CELL & GENE THERAPY INSIGHTS

ANALYTICS CHANNEL: ACCELERATING QC & RELEASE TESTING

INNOVATOR INSIGHT

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

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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 QC.

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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

FIGURE 1

LVV manufacturing and analytics considerations.

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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	

the validation of methods used for analytical 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

FIGURE 2

ViralSEQ Lentivirus Titer Kits.

conserved long terminal repeat (LTR) region of 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,

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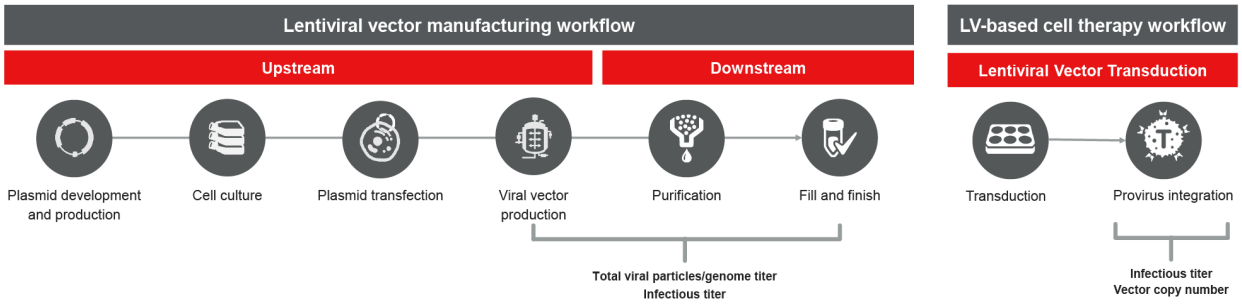
FIGURE 3

LVV manufacturing and transduction workflow.



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.

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?

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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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AUTHORSHIP & CONFLICT OF INTEREST

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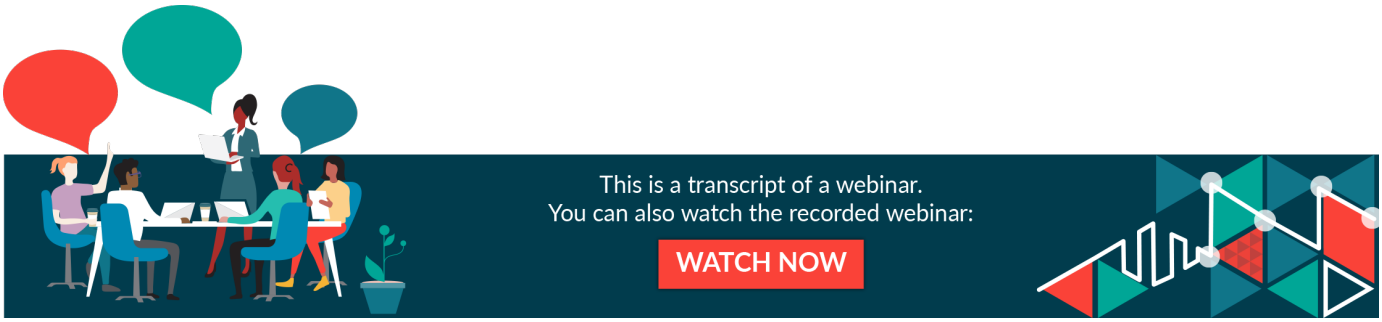

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Applied Biosystems™ ViralSEQ™ lentivirus titer kits are easy-to-use, robust, real-time PCR (qPCR) assays for lentivirus (LV) characterization that enable validation to meet regulatory expectations.

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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