Amplification-free analysis of lentiviral vector genome integrity on the BioPhase 8800 system

Using CE-LIF

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An amplification-free workflow has been developed for the genome integrity analysis of lentiviral vectors (LV) using capillary electrophoresis with laser induced fluorescence detection (CE-LIF). The high sensitivity and high resolving power of CE-LIF coupled with multi-capillary separation on the BioPhase 8800 system enable high-throughput genome integrity analysis of multiple LVs, simultaneously, to accelerate analysis time and potentially speed up lentiviral vector-related product development processes for gene therapy.

Lentivirus belongs to a genus of retroviruses that includes the human immunodeficiency virus (HIV). At 80-100 nm in diameter, it has an envelope on the outside and a capsid inside, composed of about 2000 copies of p24. Within the capsid, its genome consists of two single-stranded RNA held together as a dimer through noncovalent interactions near the 5′-end. Over recent years, recombinant LV has become a valuable gene delivery tool in clinical trials for treatment of various diseases. It can deliver transgene as large as 9kb and maintain long-term expression in target cells through integration into host cell genome. It infects dividing and no-dividing cells with broad tropism and low immunogenicity.[1-2] With the demand of LV growing, so are needs for better characterization of these vectors to ensure their safety and efficacy. Many methods have been established for LV titer determination. However, a technology with the ability to analyze the integrity of RNA genome within LVs is lacking. For example, reverse transcription-quantitative PCR (RT-qPCR) can detect the presence of short fragments on RNA genome, but it lacks the ability to detect mutants or impurities. In addition, both RT and PCR amplification are known to cause variability.[3-7] In this technical note, an amplification-free genome integrity analysis of LVs by CE-LIF on the BioPhase 8800 system is demonstrated.

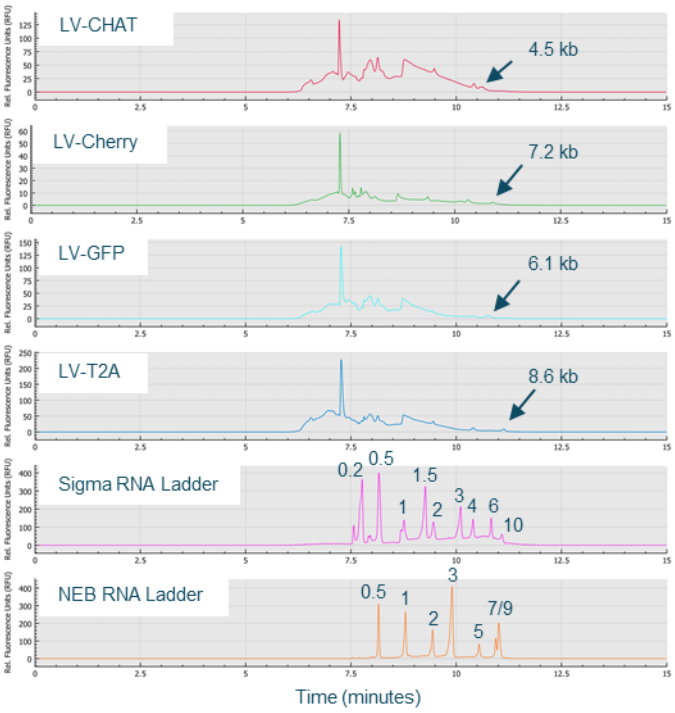

Figure 1. Genome integrity analysis for four lentiviral vectors with different genome sizes. RNA from lentivirus samples at 25 µL in volume, around 1x10^9 TU/mL in titer, were extracted using QIAamp Viral RNA Mini Kit following manufacturer's instructions. Eluted RNA samples were analyzed on the BioPhase 8800 system as described in the methods section. The Y-axis is relative fluorescence units and the X-axis is migration time. Arrows point to intact genome peaks. Fragment sizes in RNA ladders are labeled with numbers in kb.

