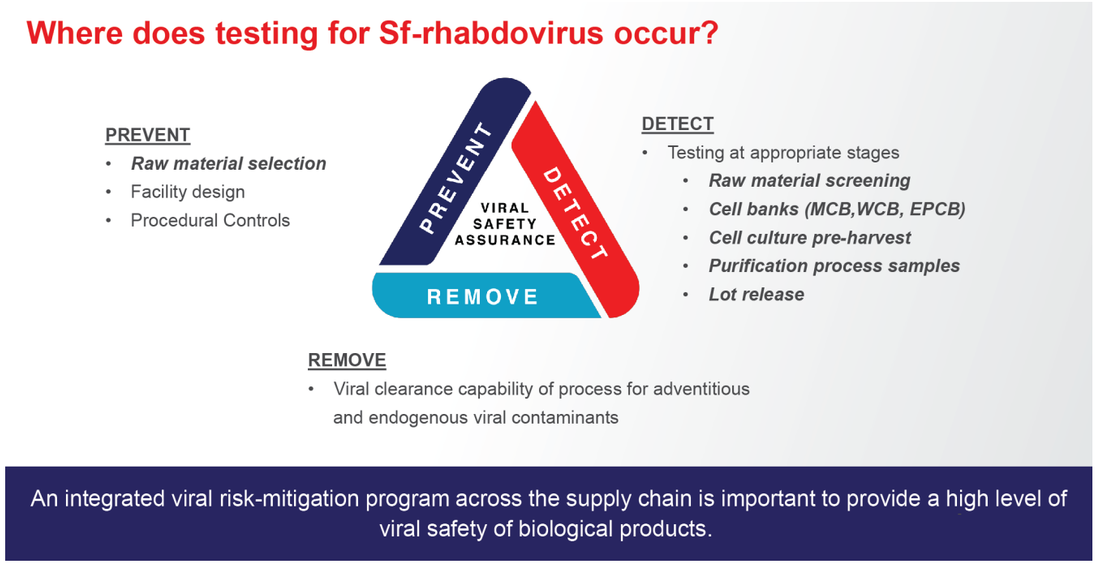
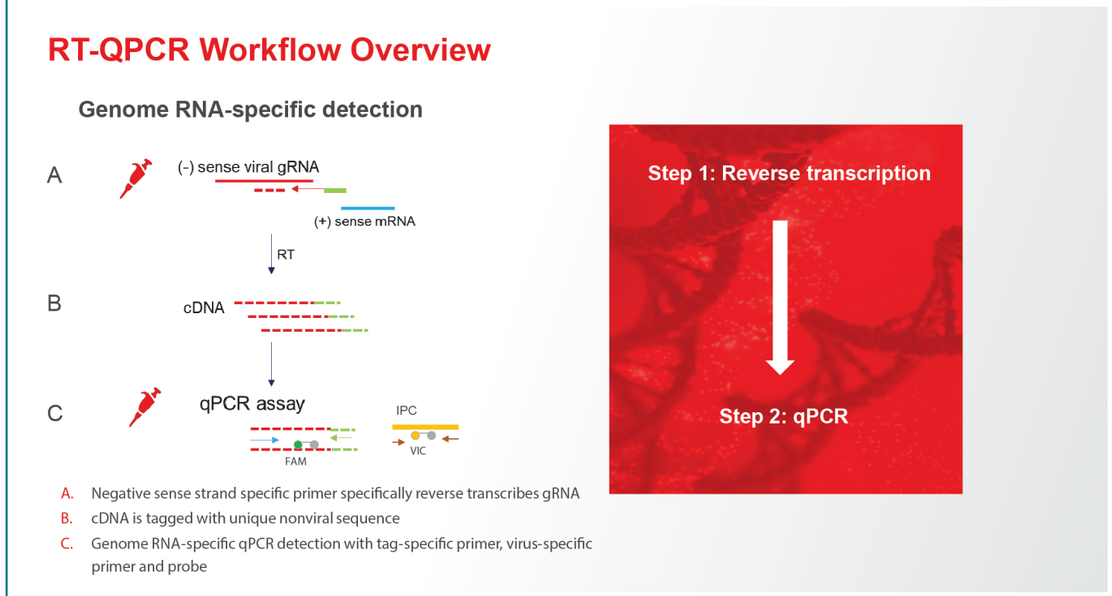


Figure sourced from [2].

FIGURE 5

RT-qPCR workflow overview.



A. Negative sense strand specific primer specifically reverse transcribes gRNA
B. cDNA is tagged with unique nonviral sequence
C. Genome RNA-specific qPCR detection with tag-specific primer, virus-specific primer and probe

automated platform KingFisher Flex, was examined. RNA controls were spiked at different varying concentrations from 500,000 copies to 500 copies in the presence of a matrix emulating the final bioprocessing purification. A good recovery of between 70 and 130% for all concentrations was observed in both manual and automated sample preparations. Different intermediate bioprocessing matrices were also tested during the RNA verification study. These matrices include constituents from the bioreactor at harvest, and after chromatography. Recovery was between 70 and 130% for all matrices through the KingFisher platform.

PCR inhibitors are often a concern in assay accuracy, so sample preparation with inhibitors that are commonly found in bioprocessing was studied: benzonase, often used in DNA degradation, tween-20, a detergent, and LV-Max, a cell culture medium. The results showed no significant difference in CT value or recovery between assay detection in PBS versus the inhibitors after our sample preparation. This shows that the sample extraction by the PrepSEQ kit is sufficient to minimize the inhibitors, and thus not affect the accuracy of assay detection.

INSIGHT

The Sf9 baculovirus expression system is an effective alternative to mammalian substrates for commercial production of AAV and other biologics. It has unique advantages for AAV production, including easy scale-up, growth in serum-free media, and no need for helper virus or plasmid raw materials for AAV production. It also comes with its own analytical challenges, including the need to demonstrate clearance and quantitate residual Sf9 host cell and baculovirus DNA, and Sf-rhabdovirus contaminant.

Thermo Fisher Scientific has simplified these challenges by staying attuned to emerging regulatory guidance and developing solutions, from sample preparation to analysis, designed to meet regulatory guidelines. We have developed solutions for residual DNA testing, with a highly sensitive and specific duplex qPCR assay for the simultaneous quantitation of residual Sf9 host cell DNA and baculovirus DNA. The ViralSEQ Quantitative Sf-Rhabdovirus Kit provides a highly sensitive and specific quantitation of Sf-Rhabdovirus via a strand-specific qPCR assay with a high dynamic range.

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2. American Pharmaceutical Review.

ASK THE AUTHORS

Yi Fang Lee
R&D Scientist, Thermo Fisher Scientific

Srinath Kashi Ranganath
Staff Scientist – Field Applications, Thermo Fisher Scientific

Q Does the validation data you provided in the presentation eliminate or shortcut the validation process required for regulatory acceptance of use of this assay in the workflow?

SKS: This is a very commonly asked question in this field. What we have covered here proves that the assay is validatable in simulated sample matrices. However, it is important that customers validate the assay in-house, using the actual sample matrices – particularly the ones that they set the item specifications on.

