Characterization of lentiviral vector quality attributes using capillary electrophoresis (CE) platform technology

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Introduction

As lentiviruses are increasingly being used as gene and cell therapy vectors, the inherent complexity of the lentiviral particle and the limited analytical methods to support the engineering, manufacturing and quality assessment of cell therapy products create challenges for clinical development. In this technical note, 2 simplified multi-capillary electrophoresis-based workflows are presented to address the key characterization and in-process attribute evaluation of lentiviral vectors (LVVs). The proteomic analysis workflow provides both protein profiling results and p24-based titer (total particle) determination. The genomic analysis workflow provides information about the genome integrity as well as the residual nucleic acids in LVV samples.

Cell and gene therapy products have the potential to offer cures for many diseases and are an area of significant focus for biotechnology and biopharma. More than 89% of cell and gene therapies in development use viral vectors for gene delivery.[1] The LVV is now one of the most commonly used viral vector platforms in pre-clinical development. It offers many advantages over other gene delivery technologies, including stable integration of genetic material into the host genome and the ability to deliver genetic sequences as large as 9 kb.[2]

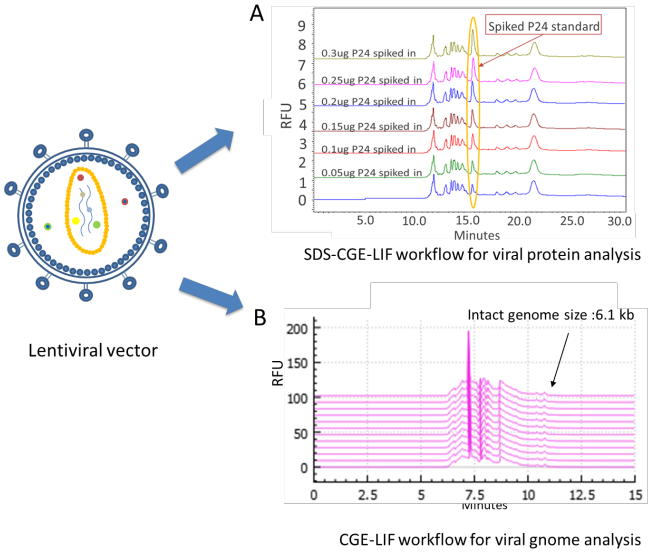

Figure 1. Two workflows for the comprehensive characterization of **LVVs** on a CE-based platform.

While these benefits make the LVV an attractive technology for cell and gene therapy applications, better analytical methods to extensively characterize and monitor multiple LVV quality attributes, including physical titer, protein profiling, genome integrity and residual nucleic acids assessment, are important to ensure product efficacy and safety.

This technical note outlines a platform-based approach that uses capillary electrophoresis (CE) to mitigate the technical difficulties in characterizing LVVs for the assessment of multiple quality attributes (Figure 1). Figure 1A illustrates the proteomic analysis of the LVVs, which can be used to monitor protein profiles and determine the p24 titer of the LVV products using a SDS-CGE-LIF workflow. Figure 1B demonstrates the genomic analysis of LVVs to determine the genome integrity of the LVV sample and the residual nucleic acids using a CGE-LIF workflow.

Key features

- Robust methods to characterize and monitor quality attributes of LVVs at both protein and genome levels using a CE-based platform
- Protein analysis using a CGE-SDS-LIF workflow provides the p24 titer and the protein profile of LVV samples
- Genomic analysis using a CGE-LIF workflow provides information on the genome integrity as well as the residual nucleic acids in LVV samples
- The multiplexing ability of the BioPhase 8800 system expedites the comprehensive characterization of LVV products to enable faster decision making during process development and streamline quality monitoring during the downstream purification process

Materials and methods

Chemicals for protein analysis: The Chromeo P503 dye (PN: 15106) was from Active Motif (Carlsbad, CA). The CE-SDS Protein Analysis Kit (PN: C30085) with the SDS-MW separation gel buffer, 10 kD internal standard, basic wash, acidic wash and sample buffer was from SCIEX (Framingham, MA).