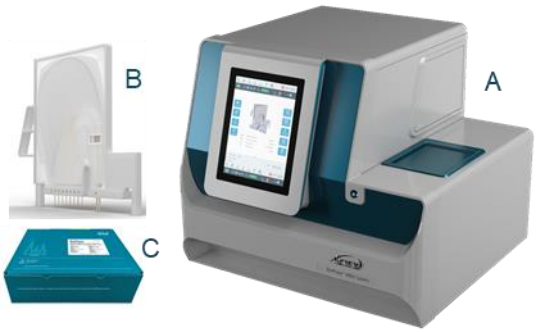

Figure 2. The BioPhase 8800 system (A), pre-assembled multi-capillary cartridge (B) and plate kit (C).

Key features of the SCIEX solution for lentiviral vector genome integrity analysis

- High sensitivity of CE-LIF enables genome integrity analysis of LV at a titer of 1 x 10^9 TU/mL without amplification
- High resolving power of CE ensures separation of intact LV genome from various impurities with good repeatability
- Multiple LVs with different genome sizes can be analyzed simultaneously
- Simple, fast workflow combined with multi-capillary separation on the BioPhase 8800 system enables rapid, high-throughput analysis of different samples and different sample treatment conditions

Materials and methods

Materials: 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 or PBS (PN AM9624) were obtained from Thermo Fisher Scientific, Waltham, MA. Polyvinyl-pyrrolidone (PVP, PN 437190), benzonase (PN E1014-5KU), 0.5 M EDTA, pH 8.0 (PN E7889-100ML), Transcript RNA markers 0.2-10kb (PN R7020) and 10x Tris Borate EDTA (TBE) buffer (PN 574795), Molecular Biology Grade, were from Millipore Sigma, St. Louis, MO. The 5 µm syringe filter (PN 4650) was from PALL Corporation, Port Washington, NY. Rainin LTS filter tips were from Mettler Toledo, Oakland, CA. QIAamp Viral RNA Mini Kit (PN 52904) was from Qiagen, Germantown, MD. Lentiviral vectors with titer of around 1 x 10^9 transduction units per mL were from SignaGen Laboratories, Rockville, MD. Single stranded RNA ladder 0.5-9kb (PN N0362S) was from New England BioLabs, Ipswich, MA. Sample Loading Solution (SLS, PN 608082), Pre-assembled, BioPhase BFS Capillary Cartridge (8 capillaries, 30 cm in total length, PN 5080121, Figure 2) and disposable BioPhase Sample and Reagent Plates (PN 5080311, Figure 2) were from SCIEX, Framingham, MA. Ethanol (200 proof) was from AAPER Alcohol and Chemical Co., Shelbyville, Kentucky.

Instrument and software: A BioPhase 8800 system (Figure 2, PN 5083590) equipped with LIF detection was from SCIEX, Framingham, MA. Excitation wavelength was at 488 nm and emission wavelength at 520 nm. Data acquisition and analysis were performed using BioPhase software V1.0 (SCIEX, Framingham, MA).

Preparation of the separation gel buffer: To make 100 mL of the separation buffer, 1 g of PVP was added to 60 mL nuclease-free water in a 250 mL glass beaker and allowed to sit at room temperature for 10 to 30 minutes. The solution was swirled gently to help PVP to be completely dissolved. Then, 24 g of urea was added. The solution was mixed with a clean stirring bar for about 20 minutes without heating. After urea was completely dissolved, 10 mL of the 10x TBE buffer was added. After one more minute of stirring, nuclease-free water was added until the total volume was 100 mL. The separation buffer contained 1% PVP, 1x TBE, pH 8.3 and 4 M urea. This buffer should be good for one month if stored at 2ºC to 8ºC in 20-30 mL aliquots. Before sample run, the required amount of gel buffer was warmed up to room temperature and filtered through a 5 µm filter. SYBR Green II dye was added at a 1 to 25,000 dilution.