To summarize, you could leverage some information from our validation. For example, the specificity of the primers remains the same, regardless of whether you test that in PBS or

whether you test that in your sample matrix. Some of the components may be leveraged, but things like LOQ, looking at spike recovery, and those kinds of parameters must be evaluated in your sample matrices that are representative of the manufacturing process at your site.

Q What did you use as the standards for the residual qPCR assays?

YF: For the rhabdovirus, we are using in vitro transcribed RNA covering the assay region. As for baculovirus, Srinath would you like to speak to that?

SKS: For Sf9, it is the Sf9 genomic DNA extracted from Sf9 cells. The baculovirus cannot live by itself, so that was cultured in Sf9 cells, followed by subsequent extraction of the baculovirus from that mix – so both genomic DNA.

Q A question from the audience: we used the Sf9 rhabdo-free cell lines and media. How would implementing this assay be useful for our process?

YF: Potential Sf rhabdovirus contamination has been expressly noted by the US FDA as a potential concern. So even though its safety and priority is still unknown, we know that reinfection of Sf-rhabdovirus cell lines has been shown to be possible.

Therefore, we encourage testing for Sf-rhabdovirus in at least the master cell bank, the working cell bank, and bioreactor points of the workflow, in a Sf-rhabdo-free Sf9 expression system. This will act as a risk mitigation strategy to monitor and ensure that these cells remain Sf-rhabdovirus free.

This will also strengthen the regulatory requirement or quality of a robust virus testing program for the Sf expression system.

Q Can the duplex Sf9 baculovirus ResDNASEQ kit be used to measure just baculovirus res DNA?

SKR: This is a problem that I encountered when I was an analyst executing the assay in the lab myself.

The assay is designed as a duplex assay. But if you get results that pass for the Sf9 target but do not meet criteria for the baculovirus target for any reason, then you are able to just run baculovirus by itself. So you can run the assay singleplex or duplex, and our validation data shows that you're able to generate similar results using either approach.

Q You mentioned that both assays can be run in a manual or semi-automated method. Could you please outline or quantify the advantages of the semi-automated method over the manual?

SKR: I have personal experience running both methods. One of the main advantages is having a shorter hands-on time, allows you to do other analyses. It also gives you the ability to spend more time on data analysis.

The other advantage is the higher throughput. You are able to extract up to 96 wells on the KingFisher Flex instrument. When you're processing the samples manually, you are working with the 16 position magnetic racks, so you can only work with 16 tubes at a time. Any more than that can get really tedious and might affect the accuracy, precision, and consistency of the data.

Another advantage of KingFisher is that it requires very little training. So when there is employer redundancy, new hires, or you're trying to scale up and you want to bring on more people to run the methods, KingFisher makes it very easy.

With a manual method you have to train each analyst in a very skillful way so that you see very little variation amongst different analysts.

I have also found that the KingFisher flex results in better and more consistent recovery in general when compared to manual methods.

Q Could you please go over the validation data for the rhabdovirus kit? Did you evaluate the specificity of the kit for other species of RNA and DNA?

YF: We have validated an exclusion panel of different species, including E. *coli* and mammalian cells. We did not detect any amplification of the rhabdovirus in the panel of different species. For the validation data, if you would like to have more details feel free to reach out to our team who can provide more details

Q What is the ratio of full versus AAV empty particles produced in insect cells?

SKR: This isn't directly related to the content we have presented, but I can touch on it. From my understanding of the process, and some of the articles and literature, it seems like the packaging efficiency is similar or better than some of the other mammalian cell-based viral vector production systems. It also depends on the particular rAAV serotype, as it's not the same for every serotype.

I have seen packaging efficiencies of greater than 90% using the Sf9 baculovirus system.

Q Are insect cells and baculovirus DNA immunogenic in humans?

SKR: Again, the literature shows that, for example, vaccines produced in using the baculovirus system have greater immune response. But is the DNA itself immunogenic to humans? There is very little in the literature that talks about the immunogenicity of the insect cell and baculovirus DNA.

One thing we have shown is that the viruses that are commonly found in the Sf9 cells do not replicate in humans, or do not infect humans by themselves. So that is one of the advantages of using this system.

Q How much of a concern is rhabdovirus contamination in insect cell production platforms?

YF: The rhabdovirus has been shown to be reported in different Sf cell lines by various different independent laboratories, so I think that it is a universal concern.

It's non-infectious to human cell lines, but because it is quite a recent discovery, the long-term immunogenic effects are still fairly unknown, so we are still advocating for testing of rhabdovirus.

Q Could you share the protocols for residual Sf9 bac-DNA for harvest, process intermediates, and final drug substance?

SKR: In general, most of our ResDNASEQ methods are geared towards using a single protocol for all sample types. That is how I recommend you design your protocols in order to have a fail-proof method when you transfer it over to your quality group.

The way we do that is somewhat of a unique approach. I think it might best help if you work with your FAS, in order to optimize your samples to get the optimized protocols that are as close to final stage as possible, and then evaluate that method.

In general, the protocol that we start out with is what is listed in the user guide. But I understand some specific sample types, especially things that start in the harvest stage, might require some optimization. The process intermediates and the final drug substance usually share the same end protocol.

Q What is the minimal sample volume required for each of the tests discussed?

SKR: For the Sf9 baculovirus, if you are testing a sample neat, and testing it by the USP method: triplicates unspiked and triplicates spiked, you need exactly 600 microliters. Our protocols start with 100 microliters per vial in the KingFisher, or 100 microliters per tube in the manual method.

So regardless of the method you use, you need exactly 600 microliters if you are testing neat. But it is always good to have retains in case you need to repeat the assay.

YF: It would be the same for the rhabdovirus. In terms of reaction and volumes, we recommend 8 microliters for the extracted samples.

Q Is there any cross-reactivity between Sf DNA and baculovirus DNA when using the Sf9 Baculo ResDNASEQ kit?

SKR: To address one of the other questions I mentioned that the baculovirus DNA is produced in Sf9 DNA, and the fact that we are multiplexing the assay to be able to detect Sf9 and baculovirus targets in the same vial.

It is important that we do not have any specificity issues as they relate to the primers themselves, so we have evaluated that. When we spiked baculovirus DNA and amplified using the Sf9 primers, we saw a signal below the lowest standard, which told us there is no non-specificity towards each other within the assay. That was one of the most important parameters we looked at when we first designed the assay. There is no cross-reactivity.

BIOGRAPHIES

Yi Fang Lee
R&D Scientist, Thermo Fisher Scientific

Yi Fang joined ThermoFisher Scientific as an RND Scientist since Jan 2020. She has led the development of the ViralSEQ Quantitative Sf-rhabdovirus assay. Her previous work experience was in cancer biology and assay development for circulating tumor cells in a microfluidics-based system. Yi Fang received her PhD education at the Genome Institute of Singapore, majoring in oncogene discovery from transcriptome- wide studies. She has published journals on cancer biology, sequencing and microfluidics systems.