Chemicals for genome analysis: Nuclease-free water (PN: AM9932), SYBR™ Green II RNA gel stain, 10,000x concentrate in DMSO (PN: S7564), RNase-free DNase I (PN: AM2222), 10x DNase I buffer (PN: AM8170G) and 10x phosphate buffered saline (PBS, PN: AM9624) were obtained from Thermo Fisher Scientific (Waltham, MA). Benzonase (PN: E1014-5KU), 0.5M EDTA, pH 8.0 (PN: E7889-100ML), transcript RNA markers 0.2-10 kb (PN: R7020) and 10x tris borate EDTA (TBE) buffer (PN: 574795) and molecular biology grade water were from Millipore Sigma (St. Louis, MO). The 5 µm syringe filter (PN: 4650) was from PALL Corporation (Port Washington, NY). The Rainin LTS filter tips were from Mettler Toledo (Oakland, CA). The QIAamp Viral RNA Mini Kit (PN: 52904) was from Qiagen (Germantown, MD). LVVs with titer of approximately 1 x 10^9 transduction units per mL (TU/mL) were from SignaGen Laboratories (Rockville, MD). The single-stranded RNA ladder 0.5-9 kb (PN: N0362S) was from New England BioLabs (Ipswich, MA). The sample loading solution (SLS, PN: 608082) and ethanol (200 proof) were from AAPER Alcohol and Chemical Co. (Shelbyville, KY).

Samples: In-process LVV samples were from an internal project at St. Jude Children's Research Hospital (Memphis, TN). Pre-made lentivirus materials (LV-CAG-GFP, PN: SL100270; LV-ChAT-GFP, PN: SL100303; LV-CAG-Cre-mCherry, PN: SL100284; LV-EF1a-GFT-T2A-fLuc-Puro, PN: SL100329) were from SignaGen Laboratories (Rockville, MD).

Sample preparation for protein analysis: Samples were prepared as previously described in a published technical note.[3] For the preparation of the Chromeo P503 working solution, 1 mg of Chromeo P503 dye (lyophilized powder) was reconstituted in 1 mL of methanol. After reconstitution, the dye label was stored at 2-8°C for up to 6 months, according to the manufacturer's instructions. For sample preparation, 5 µL of lentivirus solution was mixed with 5 µL of sample buffer provided in the CE-SDS Protein Analysis Kit and incubated at 70°C for 10 minutes. The sample solution was incubated for another 10 min at 70°C after mixing with 0.5 µL of 1 mg/mL Chromeo P503 dye working solution. After cooling to room temperature, 39.5 µL of DI water was added to the reaction mixture and the diluted sample was transferred to the sample vial for protein-level analysis using SDS-CGE-LIF.

Sample preparation for genome analysis: Preparation of the separation gel buffer and RNA ladder was performed as previously described in a published technical note.[4] For the preparation of the LVV RNA genome, RNA was extracted using the QIAamp Viral RNA Mini Kit. First, 25 µL of each LV sample was diluted with 45 µL of 1x PBS and mixed thoroughly with 280 µL of the lysis buffer containing 10 ng/mL carrier RNA. After a brief spin, 280 µL of 100% ethanol was added, the sample was thoroughly mixed and the resulting solution was loaded onto the spin column. The column was washed with buffers from the kit. The LVV RNA genome sample was eluted with 40 µL of nuclease-free water. Before loading the sample onto the instrument for analysis, 20 µL of the eluted LV genome sample was mixed with 30 µL of SLS, heated at 70°C for 2 minutes and then immediately cooled on ice for at least 5 minutes. The benzonase and DNase I treatment procedures were implemented to remove nucleic acid impurities outside of LV. Samples were digested in a 30 µL reaction that contained 25 µL of LV sample in nuclease-free water, 1 µL of 1x PBS, 3 µL of 10x DNase I buffer and 1 µL of benzonase that was diluted 10-fold with 1x DNase I buffer (10mM Tris-HCl, pH 7.5 at 25°C, 2.5mM $MgCl_2$, 0.1mM $CaCl_2$) or 1 µL of DNase I. The digestion was carried out at 37°C for 30 minutes and terminated by adding 3 µL of 50mM EDTA and heating at 65°C for 10 minutes. The resulting sample was subjected to RNA extraction using the QIAamp Viral RNA Mini Kit, according to the manufacturer's instructions.