Preparation of RNA ladders: Single stranded RNA 6000 ladder from NEB was diluted 200 fold with a 1:1 mixture of nuclease-free water and SLS, heated at 70°C for 2 minutes in a thermal cycler, and then immediately placed on ice for at least 5 minutes. For separation on the BioPhase 8800 system, 50 µL of treated RNA ladder was transferred to each well on the sample plate before the sequence was run. Same conditions were used for the Transcript RNA markers from Sigma except that it was diluted 250 fold with a 1:1 mixture of nuclease-free water and SLS.

Preparation of LV RNA genome: RNA was extracted using QIAamp Viral RNA Mini Kit. Briefly, 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 quick spin, 280 µL of 100% ethanol was added, followed by a thorough mixing and loading of the entire mixture onto the spin column. The column was washed with buffers from the kit. LV RNA genome sample was eluted with 40 µL of nuclease-free water. Before loading 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 immediately cooled on ice for at least 5 minutes. Samples were transferred to wells on the sample plate before the sequence was run on the BioPhase 8800 system.

Benzonase and DNase I treatments: 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 in 1x DNase I buffer (10 mM Tris-HCl, pH 7.5 at 25°C, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$) or 1 µL of DNase I. The digestion was carried out at 37°C for 30 minutes and terminated by addition of 3 µL of 50 mM EDTA, followed by heat treatment at 65°C for 10 minutes. The resulting sample was subjected to RNA extraction using QIAamp Viral RNA Mini Kit.

Method and sequence creation: Methods were created using the intuitive tile-based drag-and-drop interface in the “Method Editor” module of the BioPhase software where desired buffers, reagents, and specific actions like “rinse” and “inject’ steps were selected to assemble into a method. Similarly, run sequences were created in the “Sequence Editor” module of the BioPhase software in which desired methods were selected and applied to each sample column, as described in the BioPhase 8800 System Operator Guide.[8] Amounts of reagents needed were calculated by the BioPhase software based on methods and number of sample injections in the sequence. Once a sequence was created, sample plate layout and reagent plate layout were generated by the BioPhase software. Settings for cartridge conditioning, sample separation and shutdown methods are provided in Figures 3, 4 and 5, respectively.

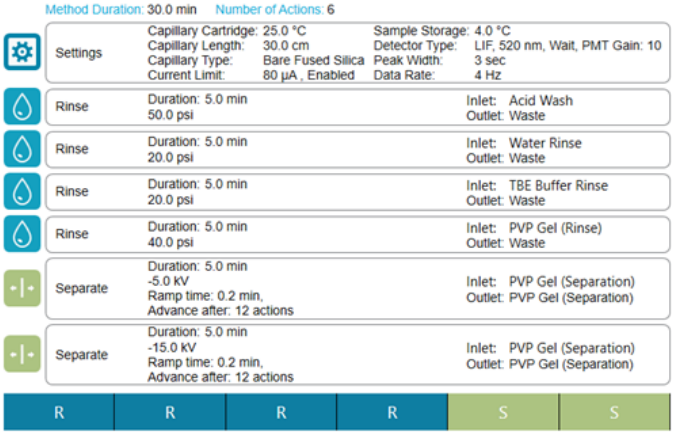

Figure 3. Settings for cartridge conditioning method as shown on the BioPhase software.

Figure 4. Settings for sample separation method as shown on the BioPhase software.

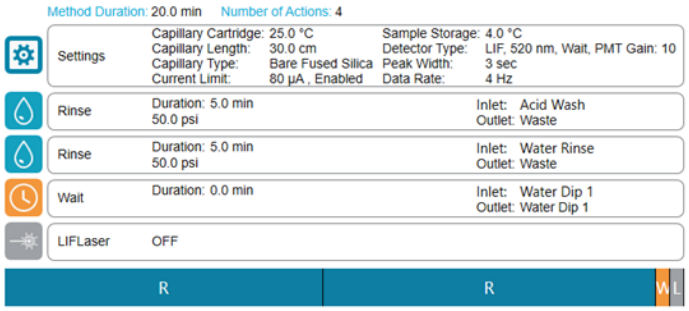

Figure 5. Settings for the shutdown method as shown on the BioPhase software.