Srinath Kashi Ranganath
Staff Scientist – Field Applications, Thermo Fisher Scientific

Srinath is a Staff Scientist – Field Applications with the Pharma Analytics group at Thermo Fisher Scientific, supporting customers in implementing, optimizing and validating the Pharma Analytics workflows for biomanufacturing processes across various therapeutic modalities. Prior, Srinath served as a Bioassay Scientist and an SME for the development and optimization of assays for residual DNA and other process impurities for 6 years. Srinath has an MS in Pharmaceutical Sciences from Campbell University, NC. His thesis is focused on understanding the expression profile of certain intracellular signaling molecules and how altering their function will affect the downstream cell signaling.

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AUTHORSHIP & CONFLICT OF INTEREST

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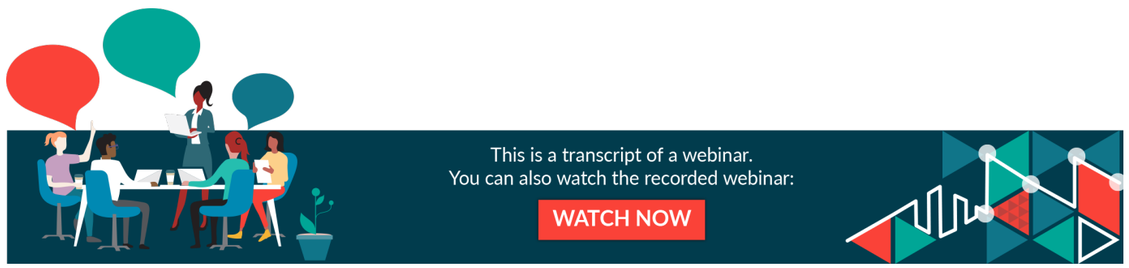


CHAPTER 3

How can we maximize the efficiency of large-scale LV vector production?

Scott Jeffers, GenSight Biologics, **Emily Jackson-Holmes,** Thermo Fisher Scientific, **Rakel Lopez de Maturana,** VIVEbiotech,
Steve Milian, Thermo Fisher Scientific, **Margherita Neri,** AGC Biologics

***Cell & Gene Therapy Insights* brought together a panel of industry experts to discuss the technological barriers to scaling up LV vector production and how they can be overcome. Here are some of the highlights...**

Cell & Gene Therapy Insights 2022; 8(7), 795. DOI: 10.18609/cgti.2022.122

How does your choice of bioreactor and upstream production platform affect LV titers, speed, and cost?

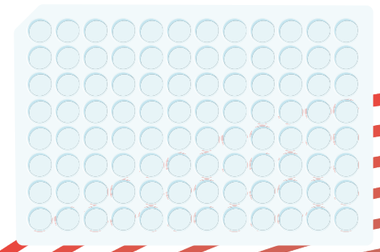
When developing products for upstream LV production, we use suspension-based systems, because they are advantageous in terms of scale-up, in addition to reducing variability and cost. To increase titer within the suspension system, we have used design of experiment (DoE) to optimize concentrations and timings of each component in the process, including the mammalian cell transfection process, cell line, transfection reagent, plasmid DNA, and any enhancers or supplements. This has resulted in a successful, optimized system that significantly increases titer and reduces cost.

Emily Jackson-Holmes,
Associate Product Manager, Cell Biology, Life Sciences Solutions Group, Thermo Fisher Scientific

What technological innovations are having the greatest impact on downstream LV processing?

The downstream side for LV is challenging, particularly because of the ~0.1µ dimensions of the LV. The most critical step is the final sterile filtration, where a large part of vector preparation is often lost. Clients frequently ask for more concentrated vectors. From a CDMO perspective, we must balance the concentration with the yield of the final sterile filtration. The more concentrated the vector, the more aggregation in the vector preparation, and the greater difficulty in balancing sterile filtration. Improvement in the analytical possibility to evaluate the vector aggregation will be important to solve the downstream challenge. Recently, many new membranes and resins for purification have become available on the market, and we are testing these to improve LV purification.

Margherita Neri, Vector Process Development Manager, AGC Biologics



How can we ensure robustness in assay selection and evaluation?

The big question is, 'do we have the assay under control'? We want low variance and high repeatability. One of the most important attributes of the assay is the ability to have different people do it – on different days, using different instruments – and still get the same answer. When assays are performed incorrectly, we should be investigating the impact of those changes on the assay itself to build a better understanding of how robust the methods are. If we notice that small changes are dramatically impacting the assays, it hints that the assay is not robust. We need to start building a library of investigations, to determine what are the critical parts of an assay and how they can be negatively impacted.

Steve Milian, Senior Staff Scientist, Science & Technology Pharma Services, Thermo Fisher Scientific

What bioprocess and analytical innovations will drive further scalability and quality/consistency improvements?

There are three key components for bioprocessing. One is the development of more producer cell lines to increase productivity. Second, automation is key, both in process and analytics. The third key point is the development of new serotyping strategies that better target the cell to be transfused, and the development of transduction enhancers. This is key to developing more cost-effective processes so that more patients can be treated.

Rakel Lopez de Maturana,
Quality Control Director, VIVEbiotec

How might the evolving regulatory landscape impact the picture?

The landscape has changed tremendously over the last 5 years. Regulators have placed great importance on quality and ensuring that we are monitoring our processes. Even from the early stages of process development, it is recommended to think about the final stages of commercialization. Understanding your process, with quality in mind from the beginning, is important to ensure you can get through the regulatory pathways. This ensures patients are safe, which is of primary importance.

