BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY
Published Online September 15, 2026

Final Processing and Quality Evaluation of a Lentiviral Vector Reference Material

By Matthew Kaleta, Zachary Cuba, Carrie Edmonds, Sujata Choudhury, Lenny Godinho, A. Ciera Albrecht, Karen Dixon, Keith L. Carson, Heather Branscome, and Heather Couch

ABSTRACT

Lentiviral vectors (LVVs) are broadly used in research and therapeutic development, yet substantial interlaboratory variability continues to hinder the comparison of vector quantity, potency, and impurity measurements, underscoring the need for standardized reference materials. To address this need, the Lentiviral Vector Reference Material (LVVRM) initiative was launched as a structured, multi-phase effort, with earlier phases focused on large-scale upstream production and downstream preparation of a GFP-encoding LVV. In the final phase of the LVVRM initiative described herein, bulk material produced during prior large-scale manufacturing runs was thawed, pooled, subjected to sterile filtration, and dispensed into single-use vials under controlled conditions designed to maintain stability and consistency. Each lot then underwent comprehensive quality control testing. Sterility, identity, purity, physical and functional titers, and safety attributes were assessed using established characterization frameworks appropriate for lentiviral vector reference materials. The resulting LVVRM (ATCC® VR-3382™) met quality standards and release criteria, providing a well-characterized reagent suitable for assay calibration, method qualification, and longitudinal performance monitoring across laboratories.

INTRODUCTION

Lentiviral vectors (LVVs) have become indispensable tools across modern molecular biology, biotechnology, and translational medicine. Their ability to stably integrate genetic payloads into dividing and non-dividing cells has enabled applications ranging from basic mechanistic studies to the manufacturing of gene-modified cellular therapeutics.[1–3] As LVV-based workflows have expanded into clinical development, regulatory evaluations, and industrial-scale platforms, the need for rigor, reproducibility, and methodological harmonization has become increasingly evident. Yet, despite the maturity of LVV technology, laboratories continue to face substantial variability in measurements associated with vector quantity, potency, identity, and impurity content. This variability stems from differences in production systems, analytical assay designs, sample handling, and lot-to-lot fluctuations in reagents.[4,5] As a result, cross-study comparisons remain difficult, and method qualification or technology transfer between organizations often requires extensive bridging work.

Efforts to address these challenges have prompted the development of standardized viral reference materials across multiple vector systems.[6] For nearly two decades, coordinated multi-stakeholder groups have generated shared adenovirus, adeno-associated virus, and retrovirus reference materials to support interlaboratory harmonization and regulatory confidence.[7] Within this context, the Lentiviral Vector Reference Material

(LVVRM; ATCC® VR-3382™) initiative was established to produce a common LVV preparation suitable for benchmarking analytical performance and enabling direct comparison of results across laboratories. The LVVRM initiative represents a coordinated effort among academic, government, and industrial contributors to generate, characterize, and distribute a research-grade lentiviral material for broad use by the LVV community.

Earlier stages of the LVVRM effort focused on large-scale upstream production and downstream preparation of a GFP-encoding LVV. These efforts included development and optimization of an inducible HEK293-based production system and execution of manufacturing runs at the 100-L scale. These upstream activities, together with associated process development steps, are described in previously published reports detailing LVVRM production and scale-up workflows.[8,9] Following vector production, downstream operations produced a purified bulk material that served as the source for subsequent fill-finish activities. The upstream and downstream phases established the identity, baseline titer, and physical characteristics of the bulk LVVRM, laying the foundation for preparing material suitable for distribution to research and manufacturing laboratories. However, generating bulk material is only part of the process required to create a usable reference material. The final transformation of bulk LVVRM into a distributed reference material involves additional steps that must be carried out under controlled conditions. These include thawing individual bulk containers, pooling to ensure homogeneity, sterile filtration to support product quality requirements, and automated dispensing into consistent vial formats. Equally important, the material must be frozen and stored under conditions that maintain stability and preserve both physical and functional characteristics. The fill-finish stage carries considerable weight in establishing reference material consistency because even minor deviations during handling, filtration, vialing, or freezing can affect vector potency, impurity profiles, stability, and suitability for long-term use.

Quality control (QC) characterization is consequently an essential component of the LVVRM workflow. The analytical expectations for a distributable lentiviral reference material align with those applied to other standardized viral reference reagents and are reflected in the characterization framework used for this LVVRM. Characterization of LVVRM includes measurement of physical particle concentration by p24 ELISA, genome copy number by digital-based PCR, functional titer via transduction of permissive cells, and evaluation of impurities such as host cell proteins (HCPs) and nuclease residuals using ELISA-based assays. Additional required QC elements include sterility testing, mycoplasma detection, adventitious agent screening, and assessment for replication-competent lentivirus (RCL). Together, these assays form a comprehensive panel that ensures reference material integrity and supports confidence in its use for assay calibration, method qualification, and longitudinal performance monitoring.

Within the LVVRM project, the fill-finish and QC qualification steps represent the final phase of a multi-year effort to generate a robust, well-defined LVVRM. This phase is critical because it bridges the gap between bulk production and practical usability: only after thorough QC assessment and verification of stability can the LVVRM be reliably deployed for consistent use across laboratories. The current manuscript describes the procedures and considerations governing the thawing, pooling, sterile filtration, automated dispensing, freezing, and storage of the LVVRM, followed by the comprehensive QC strategy applied prior to release. By documenting these final steps, this report completes the LVVRM publication series and provides transparency into the processes required to produce a standardized lentiviral reference material for community use.

MATERIALS AND METHODS

Processing of LVVRM

Source Material and Storage

Bulk LVVRM was generated and cryopreserved during earlier manufacturing phases. The material was filled into 1 L CryoStore freezing bags (OriGen Biomedical), frozen at −80°C, and shipped on dry ice. Upon receipt, all bags were inspected for physical integrity, verified against chain-of-custody documentation, and transferred directly into −80°C storage. Bags remained at −80°C until processing to maintain material stability.