Capillary electrophoresis methods: The methods used for CE separations were described in previous technical notes.[3,4]

Instruments and software: The BioPhase 8800 system equipped with a laser-induced fluorescence (LIF) detector was from SCIEX (Framingham, MA). The excitation wavelength of the LIF detector was 488 nm. The emission wavelength was set to 520 nm for genome integrity analysis and 600 nm for protein analysis. The BioPhase BFS capillary cartridge (8 x 30 cm, PN: 5080121) and Sample and Reagent Plates (PN: 5080311) were from SCIEX (Framingham, MA). The Multi-Therm shaker incubator (PN: H5000-H) was from Benchmark Scientific (Sayreville, NJ). Data acquisition and analysis were performed using BioPhase software, version 1.0 (SCIEX; Framingham, MA).

Results and Discussion

Analysis of lentiviral proteome

Workflow of lentiviral protein analysis

The proteome of the lentivirus is composed of 5 structural and several non-structural proteins (Figure 1). Each protein plays a

vital role in the life cycle of the lentivirus.[5] Glycoprotein complexes in the envelope enable the virus to attach to and fuse with target cells to initiate the infectious cycle. The p24 protein in the capsid is usually used to determine total particle titers.[6]

Here, a separation-based method for lentiviral protein analysis was developed with 2 purposes. First, the accurate total particle titer of the lentivirus sample was determined by quantifying the separated p24 capsid protein without interference from other proteins. Second, the well-separated protein profile was utilized for purity assessment of lentiviral proteins and for process optimization or process monitoring in LVV manufacturing. [3]

The protein analysis workflow uses a 2-step sample preparation protocol that includes the disassembly of the lentivirus particles and covalent labeling with a fluorophore. The resulting p503 dye-tagged lentivirus proteome was loaded onto the BioPhase 8800 system for quantitative SDS-CGE-LIF separation and analysis. This workflow and the resulting electropherogram are shown in Figure 2. The evaluation results of the workflow in a previous study[3] demonstrated that it provided sensitive titer determination based on precise quantification of the separated p24 protein peak with 4 orders of magnitude for detection, a linearity of $r^2 = 0.9953$ and LOQ of 0.4 ng/mL. It also provided a protein purity assessment of the lentiviral vector samples with excellent reproducibility.

Protein analysis of in-process samples

To further evaluate how this workflow can be applied in LVV production, 5 in-process LVV samples from an internal project were analyzed using the protein analysis CGE-SDS-LIF workflow. The 5 in-process elute dilute samples were from the same cell harvest but processed under different conditions in the downstream purification process of the lentivirus production with titer ranging from 10^7 -10^8 TU/mL. These in-process samples, together with a LV-CAG-GFP reference material with a titer of about 10^9 TU/mL, were prepared and loaded onto the BioPhase 8800 system, which allowed up to 8 samples to be analyzed in parallel. Analysis of the 5 in-process samples and the reference material was completed within 1 hour (Figure 3). The higher peak area of protein peaks in the LV-CAG-GFP relative to the 5 in-process samples was due to the relatively higher titer of LV-CAG-GFP sample. The difference in the protein profiles between the commercial LV-CAG-GFP sample and the internal in-process samples might be due to their variations in production processes.

The identification of the p24 protein and VSV-G proteinwere confirmed by spiking p24 standard in the LVV samples when analyzing using the protein analysis CGE-SDS-LIF workflow and LC-MS analysis of the peak fractions (data not shown in this technical note). The formulation protein in the elute dilute sample #3 (Figure 3A) was a matrix protein added to the formulation buffer to stabilize the LVV. The quantification of the p24 peak in each in-process sample can be used to monitor the downstream purification steps (Figure 3B). The relative quantification of p24 and VSV-G can be used to monitor the presence of free p24 in in-process LVV samples. Figure 4A shows the zoomed-in electropherogram of the 5 in-process samples. The areas of the p24 peak and the VSV-G peak of each sample were plotted to reveal a strong correlation between p24 and VSV-G (Figure 4B). These results indicate that there was no free p24 present in these in-process samples.