Scott Jeffers, Chief Technology Officer, Gensight Biologics

Watch the webinar here

Read the full transcript here

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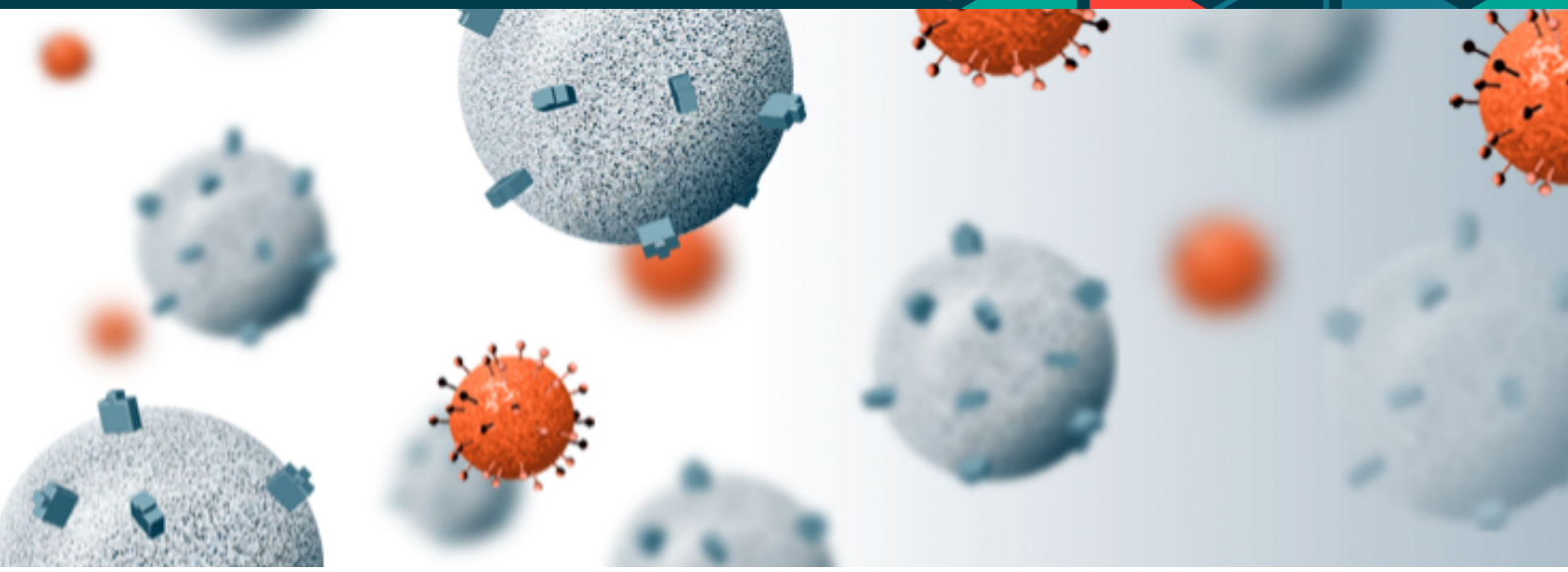

Simplifying lentiviral downstream processing with a novel affinity resin and robust analytical tools

Recombinant lentivirus has become a vector of choice for many gene-modified cell therapies, including several FDA-approved cell therapies, due to the broad tropism and long-term, stable gene expression in non-dividing cells. The safety and efficacy of lentiviral-based therapies depends greatly on optimized and controlled lentiviral production. Downstream purification of lentiviral particles presents unique challenges, and robust analytics are critical to verify both the recovery and infectivity of the purified product.

In this webinar we present an overview of challenges and newly developed solutions for robust lentiviral purification and rapid analytical methods for titer determination and impurity quantification. Details of a new affinity chromatography resin, based on CaptureSelect technology, to purify VSV-G pseudotyped lentivirus, as well as two q-PCR based genomic and proviral infectious titer assays will be discussed.

Attendees will learn about:

- Current challenges and limitations in lentivirus purification and characterization
- Development of an affinity resin to purify VSV-G pseudotyped lentivirus to address these challenges
- Regulatory guidance for lentiviral titers for both cell therapy and viral vector production
- Internal validation data for two rapid, robust, and QC-amenable assays for lentiviral titers

SPEAKERS

Chantelle Gaskin
Field Applications Scientist, Thermo Fisher Scientific
Suzy Brown
Senior Field Application Specialist for Pharma Analytics, Thermo Fisher Scientific

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ANALYTICAL INNOVATION:
Meeting the Demands of Commercial Viral Vector Manufacture

CHAPTER 4

Lentiviral titer determination: rapid and robust molecular methods suitable for validation

Unnati Dev

High-quality recombinant lentiviral vectors (LVV) are key for transgene delivery in many cell and gene therapies, including several of the FDA-approved CAR-T cell products. Critical to the success of these biologics are reliable methods to characterize and quantitate LVV. This article provides an overview of quality attributes, regulatory expectations, and challenges in LVV titer determination and characterization. Quantitation of total genome-containing particles and infectious particles is valuable to optimize the vector production process and to appropriately dose for cell transduction at the desired multiplicity of infection. Vector copy number is also a critical quality attribute to assess the integration and safety of transduced cell products. Two qPCR assays are described that enable quantitation and correlation of total and infectious lentivirus particles designed to facilitate LVV analytics in process development and manufacturing quality control.

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DOI: 10.18609/cgti.2022.234

LVV IN CELL & GENE THERAPY

As of the first half of 2021, there were an estimated 288 lentiviral vector (LVV) cell and gene therapy clinical trials in the pipeline [1]. Of the US Food and Drug Administration (FDA)-approved cell and gene therapies, four CAR-T cell therapies and two *ex vivo* gene therapies use LVV [2]. Characteristics of the recombinant LVV system, such as long-term transgene expression, high packaging capacity, and the ability to transduce both actively dividing and non-dividing cells, have advanced its use in *ex vivo* gene-modified cell therapies [3]. LVVs have also found application in therapeutic gene

editing and genetic vaccine platforms [3]. As a result, there is a strong demand for high-quality LVV for therapeutic applications.

To meet the growing demand, advances are being made in large-scale production to improve yields and turnaround and develop robust analytics to ensure vector quality and safety. While the manufacturing process is similar to that of other vectors, a few characteristics of the LVV are unique and need to be considered in downstream processing and analytics (Figure 1). Harvests typically have a range of impurities that should be accounted for in downstream processing. The LVV particles are fragile and sensitive to temperature. Also, the bioproduction matrices are complex and often require optimization for improved yields and quality.

QUANTITATION OF LV VECTORS

As LVVs are used to transduce cells, they are an active ingredient in drug substances, meaning they must be tested for identity, purity, strength, safety, and quality according to the FDA's chemistry, manufacturing and controls (CMC) guidelines [4,5]. There are several critical quality attributes (Table 1) for LVV, including titer, which is important for strength, quality, and safety testing.

Commonly used methods of LVV quantitation include p24 ELISA, reverse transcriptase (RT)-qPCR or digital PCR, and particle counting. Regardless of the method used, manufacturers have identified quantitation challenges including poor reproducibility, high variation, difficulties optimizing the assays to complex matrix conditions, and inefficient recoveries. These challenges, coupled with the lack of an LV reference standard, make it difficult to accurately quantify yields.

Furthermore, before patient cells are transduced, the infectious titers are tested in cell lines or healthy donor cells and the resulting integration frequency is measured as vector copy number (VCN) [4]. VCN is also measured in the final cell therapy product since high integration frequencies may pose a safety risk [4,6].

Methods for integration analysis include flow cytometry or fluorescent-activated cell sorting (FACS) for transgene expression, qPCR, or digital PCR to measure provirus integration, and cell-based assays to calculate infectivity. Here, assays must be amenable to use in a quality control (QC) environment with a rapid turnaround and minimal manual intervention. Cell lines and healthy donor cells used for infectious titer may have different transduction efficiencies to patient cells, which poses additional challenges.

In addition to the regulatory expectations for drug substance and drug product testing, there are also considerations around the validation of methods used for analytical

FIGURE 1

LVV manufacturing and analytics considerations.

TABLE 1

Select critical quality attributes (CQAs) and validation characteristics.