Thawing of Material

Immediately prior to fill-finish operations, individual LVVRM CryoStore freezing bags were thawed using a controlled thawing device (CPSI Biotech). The device was pre-equilibrated and disinfected before use. Bags were placed into the chamber, ensuring full contact between the bag and the heating surface. Thawing proceeded at approximately 42°C with oscillation to promote uniform heat transfer and prevent the formation of localized warm zones. Bags were visually inspected throughout thawing to verify complete thawing and the absence of residual ice crystals. Once thawed, bags were dried, surface-disinfected with 70% ethanol, and transferred onto ice packs. The LVVRM remained on ice for all subsequent operations, and bags remained sealed until connected to the pooling assembly. All manipulations were performed in a biosafety cabinet (BSL-2).

Pooling and Sterile Filtration

A custom, pre-sterilized, single-use fluid handling assembly (Sartorius) was used for pooling and filtration. The setup included four disposable bags, a dedicated 0.2 µm sterilizing-grade filter, a filtration buffer bag, a pressure sensor, and tubing. Thawed LVVRM was transferred from CryoStore freezing bags into the pooling bag via a spike port using a peristaltic pump. In parallel, pre-chilled formulation buffer (20 mM HEPES, 75 mM NaCl, 2.5% sucrose; pH 7.0) was sterile-filtered into a second bag and used to flush and pre-wet all lines and the filtration membrane. Excess buffer was diverted to waste to ensure the membrane remained wetted without excessive dilution of the vector material. The pooled LVVRM was then passed through the sterilizing filter into the final collection bag under controlled pressure. A post-filtration buffer flush was performed to recover residual vector material from the lines and filter housing. The final bag was aseptically disconnected from the assembly, and in-process samples were collected at predefined points and stored on ice until preservation.

Automated Dispensing and Preservation

Filtered LVVRM was dispensed into individual vials using a custom-configured robotic system (Dispense Works) installed in a BSL-2 cabinet. The LVVRM collection bag was mounted onto a rocking platform to maintain gentle agitation and prevent sedimentation. Fluid transfer into the dispense manifold was achieved through a sterile connector (Sartorius). The dispenser was calibrated before each run using gravimetric measurements to confirm accuracy within the specified tolerance. For all dispensing cycles, pallets were pre-chilled between runs to minimize thermal excursions of the LVVRM. Following dispensing, capped vials were immediately placed on ice and subsequently flash-frozen in a liquid nitrogen, vapor-phase cryocart for approximately one hour to ensure rapid freezing. The vials were then transferred to liquid nitrogen, vapor-phase cryotanks for long-term storage.

Quality Control Testing of LVVRM

Sterility Testing

Sterility testing was performed using the BACT/ALERT 3D system (bioMérieux). Vials were thawed once, and aliquots were inoculated directly into aerobic and anaerobic culture bottles. Bottles were incubated at 32.5°C for 14 days with continuous colorimetric monitoring for CO_2 production as an indicator of microbial growth.

Mycoplasma Testing

Mycoplasma contamination was assessed using a dual-mode strategy that combined culture-based detection with molecular screening to ensure high analytical sensitivity and specificity. All assays were performed on final frozen LVVRM vials following a single controlled thaw. Test runs included positive controls, no-template controls, and media blanks, where applicable, to confirm assay performance.

Direct Culture Assay: After thawing, 0.5–1.0 mL of LVVRM was inoculated into mycoplasma-selective broth and onto SP4 glucose agar plates. Broth cultures were incubated aerobically while inoculated agar plates were placed in sealed anaerobic jars containing indicator strips and appropriate gas-generating packs. Broth cultures were sampled and subcultured to fresh SP4 agar at multiple intervals (*e.g.*, days 0, 3, 7, and 14). Each newly plated set was returned to anaerobic incubation for at least 14 days. At the end of each incubation interval, plates were examined under a microscope for characteristic mycoplasma colony morphology, including the "fried-egg" appearance typical of many species. Positive control plates were first examined to establish the appropriate focal plane before scanning test plates. Results were recorded qualitatively as "no colonies detected."

PCR-Based Detection: An endpoint PCR assay (Universal Mycoplasma Detection Kit, ATCC® 30-1012K™) was used to detect mycoplasma. PCR was performed using universal primers targeting conserved regions of the mycoplasma 16S rRNA gene, along with a supplied positive control to verify assay performance. Amplified products were resolved by agarose gel electrophoresis and visualized by DNA staining. Samples were classified as positive or negative based on the presence or absence of a characteristic mycoplasma-specific amplicon within the expected size range.

Adventitious Agent Testing

Adventitious agent testing was performed on final LVVRM vials using a combination of next-generation sequencing (NGS) and an outsourced PCR-based screening service. For the NGS analysis, nucleic acids from thawed vials were processed by the ATCC Sequencing and Bioinformatics Center (SBC). Libraries were prepared according to established SBC procedures for adventitious agent detection and sequenced on an Illumina MiSeq platform. Quality-filtered reads were analyzed using Kraken2 for taxonomic classification. In parallel, an external PCR-based adventitious agent screening panel was performed as an added orthogonal safety check. This service tests a broad panel of viral and microbial contaminants using validated qPCR assays (IDEXX BioAnalytics).

Endotoxin Quantification

Endotoxin levels in final LVVRM vials were measured using a recombinant Factor C (rFC) fluorescence-based assay (Lonza). Frozen vials were thawed once on ice, and samples were diluted in endotoxin-free buffer to ensure readings fell within the linear dynamic range of the assay. Each plate included a full set of endotoxin standards, reagent blanks, and spike-recovery controls to monitor assay performance and potential matrix interference. Reconstituted rFC reagent and fluorogenic substrate were prepared according to the manufacturer's instructions and combined with diluted LVVRM. Fluorescence was measured kinetically using excitation and emission settings appropriate for the rFC substrate. Sample endotoxin concentrations were calculated from standard-curve regression and reported in endotoxin units per milliliter (EU/mL). Triplicate measurements were performed for all samples.