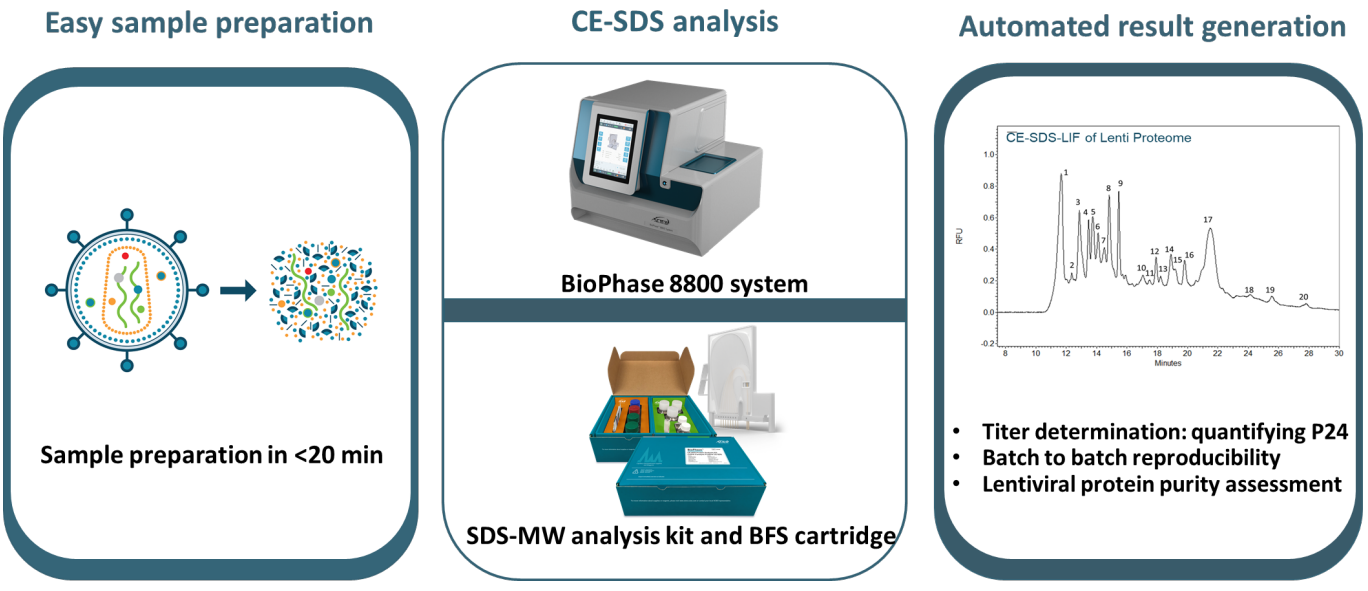

Figure 2. Workflow for LVV protein profiling and p24 titer analysis.