Preparation of sample and reagent plates: Recommended fill volumes are shown in Table 1, below. An example of plate layouts for running 16 samples is shown in Figure 6. Wells needed to be filled are indicated in the plate layouts. For sample and reagent outlet plates, reagents were added to indicated wells on the lower side of the plate, away from the chamfered corner.

Plate	Sample Inlet	Sample Outlet	Reagent Inlet	Reagent Outlet
Volume per well (µl)	*50*	*1500*	*800*	*2800 (cap protect)* *2800 (gel buffer)* *2800 (water dip)* *1500 (waste)*

Table 1. Recommended volumes for filling sample and reagent inlet and outlet plates.

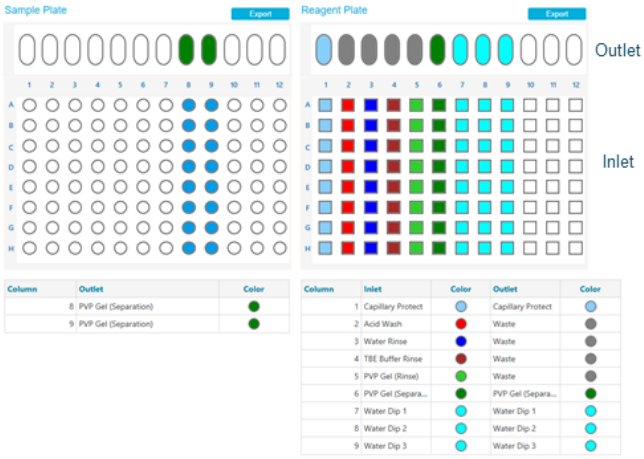

Figure 6. An example of sample plate (left panel) and reagent plate (right panel) layouts for running 16 samples in two columns. Wells selected were indicated by colored circles on sample plate, ovals on outlet plates and squares on reagent inlet plate. Tables below the plate layouts provided legends for color codes used in plate layouts.

Preparation of the BioPhase 8800 system for the sequence run: After user login, a BFS capillary cartridge was installed, sample plate, reagent plate and outlet plates prepared as described above were loaded onto the BioPhase 8800 system using the touchscreen on the front panel. A run sequence was selected from a project folder on the network. The run was started by pressing the “Run Sequence” button.

Results and discussions

Amplification-free workflow for LV genome integrity analysis: Although LV is becoming increasingly used as a gene delivery tool, one caveat is its low titer. Consequently, analysis of LV RNA genome usually requires amplification by RT-PCR. However, both RT and PCR introduce variations. Therefore, the amplification-free workflow presented here for LV genome integrity analysis should be very beneficial for rapid and consistent LV vector analysis. A simple diagram of the amplification-free workflow for LV genome integrity analysis is shown in Figure 7. In this workflow, LV RNA genome is extracted from LV samples with the use of lysis buffer containing a potent chaotropic salt and a detergent, and purified using a silicone-based spin column. RNA eluted from the spin column is directly analyzed on the BioPhase 8800 system by CE-LIF without any further amplification, as described in the methods section.

Figure 7. Amplification-free workflow for LV genome integrity analysis.

Analysis of LVs with different RNA genome sizes: RNA was extracted from 4 LVs: LV-ChAT-GFP, LV-CAG-Cre-mCherry, LV-CAG-GFP and LV-EF1a-GFP-T2A-fluc-Puro as described in the methods section. After elution with 40 µL nuclease free water, 20 µL of it was mixed with 30 µL SLS, heated at 70°C for 2 minutes. After a quick chill on ice, samples were loaded on the BioPhase 8800 system for CE-LIF analysis. Results are shown Figure 1. The pink trace is the Sigma RNA size standards with sizes marked in dark blue font while the orange trace shows the NEB RNA ladder. Electropherograms obtained with LV-ChAT-GFP, LV-CAG-Cre-mCherry, LV-CAG-GFP and LV-EF1a-GFP-T2A-fluc-Puro are shown in red, green, turquois and blue traces with intact RNA genome sizes of 4.5 kb, 7.2 kb, 6.1 kb and 8.6 kb, respectively. In addition to peaks corresponding to expected intact genome sizes, smaller sized species were also detected for each LV sample. These might be a combination of impurities outside of the LV particles, partial RNA genomes and smaller RNA fragments inside the LV particles.