Endonuclease Quantification

Residual endonuclease was measured using an immunoenzymatic ELISA (Cygnus Technologies). Each plate included a full set of endonuclease standards (0–20 ng/mL) and blank wells for background correction. LVVRM vials were thawed once on ice, and samples and standards were added to microplate wells pre-coated with capture antibody and incubated with an HRP-conjugated detection antibody. After washing, substrate was added, and color development proceeded at room temperature before stopping the reaction. Absorbance was measured at 450 nm with a 650 nm reference filter. Endonuclease concentrations were interpolated from a non-linear standard curve (four-parameter logistic fit) and reported in ng/mL. All samples were assayed in duplicate.

Host Cell Protein Quantification

Residual HCP was measured using an immunoenzymatic ELISA (Cygnus Technologies). LVVRM vials were thawed once on ice, and samples were diluted in the kit-recommended diluent to fall within the quantifiable range of the assay. Each microplate included a complete set of HEK293 HCP standards (0–200 ng/mL) and blank wells for background correction. Diluted samples and standards were added to microplate wells pre-coated with anti-HEK293 capture antibodies and incubated with an HRP-conjugated detection antibody. Following plate washes, substrate was added and allowed to develop at room temperature before stopping the reaction. Absorbance was measured at 450 nm with a 650 nm reference filter. HCP concentrations were calculated from a non-linear standard curve (four-parameter logistic fit) and reported as ng/mL of immunoreactive HEK293 HCP equivalents. All samples were assayed in duplicate.

Western Blot

Identity of the LVVRM was assessed by detecting the lentiviral gag/p24 protein via western blot. Vials were thawed once on ice, and lysates were prepared by mixing LVVRM 1:1 with NP-40 lysis buffer containing protease and phosphatase inhibitors and 1 mM dithiothreitol (DTT). Positive-control lysate was generated in parallel from HIV-1-infected Jurkat E6 cells (J1.1; BEI Resources ARP-1340) using the same buffer formulation. Total protein concentrations were determined using a bicinchoninic acid (BCA) assay to ensure uniform loading. Equal amounts of protein were denatured in Laemmli sample buffer containing β-mercaptoethanol and then heated at 95°C. Samples were resolved on 4–20% Tris-glycine SDS-PAGE gels and transferred to PVDF membranes using a semi-dry transfer system (Bio-Rad). Membranes were blocked and then incubated with primary antibody (abcam # ab63917; 1:500 dilution), followed by an HRP-conjugated secondary antibody (1:2000 dilution). After washing, chemiluminescent substrate was applied and signal was captured using a digital imaging system.

p24 ELISA

Physical lentiviral particle concentration was measured using a p24 ELISA kit (GenScript), which incorporates a proprietary free-p24 removal step to quantify lentivirus-associated p24 only. LVVRM vials were thawed, once on ice, and samples were processed according to the manufacturer's recommended workflow. Briefly, free p24 was removed using the kit's magnetic bead-based depletion reagent. The clarified, p24-depleted sample was then diluted in complete medium to fall within the linear range of the assay. A p24 standard curve (31.25–2000 pg/mL) was generated in parallel. For ELISA detection, diluted samples and standards were combined with lysis solution and added to capture antibody-coated wells. After incubation and washing, plates were sequentially treated with biotin-conjugated anti-p24 antibody and streptavidin-HRP, followed by substrate. Absorbance was measured at 450 nm, and p24 concentrations were calculated using a four-parameter logistic fit and multiplied by the dilution factor to determine virus-associated p24 (pg/mL). Lentiviral particle numbers (LP/mL) were estimated using the conversion factor of 1 ng p24 = 1.25×10^7 LPs. All samples were assayed in duplicate.

Genome Copies by Digital-Based PCR

Genome copy number was determined using digital-based PCR. LVVRM vials were thawed, once on ice, and nucleic acid was extracted using the QIAamp Viral RNA kit (QIAGEN). Extracted RNA was reverse transcribed to cDNA prior to digital-based PCR analysis. Reactions were set up using the ddPCR Supermix for Probes (Bio-Rad), forward primer (5′-TGAAAGCGAAAGGGAAACCAG-3′), reverse primer (5′-CACCCATCTCTCTCCTTCT AGCC-3′), and a FAM-labeled hydrolysis probe (5′-AGCTCTCTCGACGCAGGACTCGGC-3′). Droplets were generated using the Bio-Rad QX200 Droplet Reader and amplified in a sealed 96-well plate. After thermal cycling, droplets were analyzed on the droplet reader using QuantaSoft software (Bio-Rad). Thresholds were set using no-template controls to distinguish positive from negative droplets. Genome copy concentrations were determined from droplet positivity rates and adjusted for dilution. All samples were run across multiple dilutions in triplicate, and mean values were reported.

RNase P by Digital-Based PCR

Residual host cell DNA was assessed using a digital-based PCR assay targeting the human RNase P gene. Nucleic acids were extracted from thawed LVVRM vials using the same extraction workflow described above. Extracted nucleic acid was analyzed directly by digital-based PCR without reverse transcription. Reactions were set up using the ddPCR Supermix for Probes and a primer-probe mix targeting the human RNase P gene (IDT), using undiluted extracted nucleic acid. Droplets were generated using the QX200 droplet generator and amplified in a sealed 96-well plate. Following amplification, droplets were read on the QX200 droplet reader, and fluorescence data were analyzed using QuantaSoft software. Thresholds were set using no-template controls to distinguish positive from negative droplets. Absolute RNase P copy numbers were determined from droplet positivity rates. All samples were run in triplicate and mean values were reported.