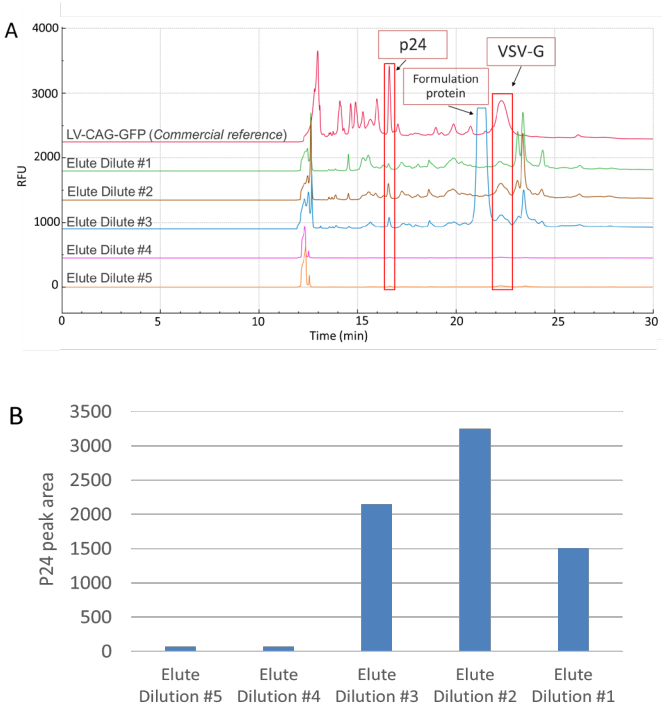

Figure 3. Protein analysis of 5 in-process LVV samples. A) Comparison of protein profiles of in-process samples and a commercial reference material. B) Bar graph of the p24 peak area of the 5 in-process LVV samples.

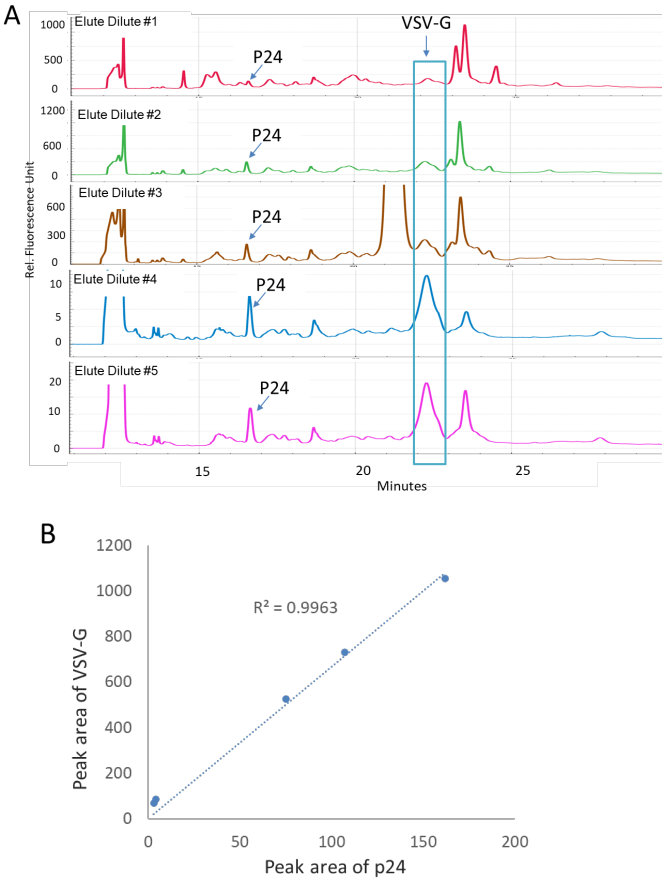

Figure 4. Correlation of p24 peak and VSV-G peak of 5 in-process LVV samples. A) Zoomed in electropherogram of the 5 in-process samples. B) Plot of peak area of p24 peak and VSV-G peak.

Analysis of lentiviral genome

Workflow for genome integrity analysis of LVV

Genomic analysis is another important quality attribute of LVV samples during manufacturing. While many methods have been established to determine viral titer, none currently analyze the integrity of the RNA genome within the lentiviral vectors.[5] Reverse transcription-quantitative PCR (RT-qPCR) can detect the presence of short fragments from the RNA genome. However, this method cannot detect deletion mutants or impurities and measurements that follow both RT and PCR amplification tend to have high variability.[7] Here, we demonstrate parallel amplification-free genome integrity analysis of LVVs by CE-LIF on the BioPhase 8800 system to accelerate analysis time and potentially speed up LVV-related product development processes.

Nucleic acids were first extracted from LVV samples with the Qiagen viral RNA extraction kit, then analyzed on BioPhase 8800 system (Figure 5). Results are shown in the electropherogram in the panel on the right. In this example, the intact genome at 6.1 kb was well-separated from the partial genome and other impurities with excellent repeatability.

Figure 6 shows results obtained from analyzing 4 commercially available, research-grade LVVs with different genome sizes. Nucleic acid extraction with the Qiagen viral RNA extraction kit was performed on 25 µL samples of each LVV with a titer of 1 x 10^9 TU/mL in the presence of the carrier RNA. The arrows indicate peaks that correspond to intact genomes. RNA ladders are shown in the 2 traces at the bottom. This genome integrity method can be used to analyze a wide range of genome sizes.