Importance of using carrier RNA during RNA extraction: In order to understand the impact of carrier RNA on the yield of RNA extraction, two vials of LV-CAG-GFP samples at 26 µL were pooled, mixed by gentle inverting and transferred to two Eppendorf tubes at 25 µL each. Both were subjected to the same RNA extraction process with the QIAamp Viral RNA Mini Kit, except one with 10 ng/mL carrier RNA in the lysis buffer, and the other one without carrier RNA. After elution with 40 µL of nuclease free water, 20 µL of it was mixed with 30 µL SLS, heated at 70°C for 2 minutes. After a quick chill on ice, samples were loaded on the BioPhase 8800 system for CE-LIF analysis. Results are shown in Figure 8. The red trace was obtained with RNA extracted with carrier RNA while the green trace was obtained without carrier RNA. Although the peak profiles were identical in the two traces, the scale in the green trace was smaller, indicating that lower RNA yield was obtained without carrier RNA. Therefore, carrier RNA should be included in the lysis buffer for RNA extraction for improved yield.

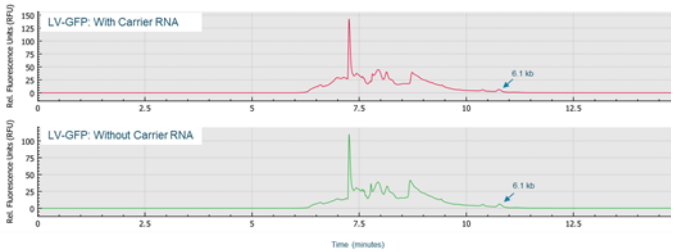

Figure 8. Comparison of RNA extraction with and without carrier RNA.

Effect of benzonase and DNase I treatments: In order to determine if some of the small sized peaks detected in Figure 1 and Figure 8 were due to impurities present outside of the LV particles, 25 µL of LV-CAG-GFP was incubated with benzonase, RNase-free DNase I or buffer control prior to RNA extraction, as described in the methods section. RNA was then extracted, and eluted with 40 µL of nuclease-free water. Half of the eluted sample was used for analysis on the BioPhase 8800 system. Figure 9 summarizes results obtained. Panel A is the control, panel B is benzonase treated and panel C is DNase I treated. In all three panels, the intact RNA genome peak for LV-CAG-GFP (Green arrow) was detected. The same is true for the peaks labeled with red stars which are located around 7.3 minutes, and for peaks labeled with blue stars located around 10.3 minutes, 9.8 minutes, 9.4 minutes and 10.3 minutes, indicating these species were from the inside of LV particles. The peaks marked with purple arrows were detected only in control samples, but not in benzonase or DNase I treated samples, suggesting that these peaks might be from residual DNA contamination present outside of the LV particles. With the disappearance of peaks marked in purple arrows in benzonase or DNase I treated samples, there were concomitant increase in smaller fragments