Functional Titer by Transduction (GFP)

Functional titer was determined using a fluorescence-based transduction assay that was adapted from a publicly available Addgene protocol for lentiviral titering.[10] Briefly, HEK293 cells (ATCC® CRL-1573™) were seeded into multi-well plates one day prior to transduction to achieve a uniform, sub-confluent monolayer. On the following day, serial dilutions of LVVRM were prepared and added directly to the cells and incubated for 24 hours. After this time, the inoculum was removed from each well and cells were fed with DMEM+2% FBS. Plates were returned to the incubator and maintained for 48–72 hours to allow GFP expression. At the endpoint, the percentage of GFP-positive cells was quantified by fluorescence microscopy. Only wells with <40% GFP-positive cells were used for titer determination. Transducing units per mL (TU/mL) were calculated using the following equation: $T = (N \times F \times D)/V$ where N = the number of cells seeded per well, F = fraction of GFP-positive cells, D = dilution factor, and V = transduction volume (mL). Two wells were assessed and the average TU/mL was reported.

Replication-Competent Lentivirus Assay

The presence of RCL was evaluated using the ARP-404 cell line (BEI Resources; derived from HTLV-1-infected C8166-45 cells). Prior to assay initiation, cells were expanded in RPMI-1640 (ATCC® 30-2001™) supplemented with 10% FBS (ATCC® 30-2020™), then transitioned and adapted to Iscove's modified Dulbecco's medium (IMDM) + 10% FBS for at least two passages to ensure consistent growth under assay conditions. For the assay, ARP-404 cells were seeded into T25 flasks and allowed to equilibrate overnight. The following day, cultures were exposed to the LVVRM, or to a lentiviral control (prepared from a dual-envelope HIV-1 construct), to confirm cell transduction and downstream detection performance. Cultures were maintained without removal of the inoculation medium and cells were passaged every 2–3 days by dilution into fresh medium to support potential amplification over a full replication cycle. Samples were collected at weekly intervals over a three-week culture period. At each timepoint, cell supernatants and cell pellets ($\geq 1\times10^6$ cells) were harvested and stored at −80°C until analysis. Supernatants were assayed by ELISA for lentiviral p24 antigen (as described above), and pellets were evaluated by quantitative reverse transcriptase PCR (qRT-PCR) for lentiviral genome sequences.

Statistical Analysis

Quantitative data were analyzed with GraphPad Prism and *p*-values were determined using a one-way analysis of variance (ANOVA) with a Dunnett's *post hoc* test. Data are expressed as mean ± SD and *p*-values were defined as statistically significant (<0.05), of greater significance (<0.01), and of greatest significance (<0.001).

RESULTS

Overall Performance of LVVRM During Fill-Finish

All fill-finish operations were completed without deviation for each lot, yielding a uniform set of cryopreserved vials with consistent appearance and no evidence of precipitation or turbidity. To verify that the processing steps preserved material integrity and did not introduce contaminants, final vials underwent a comprehensive QC panel spanning sterility, adventitious agent detection, purity attributes, vector identity, genome integrity, and titer determination. The outcomes of these analyses collectively demonstrate that the LVVRM met all release specifications and maintained expected biological properties following thaw, pooling, filtration, and dispensing. The subsequent sections summarize the testing results obtained from three independent LVVRM production lots evaluated in this study.

Microbiological and Adventitious Agent Testing

To confirm the absence of microbial contaminants introduced during fill-finish, sterility and mycoplasma testing were performed on the final cryopreserved vials. As shown in **Table 1**, no bacterial or fungal growth was observed during 14 days of aerobic and anaerobic incubation. In parallel, both direct-culture and PCR-based mycoplasma assays were negative, with no colony formation or detectable amplification in any vial tested. To evaluate the presence of unintended viral agents, LVVRM lots were assessed using both NGS and an independent, outsourced PCR-based viral screening panel. Sequencing revealed only LVV-derived reads. A small cluster mapping to a woodchuck hepatitis polymerase fragment was observed, but alignment patterns and database comparison confirmed that these sequences correspond to a vector-associated sequence element, not a biological contaminant. No additional exogenous agents were detected by sequencing or by the IDEXX viral PCR screen (**Table 1**). Together, these orthogonal methods confirm that the LVVRM contains no detectable adventitious agents.

TABLE 1. Summary of microbiological and adventitious agents testing results.

Tests	Specifications	Lot 1	Lot 2	Lot 3
Sterility				
iAST (aerobic)	No growth	No growth	No growth	No growth
iNST (anaerobic)	No growth	No growth	No growth	No growth
Mycoplasma				
Broth and agar (direct)	No growth	No growth	No growth	No growth
PCR	None detected	None detected	None detected	None detected
Adventitious Agents				
NGS	None detected	None detected	None detected	None detected
PCR (IDEXX)	None detected	None detected	None detected	None detected

TABLE 2. Residual impurity testing results.

Tests	Specifications	Lot 1	Lot 2	Lot 3
Endotoxin	Report results	1.8 EU/mL	1.5 EU/mL	2.3 EU/mL
Endonuclease	Report results	BLQ	BLQ	BLQ
HEK293 HCP	Report results	196.0 ng/mL	240.5 ng/mL	208.7 ng/mL
RNase P	Report results	3.4×10^3 copies/mL	9.2×10^3 copies/mL	7.8×10^3 copies/mL

BLQ = below limit of quantification

Purity Evaluation

Analytical purity testing was performed to confirm that process-related impurities remained at levels appropriate for a reference-grade lentiviral material (**Table 2**). Endotoxin was measured using a recombinant Factor C assay, which was selected for its high specificity toward lipopolysaccharide (LPS) and its reduced susceptibility to β-glucan cross-reactivity or other non-LPS contaminants that can confound traditional Limulus amebocyte lysate (LAL)-based methods.[11] The measured endotoxin values across all lots remained < 2.5 EU/mL. Residual endonuclease levels were also low across all LVVRM lots tested. Benzonase-type endonucleases are intentionally introduced during upstream processing to degrade host cell-derived DNA and RNA; therefore, their efficient removal during downstream purification is an important indicator of process performance. The low residual nuclease activity observed following purification and fill-finish demonstrates effective clearance of these enzymes and indicates that they were not carried through into the final cryopreserved vials.