CQAs for viral vectors	CQAs for transduced cells
Strength/potency: total viral particles (genome titer)	Safety: vector copy number (VCN)
Strength/potency: infectious viral titer	Safety: mycoplasma
Strength/potency: potency assays	Safety: sterility
Purity: residual host cell DNA	Safety: endotoxin
Purity: residual plasmid DNA	Safety: replication competent viruses
Purity: host cell proteins	Strength/potency: potency assays
Safety: mycoplasma	Strength/potency: % or # of genetically modified cells
Safety: sterility	Identity: cell composition
Safety: endotoxin	Identity: transgene identity
Safety: replication competent viruses	Identity: HLA typing
Safety: adventitious agents	Purity: unintended cell population
Validation characteristics for analytical assays	
Specificity	Linearity
Assay range	Accuracy
Precision	Detection limit
Quantitation limit	Robustness
System suitability	

testing [7,8]. Performance characteristics evaluated for content/potency assays (e.g., titer) include specificity, working range, accuracy, repeatability, and intermediate precision [7,8].

Given the many demands and expectations for analytical testing and method validation, manufacturers must take these into account when planning scope, time, and budgets. Analytical assays could be developed in-house, which requires specialist technical expertise to design and develop them for achieving the robust performance required for validation. In addition, sourcing and qualifying critical reagents and maintenance post-validation also requires resources. Testing could also be contracted out to service labs, which may reduce resource dependence but may also reduce control of data, delay actionable results, and extend timelines and costs. Alternatively, commercially available solutions can be evaluated and implemented quickly to help reduce development timelines and leverage vendor expertise for implementation and validation.

APPLIED BIOSYSTEMS™ VIRALSEQ™ LENTIVIRUS TITER KITS

Thermo Fisher Scientific has developed robust integrated assay solutions that can be validated to give rapid, actionable results in-house. For LVV quantitation (total genomes) and integration analysis (proviral copy numbers), two qPCR assays have recently been introduced: the ViralSEQ™ Lentivirus Physical Titer Kit and the ViralSEQ™ Proviral DNA Titer Kit respectively (Figure 2).

In a typical LVV manufacturing process, there may be a series of purification and downstream processing steps between harvest and fill/finish. The Lentivirus Physical Titer Kit can be used to measure and compare yields across these different phases, for example at harvest after nuclease treatment or after tangential flow filtration and/or ion exchange chromatography.

This is a one-step RT-qPCR assay for genome-based LVV titers. It targets the conserved long terminal repeat (LTR) region of

FIGURE 2

ViralSEQ lentivirus titer kits.

LVV. TaqMan™ assay-based chemistry provides high target specificity, preventing overestimation of titer due to background signals from cross-contaminants, such as residual plasmid and host cell DNA. The assay has a dynamic range from 50 to 10^9 copies per reaction, which provides over 7 log dynamic range for measurement without needing to dilute samples. The kit includes all reagents required for the RT-qPCR reaction and comes with an RNA standard. The total assay runtime with sample preparation included is under 6 hours.

Once a suitable cell line has been transduced with the LVV, the Proviral DNA Titer Kit can be used to measure integrated LVV (provirus) in the cell line genome. This is a qPCR assay to measure proviral copies in transduced cells, and subsequently, calculate viral infectious titers and VCN. It also targets the LTR region of the provirus. TaqMan assay-based chemistry provides high target specificity, and the assay range enables proviral copy number determination for a range of transduction efficiencies. The assay has excellent sensitivity with a limit of quantitation (LOQ) of 25 copies per reaction for lower efficiencies. The kit includes all reagents required for qPCR and a DNA standard. The total assay runtime with sample preparation is around 5 hours.

Combined, the two qPCR assays provide a convenient method to compare and correlate data for total and infectious titers, and measure VCN, for analytics across the LVV workflow (Figure 3). Both assays are designed to provide robust performance, facilitating LVV analytics for process development and manufacturing QC.

ASSAYS DESIGNED FOR PROCESS DEVELOPMENT & QC

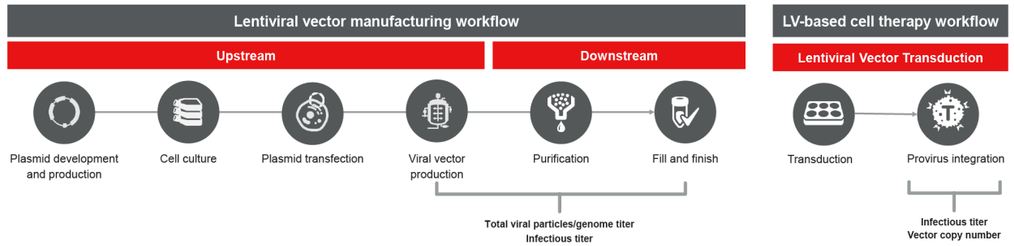
There are many demands and regulatory expectations for the characterization of viral vectors, transduced cells, and even the methods used for analytical testing of these products. Thermo Fisher Scientific understands the rigors of assay validation and the specific processes that manufacturers must perform as part of the CMC filings. Hence assays are developed with extensive verification and internal validation testing (Box 1) to help ensure that they perform to the high standards required to meet validation criteria and regulatory expectations.

In the development testing for these titer assays, bioproduction and cell culture matrices were evaluated to mimic the representative sample conditions and assays were tested against potential inhibitors. For the physical titer kit, assay performance was verified with harvest and purified bioproduction LVV samples. Transduced cell lines were used for verification of the proviral DNA titer assay. Additionally, qPCR data were correlated with orthogonal titer methods.

For assay validation, specificity, precision, repeatability, working range, and relevant qPCR parameters, in line with the ICH Q2 R2 guidelines, were evaluated. Multi-lot and multi-site testing were performed to ensure robust, reproducible assay performance. A detailed report outlining the criteria, experiments, and results of validation is available upon request. With extensive development and validation testing, Thermo Fisher Scientific assays and solutions are QC-amenable and ready for validation, supporting process development and manufacturing QC environments.

FIGURE 3

LVV manufacturing and transduction workflow.



SUMMARY

The ViralSEQ Lentivirus Titer Kits provide a rapid, robust, and reliable solution for measuring total (genomic) and infectious titers for lentivirus characterization. Both assays quantitate based on LTR, allow seamless correlation of qPCR data in the downstream phases, and facilitate analytics across the workflow from viral vectors to transduced cells. The assays have been internally validated to support validation at user sites as per regulatory expectations.

Additionally, through Thermo Fisher Scientific's support programs, field application scientists can guide method optimization, qualification, and validation to accelerate the implementation and integration of methods into processes and help meet specific regulatory needs. A growing team of global regulatory specialists can assist customers with attendance at type C meetings and detailed responses to regulatory queries during reviews. For relevant products, drug master files are in place to allow access for regulators should they need additional proprietary information. Special regulatory support packages are available to assist with any additional customer needs.

BOX 1

Assay development and validation testing.

ViralSEQ lentivirus titer assays testing

- Bioproduction and cell culture matrices
- Bioproduction and cell culture representative samples
- Potential inhibitors
- Correlation with orthogonal methods
- Internal assay validation: lot-to-lot (3 lots)
- Internal assay validation: site-to-site (2 sites)
- Internal assay validation: operator-to-operator (5+)
- Internal assay validation: 2 PCR systems, multiple instruments
- Internal assay validation: 2 sample prep methods

Q&A

Charlotte Barker, Editor, BioInsights speaks to **Unnati Dev, Product Manager, Pharma Analytics Team, Thermo Fisher Scientific** (pictured)

Q With a variety of LVV designs out there, how do I know this assay will work for our recombinant lentivirus platform?