Genome integrity analysis of in-process samples

Figure 7 shows the results obtained for genome integrity analysis of the 5 in-process LVV samples together with a commercial LV-GFP reference material. Samples were injected using pressure injection and separated at a capillary temperature of 30°C. The intact genome that separated from the small molecule nucleic acids can be used to monitor the genome integrity and the

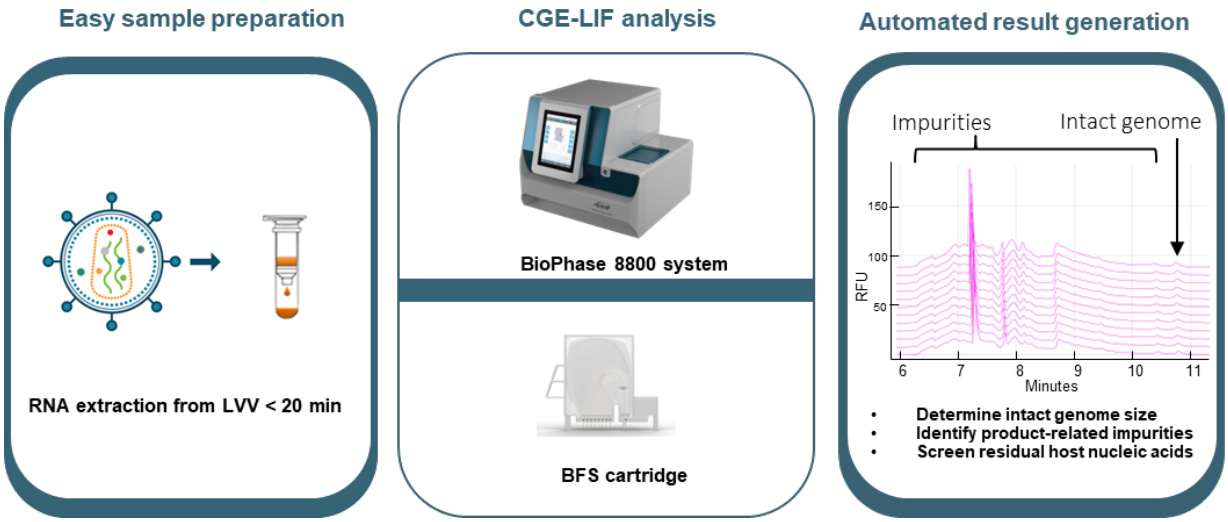

Figure 5. Workflow for LVV genome integrity analysis.

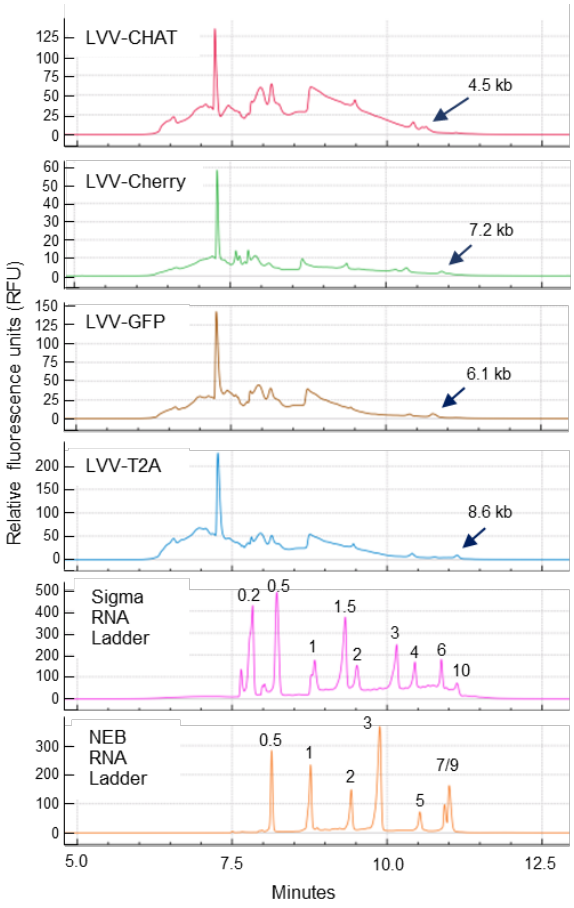

Figure 6. Analysis of LVVs with various theoretical genome sizes. Nucleic acid extraction with the Qiagen viral RNA extraction kit was performed in the presence of the carrier RNA on 25 µL samples of LVVs with a titer of 1 x 10^9 TU/mL.