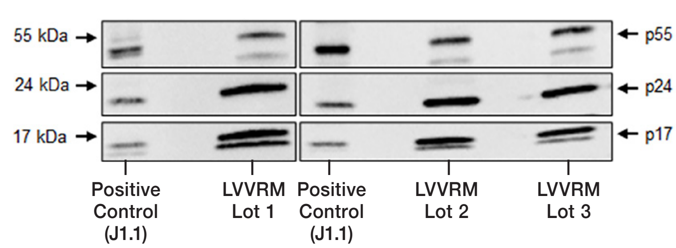
Because the LVVRM was produced in HEK293-derived cells, HCPs represent an expected process-related impurity and must be quantified to confirm their removal during purification. Measurement of HEK293 HCP therefore serves as a direct indicator of producer-cell clearance and verifies that residual cell-derived proteins do not persist in the final LVVRM.[12] Levels were relatively low across batches (< 250 ng/mL). Residual host cell DNA was further assessed by RNase P digital-based PCR. Low RNase P copy numbers (within the 10^3 copies per reaction order of magnitude) were detected across all LVVRM lots, indicating minimal carryover of producer cell-derived genomic material following purification. Together, with the low endotoxin and residual endonuclease levels, these findings suggest that no step in the fill-finish workflow, including thawing, pooling, filtration, dispensing, or cryostorage, introduced substantial impurities and that the LVVRM meets expectations for a distributed reference material.

Identity Confirmation

Identity of the LVVRM was confirmed by western blot detection of the lentiviral gag polyprotein and its expected cleavage products. The assay used an anti-HIV-1 p55/p24/p17 antibody, which recognizes the gag precursor (p55) and its major processed products, capsid (p24) and matrix (p17), enabling detection of multiple lentiviral structural components.

To ensure assay specificity, LVVRM lysates were analyzed alongside lysate from HIV-1-infected Jurkat E6 cells, a well-characterized positive control that expresses authentic HIV-1 structural proteins. The J1.1 line provides a biologically relevant benchmark for expected gag/p24 banding patterns[13] and confirms that the antibody is detecting lentiviral capsid and matrix proteins rather than nonspecific cellular proteins. As shown in **Figure 1**, western blot analysis of LVVRM lots revealed a distinct p24 band at the expected molecular

FIGURE 1. Western blot confirmation of LVVRM identity.



Western blot of LVVRM lots and a positive control lysate from HIV-1–infected cells (J1.1) was performed using an anti-HIV-1 gag antibody recognizing gag precursor (p55), the processed capsid (p24), and matrix (p17) proteins.

weight, together with bands corresponding to the gag p55 precursor and the p17 matrix protein. The presence of all three gag-associated species, with banding patterns comparable to the J1.1 positive control, confirms detection of the full gag polyprotein and its expected processing products. These results verify the identity of the LVVRM and demonstrate retention of the expected HIV-1-derived lentiviral structural proteins throughout the manufacturing process.

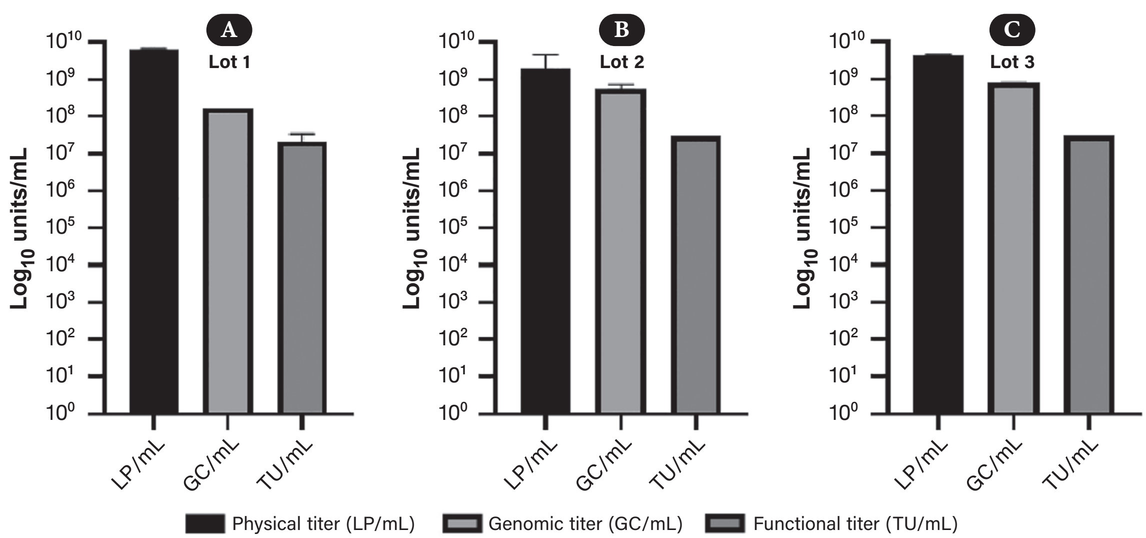
Physical, Functional, and Genomic Titers

Physical, genomic, and functional titers were measured to assess the integrity and biological activity of the LVVRM. Physical titer was determined by p24 ELISA using a magnetic bead-based step to remove free p24, ensuring only virion-associated capsid protein was measured. Genome copy number was quantified by digital-based PCR targeting the (Ψ) packaging signal to assess the concentration of encapsulated lentiviral RNA. Finally, functional activity was evaluated by measuring GFP expression in transduced HEK293 cells. Across all three metrics, values remained highly consistent between independently produced lots (**Figures 2A–C**). The close alignment between physical, genomic, and functional titers indicates a high-quality, reproducible population of properly packaged infectious virions. These results indicate that the fill-finish process successfully preserved both the structural integrity and biological potency of the LVVRM.