UD: The assay targets one of the conserved regions of the long terminal repeat (LTR) sequence, and we have tested it *in silico* with several LTR sequences on various transfer plasmids, including self-inactivating (SIN) modifications. The assay should be compatible with most transfer plasmid systems, as long as the LTR is not specifically modified. If you have any questions regarding design compatibility, please do contact us – we are always able to check the sequences of your LTRs to confirm compatibility.

Q Are there any regulatory issues with using a platform assay compared with target-specific assays?

UD: Regarding lentiviral vectors, so far there has not been specific guidance or recommendations about targets that can or cannot be used. LTR is commonly used for titer assays, along with other similar platform assays targeting conserved backbone elements. So it should be an acceptable approach.

Q Is there a reference gene included in the proviral titer assay?

UD: The assay does not target a reference gene. Instead, the recommendation in the protocol is to quantitate DNA extracted from the cells using a Qubit fluorometer and use the mass conversion factor provided for diploid cells, to estimate the cell number. If your cell line is polyploid, you may need to establish that conversion factor separately. A reference gene can be used but it will need to be run in a separate reaction and then the data from the two assays can be correlated.

Q Can the physical titer assay distinguish between plasmid and viral genome?

UD: The assay isn't designed to select against plasmid DNA directly, but the protocol includes a DNase step to degrade residual plasmids prior to RT-qPCR. The DNase treatment is optimized to remove most residual plasmid DNA and host cell DNA. Then, you would have the primer-probe designed to bind specifically to the viral cDNA and amplify those genomic sequences.

Q Do any of your titer assays use Jurkat cells?

UD: The proviral titer assay kit does not include a cell line. You can use Jurkat or any other cell line for viral transduction and then use our assay to measure integration and/or infectious titer.

Q What about the RNA genomes that are not encapsidated?

UD: The assay will not be able to distinguish between encapsidated and non-encapsidated genomes. However, an initial RNAse step can be performed to remove any non-encapsidated RNA, if this is expected in harvest samples. The RNase treatment can be performed first, followed by sample extraction, DNase treatment, and RT-qPCR.

Q What is the range of lentivirus titers that can be measured with this assay?

UD: The physical titer assay has over 7 logs of dynamic range from the limit of quantitation (LOQ) of 50 copies to the upper LOQ of 10^9 copies per reaction. Numbers can vary depending on the experimental conditions, but our testing covered the range between 10^4–10^{11} viral particles per mL.

Q Is there correlation between physical titer assays and transduction/bioassays?

UD: Both the physical and proviral DNA titer assays are qPCR-based and hence easier to correlate than using two different methods, such as ELISA for viral titer and plaque assays for infectious titer. Typically, we have observed that the total titer is about 2–3 log greater than the infectious titer. But this depends on the manufacturing yield, the quality of LVV produced, and how the production process has been optimized.

Q Will these lentivirus qPCR assays work on digital PCR platforms?

UD: The kit has been developed and optimized for quantitation on a qPCR system. We have run a few experiments on the Absolute Q digital PCR system, and it can be used with some protocol modification. But we have not tested it on other digital PCR platforms. With the appropriate reagents, they are expected to work, but performance cannot be guaranteed.

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BIOGRAPHY

UNNATI DEV is a Product Manager with the Pharma Analytics team at Thermo Fisher Scientific. She supports customers in bioproduction with solutions for detection and quantitation of adventitious viruses and viral vectors across various therapeutic modalities. While identifying analytical testing needs in vector characterization for cell and gene therapies, she supports development and implementation of new assays to improve analytics. Unnati has a Master's degree in Biotechnology from Texas A&M University.

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Pharmaceutical analytics

The way forward in lentivirus quantitation.

Rapid analytical solutions for lentiviral vector titer and characterization

Applied Biosystems™ ViralSEQ™ lentivirus titer kits are easy-to-use, robust, real-time PCR (qPCR) assays for lentivirus (LV) characterization that enable validation to meet regulatory expectations.

The Applied Biosystems™ ViralSEQ™ Lentivirus Physical Titer Kit measures genome-containing LV particles, while the Applied Biosystems™ ViralSEQ™ Lentivirus Proviral DNA Titer Kit quantifies integrated proviral DNA copies in transduced cells for infectious titer and vector copy number assessment.

These kits provide a convenient method to quantitate and correlate qPCR data for total and infectious LV particles, facilitating analytics in process development, optimization, and manufacturing quality control (QC).

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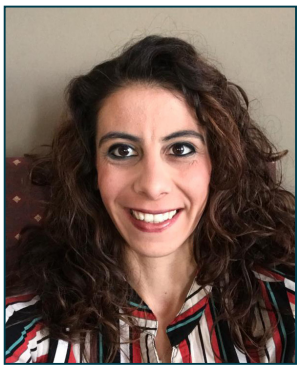
CELL & GENE THERAPY INSIGHTS

ANALYTICAL INNOVATION:
Meeting the Demands of Commercial Viral Vector Manufacture

CHAPTER 5

Residual DNA testing in viral vector manufacture: exploring the challenges and solutions



Ilaria Scarfone, Field Application Specialist, Pharma Analytics, Thermo Fisher Scientific Scientific **speaks to**
Mike Brewer, Director and Global Principal Consultant, BioProduction Division, Thermo Fisher Scientific

Cell & Gene Therapy Insights 2022; 8(1), 47–52

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Q IS: Mike, to begin, could you give a brief introduction and background on the residual DNA testing regulatory requirements in general, and why gene therapy products present some particular challenges?

MB: For recombinant viruses used in gene therapy treatments, there are additional challenges compared to monoclonal antibody (mAb) manufacturing and purification processes.

Specifically, the regulatory guidance on host cell DNA is that there should be less than 10 nanograms of host cell DNA per therapeutic dose. Additionally, at present you should be able to demonstrate that the DNA is less than 200 base pairs in length.

This guidance was essentially carried over from the older guidance on host cell DNA regarding manufacturing of cell-culture based vaccines. Some manufacturers that have been in that space may already be familiar with this guidance.

Additionally, for recombinant viruses such as AAV, there are multiple DNA residuals of concern depending on the processes. It can be host cell DNA, plasmid vector, or helper virus DNA that is part of the process. Multiple DNA assays may be required for full characterization at the levels and the capability of your purification process to reduce the levels of these DNAs.

You also need to have a good size assessment assay. DNA fragment size determination is expected, and that may need to be done at different points in the process, along with quantitation of the remaining host cell DNA at the end of production. In some cases the level of that DNA may be too low to enable accurate size assessment.

An additional challenge with AAV and host cell and vector DNA is recombinant AAV has been shown to encapsidate fragments of both vector and host cell DNA. The size of these encapsidated fragments can be significant; up to 5 kB. That can create challenges, including being able to reach that 10 nanogram per dose limit for high dosage formulations in these gene therapy applications.