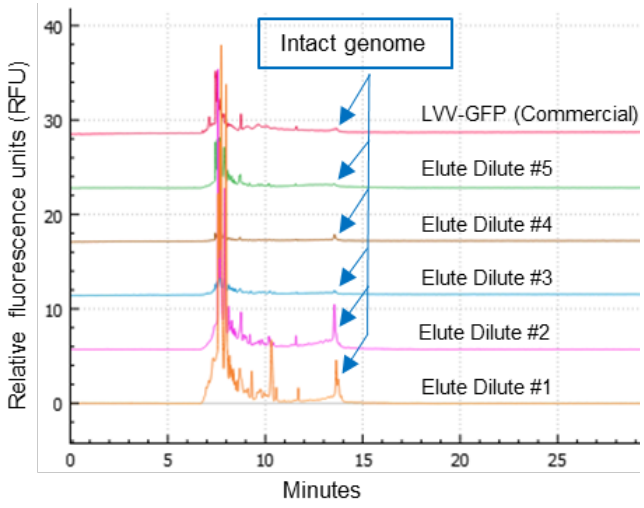

Figure 7. Genome integrity analysis of in-process samples.

presence of residual nucleic acids in the samples during process development.

Residual nucleic acids screening workflow

Figure 8 shows results obtained from an in-depth genome integrity analysis of LV-GFP obtained from a commercial vendor. The top panel shows control results that were obtained without treating the LVV sample before nucleic acid extraction. The middle panel shows results obtained when samples were treated with benzonase before extraction. The sample in the lower panel was treated with DNase I before extraction. The areas in purple circles indicate the impurities that were present outside of the LVVs, as

these peaks were present in the control condition but not in the benzonase- or DNase I- treated samples.

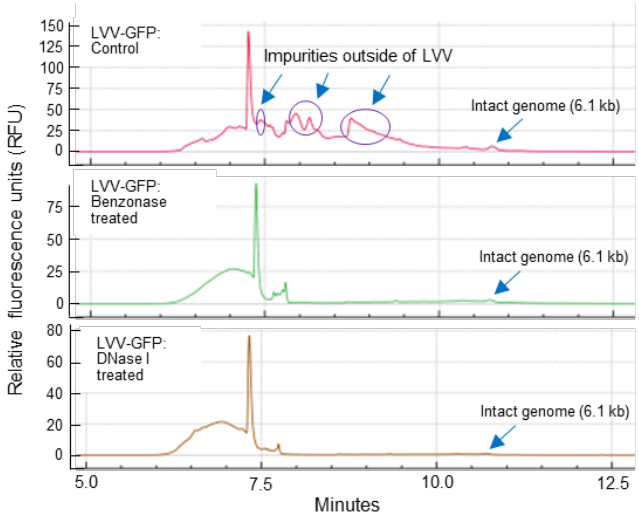

Figure 8. Residual nucleic acids screening workflow.

Conclusions

- CE technology demonstrates unique capabilities for quality attribute monitoring of LVVs at both protein and genome levels
- Precise quantification of key proteins and informative proteome profiles were utilized for characterization and monitoring of the in-process LVV products using the SDS-CGE-LIF workflow for protein analysis
- Genome integrity and residual nucleic acids were monitored for the eluting dilute of LVVs in different purification steps using the CGE-LIF genome analysis workflow
- Both the protein- and genome-level analyses were performed on a high-throughput BioPhase 8800 system to provide a comprehensive, ultrafast characterization of the quality attributes of the LVVs

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