Replication-Competent Lentivirus Testing

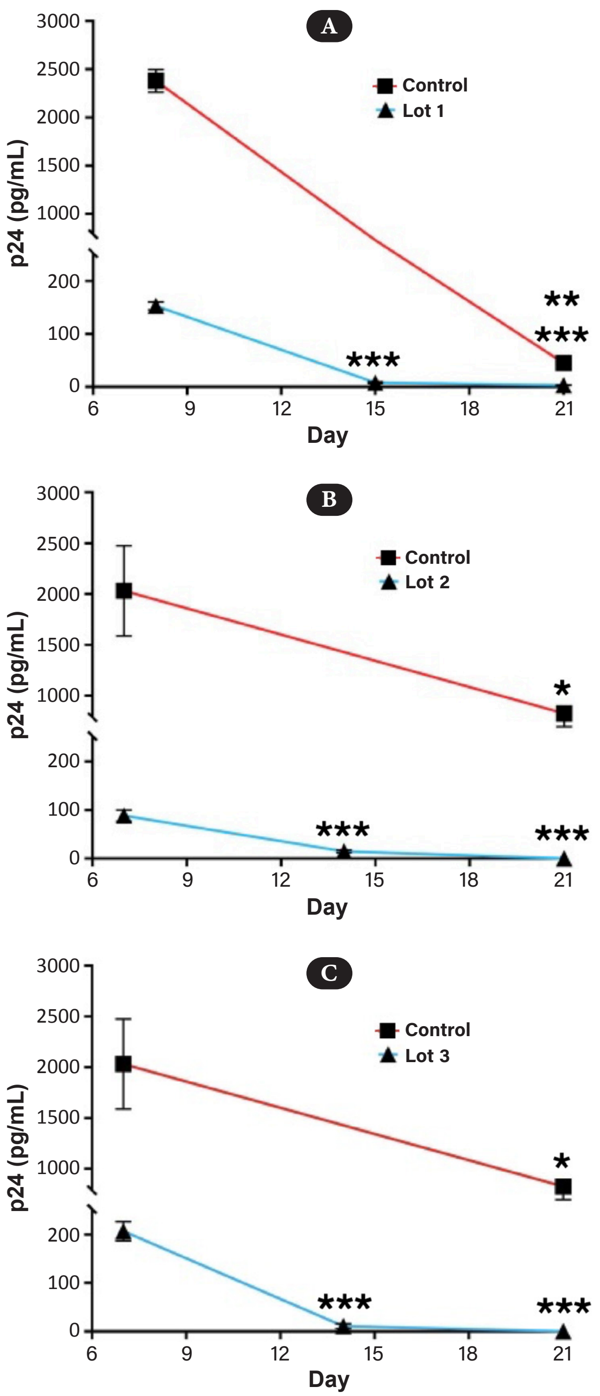
Safety testing for RCL was performed using extended culture of ARP-404 cells[14], a sensitive indicator cell line for detecting replication-competent HIV-1-derived vectors. Cells were exposed to LVVRM lots, or to a RCL control, and were monitored over a three-week period. Culture supernatants were collected at approximately weekly intervals for p24 analysis, whereas samples for qPCR analysis were collected at additional early timepoints to improve detection of residual lentiviral genomes and potential amplification events. At each designated timepoint, culture supernatants and/or cell pellets were evaluated by p24 ELISA and qPCR, targeting lentiviral genome sequences. As expected for replication-incompetent lentiviral material, both the lentiviral control and all three LVVRM lots showed a progressive decline in p24 levels over the culture period, consistent with the dilution of input capsid protein in the absence of *de novo* particle production (**Figures 3A–C**). Correspondingly, qPCR analysis of all samples demonstrated increasing

FIGURE 2. Physical, genomic, and functional titers across three independent lots of LVVRM.



Three independently filled LVVRM lots **(A–C)** were characterized across titer metrics to assess particle composition, genome content, and biological activity. Physical particle concentration was determined by p24 capsid ELISA, genome copy number was quantified by digital-based PCR, and functional titer was assessed by GFP expression in HEK293 cells following transduction.