Finally, if using cell lines that are known to contain potential oncogenes such as *E1A*, or SV40 T antigen in the case of 293-based processes, the presence of these genes and potentially the size of the genes that are present should be characterized.

These are all the additional considerations that manufacturers must take into account for these viral vector manufacturing processes.

Q IS: Before we analyze each of these particular concerns, let's discuss some of the challenges in the development and validation of residual DNA analytical assays in general, and in particular for gene therapy.

MB: Let's start with development. Development of these assays for sensitive and accurate quantitation of host cell DNA and other DNA residuals requires specialized expertise, it takes considerable time, and you have to develop multiple documents including method standard operating procedure (SOP) preparation qualification of critical reagents, equipment SOPs, and development and qualification reports for the new method. Plus validation protocols, execution of that validation, and generation of a report.

If an organization has the time and expertise, then certainly in-house development can be considered. However in this era they are fully integrated, and that includes sample preps, standard DNA, quantitation assay, even application-specific software and kit-based solutions for host cell and other DNA residuals. In the case of the Applied Biosystems™ ResDNASEQ™ kits, they are also supported by experienced application scientists and regulatory support teams. Therefore the process of implementation of a host cell DNA assay purchased from a vendor can be accelerated dramatically. A new user can be generating valid results in a few weeks, as opposed to the months or even years required for in-house development.

"Development of these assays for sensitive and accurate quantitation of host cell DNA and other DNA residuals requires specialized expertise, it takes considerable time..."

My view on this, and I am sure it's shared by many others, is that if a high-quality kit is available for your application, then use it. Spend your time and resources on generating results, not on development of a method.

One other thing to keep in mind is that a rigorous, well-designed study to demonstrate a robust host cell DNA clearance and purification process can require analysis of a large number of samples. A method that can be automated, such as sample preparation, can be a huge value and efficiency driver.

The sample preparation method used in the ResDNASEQ kits is based on magnetic beads, so there are two options for automating that workflow. That is an important consideration as you look to the future.

Finally, moving to validation. For most applications, host cell DNA testing is considered a quantitative test for impurities and should be validated as such, per the ICH Q2 (R1) guidance on validation of analytical procedures.

Typically the test performed for a quantitative method should include accuracy, both repeatability and intermediate precision, limit of quantitation, specificity, range, and linearity. Robustness of a method should also be demonstrated, but it is acceptable to do that as part of method development – or that data can be supported by the vendor if you are using a solution such as ResDNASEQ. We have extensive data demonstrating robustness that was done as part of our method development process, and this certainly can be shared during a regulatory review.

Q IS: Looking at validation and qualification, how does the Thermo Fisher Scientific development validation study help ensure the quality of the kits, and how does this differ from the validation of the method for regulatory approval?

MB: As part of our development process we have a very well established and defined process. There are tests we put a new method through as we develop it and then as we get near completion, to demonstrate the robustness of it and assess how small deviations to

the recommended method affect performance. We keep those all well documented, and as I mentioned before it can be available for referencing during a regulatory review.

When I use or hear the term qualification when discussing analytical methods, I consider that step as the initial demonstration, in a fairly rigorous manner, that the method will perform.

Can it accurately quantitate DNA recovered from key sample types, or from key sample matrices? Can it detect a contaminant in a key process sample? Qualification can also be referred to as pre-validation, generating a set of data that enables design of a validation study, and importantly, the health and setting appropriate acceptance criteria for that validation study.

Then importantly, the development stage of your manufacturing process and your clinical process should be considered; where and when use of a qualified method is acceptable for testing, and when a validated method is required.

It can be acceptable to use a qualified method for testing at the preclinical and early clinical stage of your product. Then, following success in advancing a product candidate through clinical trials, at some point validation will be required.

When you are selecting analytical methods, choose a method early that offers the performance required and looks able to be validatable, and critically, a method that typically regulatory have accepted in the past, following validation of submission and inclusion in your CMC package.

Choosing the right method early is critical so that you don't have to go back and redevelop or switch to a new method late in the process where there are lots of tasks that need to be accomplished in order to file an application for approval of a new product.

When working closely with an experienced vendor, key considerations should be that they can provide analyst training and workflow, equipment validation support, and provide examples of validation study design and drug master files when appropriate. The ability to support on regulatory use and having a record of success should also be key considerations when selecting an analytical solution. This is where Thermo Fisher in particular is very strong. In my opinion, the team we have supporting our products is unmatched in the industry.

In certain cases, such as here where we are talking about host cell DNA and other DNA residual testing with the ResDNASEQ product, the vendor can be a collaborator, and that will accelerate timelines and provide confidence in success.

"When working closely with an experienced vendor, key considerations should be that they can provide analyst training and workflow, equipment validation support, and provide examples of validation study design and drug master files when appropriate."

Q IS: Focusing on gene therapy, and residual DNA testing in particular, let's address a couple of specific questions I am often asked by customers. How do we help simplify the process of measuring vector DNA, and how do we address the challenges of fragment sizing an oncogene?

MB: In the past people hadn't given as much consideration to testing for these DNA residuals, as well as sizing. To support that we have recently introduced three new products for recombinant virus manufacturing.

One of these is a combination SF9 and baculovirus residual DNA assay, and this is for insect cell culture-based manufacturing processes. We also now have an assay for residual vector DNA for vectors that are used in the recombinant AAV workflow, and this assay targets an element common in many plasmid vectors, the kanamycin resistance gene.

Finally we have an assay for detection of the *E1A* gene, that is present in 293 cell-based processes. Certainly that gene would be undesirable if it was present in the final product, but additionally the *E1A* assay enables size assessment of the *E1A* gene, and can also be used to assess the general size and quantity of host cell DNA fragments as you go through the purification process.

This is accomplished by use of a primer design for detection and quantitation of three distinct size classes of the DNA in the sample: the larger size of 476 base pair amplicon, an intermediate size of 200 base pair amplicon, and finally an assay specific for small fragments. This is important because this is an 86 base pair amplicon, which is below the regulatory guidance of less than 200 base pair fragments.

Q IS: Do you have any suggestions on how to address copy number versus mass concerns?

MB: For some analytes such as host cell DNA, regulatory guidance has always been the mass of the analyte in the sample. For other analytes such as the residual vector or *E1A* fragments, it may be more appropriate to report that result than copies of the analyte. The most important consideration here is to use a method that can accurately generate results and data that is aligned with the regulatory guidance for requirements. If you are using an assay like quantitative real-time PCR, the results can be generated and reported in either copy or mass of DNA, depending on how you design the experiment.

BIOGRAPHIES

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Field Application Specialist, Pharma Analytics, Thermo Fisher Scientific

Ilaria has been in her current role since September 2019. She has a strong academic research background in industrial biotechnology and has completed an MBA at the IAE of Montpellier.

Mike Brewer

Director and Global Principal Consultant, BioProduction Division, Thermo Fisher Scientific

In his current role, Mike is responsible for providing global support to BioProduction customers and serving as the regulatory thought leader and expert across various technology areas. Mike has over 30 years' experience in the Biopharma industry in a variety of roles including Quality Control, Discovery Research and Analytical sciences.