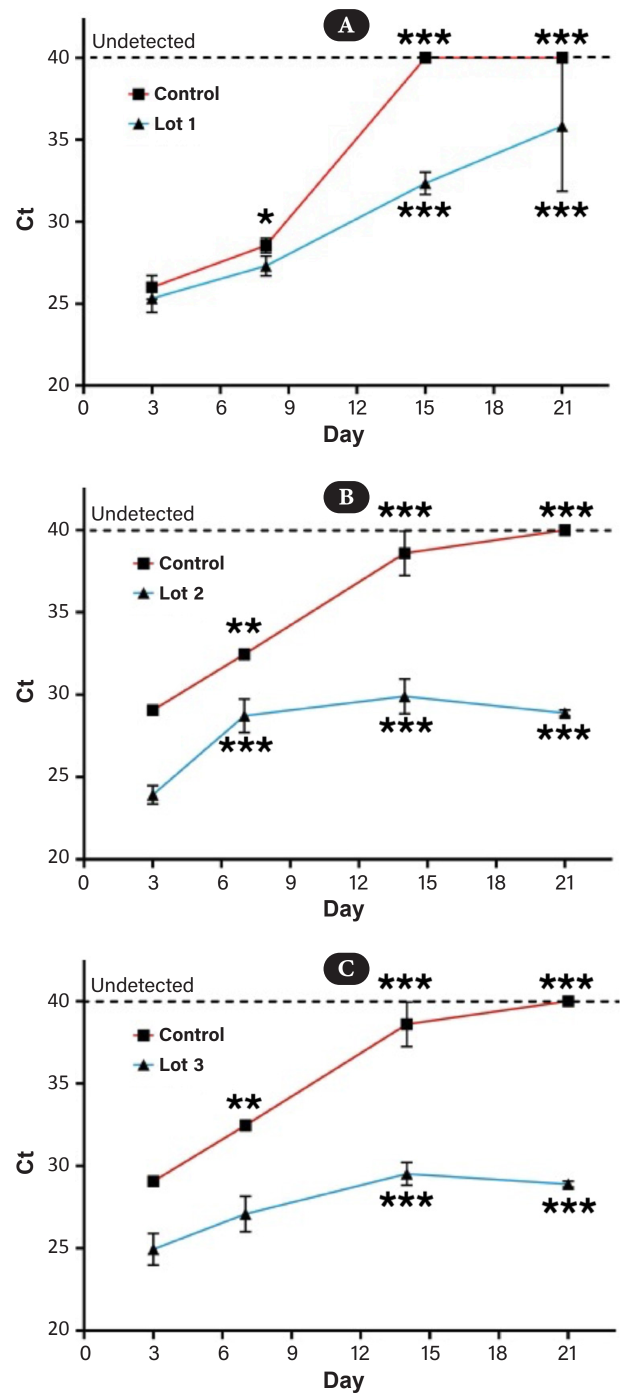
FIGURE 3. p24 levels during extended ARP-404 culture for RCL assessment.



ARP-404 indicator cells were cultured following exposure to LVVRM lots **(A–C)**, or a replication-incompetent lentiviral control, and monitored over a three-week period. p24 capsid levels in culture supernatants were measured by ELISA.

* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$ (relative to days 7–8)

FIGURE 4. qPCR analysis of lentiviral genome sequences during extended ARP-404 culture for RCL assessment.



ARP-404 indicator cells were cultured following exposure to LVVRM lots **(A–C)**, or a replication-incompetent lentiviral control, and monitored over a three-week period. Cell pellets were harvested and analyzed by qRT-PCR for lentiviral genome sequences.

* = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$ (relative to day 3)

cycle threshold (Ct) values over time, reflecting loss of detectable genomic material rather than amplification (**Figures 4A–C**). Together, the lack of increasing p24 signal, and the absence of decreasing Ct values across all LVVRM lots, demonstrate that no RCL was detected following extended culture in a permissive cell line.

DISCUSSION

This work describes the final processing and quality evaluation of a LVVRM generated to support assay harmonization and interlaboratory comparability within the LVV field. Building upon previously reported large-scale upstream production and downstream purification efforts, this study focuses specifically on the fill-finish operations and associated quality control testing to convert bulk material into a distributable reference material. Collectively, the results demonstrate that controlled thawing, pooling, sterile filtration, automated dispensing, and cryopreservation can be performed without compromising LVV integrity, quantity, or safety attributes.

A key objective of the fill-finish workflow was to minimize handling-induced variability while preserving the physical and functional characteristics of the LVV. Importantly, orthogonal analytical measurements demonstrated strong lot-to-lot concordance. Physical particle concentration (p24 ELISA), genome copy number (digital-based PCR), and functional titer (GFP transduction) aligned closely across lots, indicating preservation of particle integrity, genome packaging, and infectivity throughout the fill-finish process. This consistency is a critical prerequisite for reference material utility, as it ensures that observed variability in downstream applications reflects assay or laboratory differences rather than material heterogeneity.

Purity and safety testing further supported the quality of the LVVRM. Endotoxin levels, residual endonuclease, HCP, and host cell DNA were all detected at low levels, consistent with expectations for a research-grade lentiviral preparation. Importantly, no microbial contamination, mycoplasma, or adventitious agents were detected using orthogonal methodologies including culture-based testing, PCR, and NGS. Together, these results indicate that the fill-finish workflow did not introduce new impurities or contaminants, and that the final product maintains a favorable impurity profile suitable for broad distribution.

Identity testing by western blot provided additional confirmation of vector integrity, with clear detection of the expected HIV-1-derived gag polyprotein and its processed products (p55, p24, and p17). The observed banding patterns were consistent with those of a well-characterized HIV-1-infected cell lysate control, demonstrating retention of key structural features following final processing. While identity confirmation alone does not establish functional performance, these findings complement titer and transduction data by verifying that the material retains expected lentiviral structural components.

RCL testing represents a particularly important attribute for lentiviral preparations, including reference materials. Extended culture of ARP-404 indicator cells revealed no evidence of viral amplification for any LVVRM lot. Both p24 measurements from culture supernatants and qRT-PCR analysis of cell pellets showed trends consistent with decay or dilution of input replication-incompetent lentiviral material rather than productive replication. The inclusion of a replication-incompetent lentiviral control confirmed assay performance while reinforcing the interpretation that no RCL was present in the LVVRM. These results further support the biosafety of the material for research and assay development applications.

Taken together, the results presented here establish that the LVVRM meets expectations for a distributed lentiviral reference material, including consistency, identity, purity, and safety. By providing a well-characterized, common benchmark, the LVVRM addresses a long-standing need in the LVV community for tools that support assay calibration, method qualification, and cross-laboratory comparison. As LVVs continue to play an expanding role in research and therapeutic development, access to standardized reference materials such as the LVVRM is expected to improve reproducibility, enhance confidence in analytical measurements, and facilitate clearer interpretation of results across platforms and institutions. In conclusion, this study completes the LVVRM production and qualification workflow by detailing the final fill-finish operations and associated quality control assessments. The resulting material provides a robust foundation for improving standardization in LVV analytics and represents a valuable community resource for supporting harmonized measurement and long-term performance monitoring.