AUTHORSHIP & CONFLICT OF INTEREST

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Development & validation of a robust commercial solution for measuring residual kanamycin-resistant plasmid DNA

Tania Chakrabarty, Senior Manager, Innovation Leader, Research and Development, Pharma Analytics, Thermo Fisher Scientific

Plasmid vectors are used in bioproduction, gene therapy, and vaccine manufacturing workflows and the final drug products must be tested for residual DNA for lot release and QC. Residual plasmid DNA is a part of the residual DNA limit of 10ng/dose per regulatory guidelines. This article will discuss the development and validation of a highly robust multiplexed qPCR assay for the quantitation of residual plasmid DNA carrying kanamycin resistance genes.

The gene therapy field is growing at a tremendous rate, with over 3000 candidates currently in clinical trials. Many of these are viral vector-based therapies, which utilize plasmids in the production process and must meet regulatory guidelines for residual DNA in their process and final drug product (10 ng total residual DNA/dose).

With few commercially available solutions for residual plasmid DNA testing, companies have been forced to develop in-house tests – a time-consuming, technically challenging, and labor-intensive process.

A NEW TOOL

To address this need, Thermo Fisher Scientific has developed the resDNASEQ™ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit. For gene therapy or vaccine makers who are currently using plasmids in their bioproduction workflow, the kit provides a robust, easy-to-use, highly sensitive multiplex qPCR assay to measure residual plasmid DNA by targeting all common alleles of kanamycin resistance genes.

The product targets multiple alleles of the kanamycin resistance gene to cover the vast majority of commonly used kanamycin resistance plasmids in bioproduction, to detect and quantify residual plasmid DNA with kanamycin resistance gene in the sample of interest. The assay uses the FAMTM dye to detect three conserved regions among different kanamycin resistance gene families.

VALIDATION STUDIES

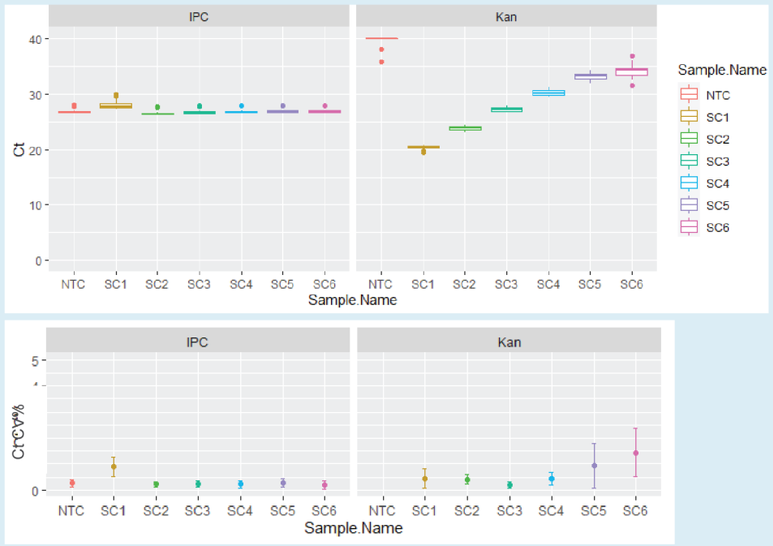
We carried out a series of validation experiments to demonstrate that the resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit is a highly specific, sensitive, and robust solution for measuring residual plasmid DNA.

To validate the assay for residual plasmid DNA testing, we focused on the following performance parameters:

- PCR efficiency (100 ±10%)
- Linearity (R^2 > 0.99)
- Range of the standard
- Accuracy
- Precision
- LOQ / LOD (30/15 copies)
- PrepSeq Spike-Recovery (70–130%)
- Specificity

We validated performance using a total of 10 operators across two continents, spanning several days and using two qPCR platforms (7500 Fast Real-Time PCR Instrument and QuantStudio 5 Real-Time PCR Instrument). We also used two different sample preparation methods – one was manual, and the other was automated using the KingFisher Flex (KFF) platform.

Figure 1. QuantStudio 5: standard curve performance across instruments, days, and operators.



The results demonstrated high overall precision down to a LOQ of 30 copies, indicating that the data are consistent and reliable across multiple operators and instruments, even when quantitating low levels of DNA (Figure 1). The IPC Ct remained steady across a wide range of kanamycin concentrations. The PCR efficiency was 100+/-10% and showed excellent linearity

We also tested the assay performance against a variety of exclusion panel substances to ensure there is no cross-reactivity. The IPC amplification remained the same in the presence and absence of a variety of cross-reactants, demonstrating that the assay was highly specific.

CONCLUSION

Our results demonstrate that the resDNASEQ Quantitative Plasmid DNA - Kanamycin Resistance Gene Kit could detect its targets quantitatively in a variety of matrices corresponding to workflows used in bioproduction, gene therapy, and vaccine manufacturing workflows.

The assay is compatible with both manual and automated extraction (KFF) workflows using PrepSEQ kit and gave robust results on both QS5 and ABI7500 platforms. It is highly sensitive (LOQ=30 copies, LOD=15 copies) and specific, showing no cross-reactivity as tested using exclusion panels (Table 1).

Table 1. Specification of resDNASEQ Quantitative Plasmid DNA – Kanamycin Resistance Gene Kit

Linearity	R^2 > 0.99
PCR efficiency	100 ± 10%
Precision	≤10% CV
Limit of detection (LOD)	15 copies
Limit of quantitation (LOQ)	30 copies
Assay range	300,000 copies to 30 copies

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FASTFACTS

FAST FACTS VIDEO

Development and validation of a robust commercial solution for measuring residual kanamycin-resistant plasmid DNA

Applied Biosystems resDNASEQ™ Quantitative Plasmid DNA–Kanamycin Resistance Gene Kit

For gene therapy/vaccine customers who are currently using plasmids in their bioproduction workflow, Thermo Fisher Scientific provide a robust, easy to use, highly sensitive multiplex qPCR assay for them to measure residual plasmid DNA by targeting all common alleles of kanamycin resistance genes.

Plasmid vectors are used in bioproduction, gene therapy, and vaccine manufacturing workflows and the final drug products must be tested for residual DNA for lot release and QC. Residual plasmid DNA is a part of the residual DNA limit of 10ng/dose per regulatory guidelines.

In this **FASTFACTS** Tania Chakrabarty, Senior Manager, Innovation Leader at Thermo Fisher Scientific discusses the development and validation of a highly robust multiplexed qPCR assay for the quantitation of residual plasmid DNA carrying kanamycin resistance genes.

- This product targets multiple alleles of the kanamycin resistance gene to cover the vast majority of commonly used kanamycin resistance plasmids in bioproduction.
- The function is to detect and quantify residual plasmid DNA with kanamycin resistance gene in the customer's sample of interest.
- The assay uses the FAM™ dye to detect three conserved regions among different kanamycin resistance gene families.
- The NEDTM IPC acts as the internal control to verify the absence of inhibition during PCR amplification.

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