References

[1] Escors, D.; Breckpot, K. Lentiviral vectors in gene therapy: their current status and future potential. *Arch Immunol Ther Exp (Warsz)* **2010**, *58*(2) 107–119. https://doi.org/10.1007/s00005-010-0063-4

[2] Bulcha, J. T.; Wang, Y.; Ma, H.; Tai, P. W. L.; Gao, G. Viral vector platforms within the gene therapy landscape. *Signal Transduct Target Ther* **2021**, *6*(1) 53. https://doi.org/10.1038/s41392-021-00487-6

[3] Milone, M. C.; O'Doherty, U. Clinical use of lentiviral vectors. *Leukemia* **2018**, *32*(7) 1529–1541. https://doi.org/10.1038/s41375-018-0106-0

[4] Barbieri, E.; Heldt, C. L. Challenges and opportunities in lentivirus viral vector manufacturing for *in vivo* applications. *Biomedicines* **2026**, *14*(2) 369. https://doi.org/10.3390/biomedicines14020369

[5] Perry, C.; Rayat, A. C. M. E. Lentiviral vector bioprocessing. *Viruses* **2021**, *13*(2) 268. https://doi.org/10.3390/v13020268

[6] Corre, G.; Seye, A.; Frin, S.; Ferrand, M.; Winkler, K.; Luc, C.; Dorange, F.; Rocca, C. J.; Galy, A. Lentiviral standards to determine the sensitivity of assays that quantify lentiviral vector copy numbers and genomic insertion sites in cells. *Gene Ther* **2022**, *29*(9) 536–543. https://doi.org/10.1038/s41434-022-00315-8

[7] He, H.-J.; He, Z.; Lund, S. P.; Turner, L.; Fan, Y.; Qiu, Y.; Corney, D. C.; Dropulic, B.; Orentas, R.; Slessareva, O.; Welch, P.; Dungca, K.; Stelloo, E.; Dijksteel, G.; Feitsma, H.; Ahmed-Seghir, S.; Makarenko, R.; Altunlu, E.; Steenmans, D.; Spanholtz, J.; Raimo, M.; Senderovich, S.; Paugh, B. S.; Li, C.-Y.; Schroeder, B.; Whale, A. S.; Yener, D.; Foy, C. A.; Nahas, S.; Tu, F.; Sheldon, M.; Ding, Y.; Kandell, J.; Lakshmipathy, U.; McDaniel, J. H.; Zook, J. M.; Miller, S.; Maragh, S.; Patange, S.; Mohiuddin, M.; Tona, A.; Cole, K. D.; Lin-Gibson, S. Interlaboratory assessment of candidate reference materials for lentiviral vector copy number and integration site measurements. *Mol Ther Methods Clin Dev* **2025**, *33*(2) 101472. https://doi.org/10.1016/j.omtm.2025.101472

[8] Manceur, A.; Rodenbrock, A.; Jacob, D.; Patel, M.; Puttaswamaiah, S. G.; Dorion-Thibaudeau, J.; Transfiguracion, J.; Tiwari, K.; Loignon, M. Large-scale production and purification of a lentiviral vector reference material. *BioProcessing Journal* **2025**, *24*. https://doi.org/10.12665/J24OA.Manceur

[9] Rodenbrock, A.; Manceur, A.; Broussau, S.; Transfiguracion, J.; Gilbert, R.; Shen, C. F.; Loignon, M. Development of a scalable fed-batch bioreactor process for high-titer production of lentiviral vector using an inducible HEK293 producer cell line. *BioProcessing Journal* **2024**, *23*. https://doi.org/10.12665/J23OA.Manceur

[10] *Addgene: Fluorescence Titering Assay.* Addgene. https://www.addgene.org/protocols/fluorescence-titering-assay/ (accessed 2026-04-02).

[11] Ding, J. L.; Ho, B. Endotoxin detection—from limulus amebocyte lysate to recombinant factor C. In: Wang, X., Quinn, P. (eds) *Endotoxins: Structure, Function and Recognition. Subcellular Biochemistry* **2010***,* vol 53. Springer, Dordrecht. https://doi.org/10.1007/978-90-481-9078-2_9

[12] Pilely, K.; Johansen, M. R.; Lund, R. R.; Kofoed, T.; Jørgensen, T. K.; Skriver, L.; Mørtz, E. Monitoring process-related impurities in biologics-host cell protein analysis. *Anal Bioanal Chem* **2022**, *414*(2) 747–758. https://doi.org/10.1007/s00216-021-03648-2

[13] Rodari, A.; Poli, G.; Van Lint, C. Jurkat-derived (J-Lat, J1.1, and Jurkat E4) and CEM-derived T cell lines (8E5 and ACH-2) as models of reversible proviral latency. In: Poli, G., Vicenzi, E., Romerio, F. (eds) *HIV Reservoirs. Methods in Molecular Biology* **2022**, vol 2407. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1871-4_1

[14] Akrigg, A.; Wilkinson, G. W.; Angliss, S.; Greenaway, P. J. HIV-1 indicator cell lines. *AIDS* **1991**, *5*(2) 153–158. https://doi.org/10.1097/00002030-199102000-00004

About the Authors

Matthew Kaleta[1], Zachary Cuba[1], Carrie Edmonds[1], Sujata Choudhury[1], Lenny Godinho[1], A. Ciera Albrecht[1], Karen Dixon[1], Keith L. Carson[2], Heather Branscome[1], and Heather Couch[1]*

1. ATCC, Manassas, Virginia USA
2. BioProcessing Journal (BPJ), Ocala, Florida USA

**Corresponding author:* hcouch@atcc.org