located between 6.0 to 7.3 minutes, suggesting these smaller fragments could be the products of benzonase and DNase I digestion or degradation of larger fragments. In addition, there were differences between the benzonase treated samples and DNase I treated samples around the 7.3 to 7.8 minutes area, indicated by blue ovals. A zoomed-in view for this area is shown as an inset figure at the upper right corner of each panel. The peak labeled with purple arrow was present only in the control indicating this peak was a DNA contaminant located outside of LV particles. The peaks labeled with pink stars were detected in all three panels, suggesting they were from inside of LV particles. The peak marked by the red circle was present in the control and in DNase I treated samples, but not in benzonase treated sample, indicating this peak might be an RNA contaminant outside of LV particles. Although benzonase and DNase I treatments are useful in identifying the contaminants present outside of the LV particles, further analysis is needed to understand the origin of the peaks that were detected in all three panels, but with sizes smaller than the intact genome. Peaks located around 7.8 minutes have estimated sizes around 200 to 300 bases. These peaks could be related to the minus strong-stop cDNA (U5/R region) fragments present within lentiviral capsids as described in published literature.[9] This minus strong-stop cDNA has been utilized for quantification of LV vector particle numbers. The minus strong-stop cDNA fragment size for LV-CAG-GFP is around 250 bases in length according to manufacturer. However, further experiments are needed to confirm this hypothesis.

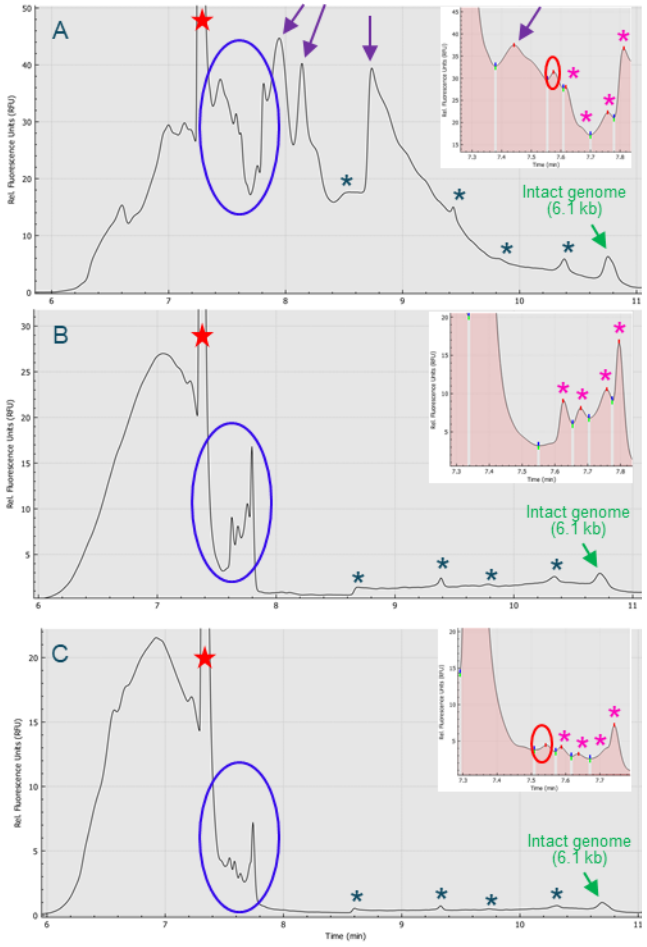

Figure 9. Effects of benzonase and DNase I treatments on genome analysis profile of LV-CAG-GFP samples. Samples were treated with control buffer (panel A) or benzonase (panel B) or DNase I (panel C) prior to RNA extraction. Extracted RNA was eluted with nuclease-free water and analyzed on the BioPhase 8800 system. Peaks labeled with red, blue and pink stars were detected in all three panels. Peaks labeled with purple arrows were only detected in the control. Peak marked with red circle was present in control and DNase I treated samples, not in benzonase treated sample.

Repeatability test for sample separation: RNA was extracted from 25 µL of LV-CAG-Cre-mCherry and LV-CAG-GFP and eluted with 40 µL of nuclease-free water. Half of the eluted sample was mixed with 30 µL of SLS, heat treated and injected at 5 KV for 10 seconds for 12 consecutive times. As demonstrated in Figure 10, peak patterns in 12 runs for both LV-CAG-Cre-mCherry and LV-CAG-GFP were very consistent. The CV for migration time for the intact genome peaks was less than 0.5%.

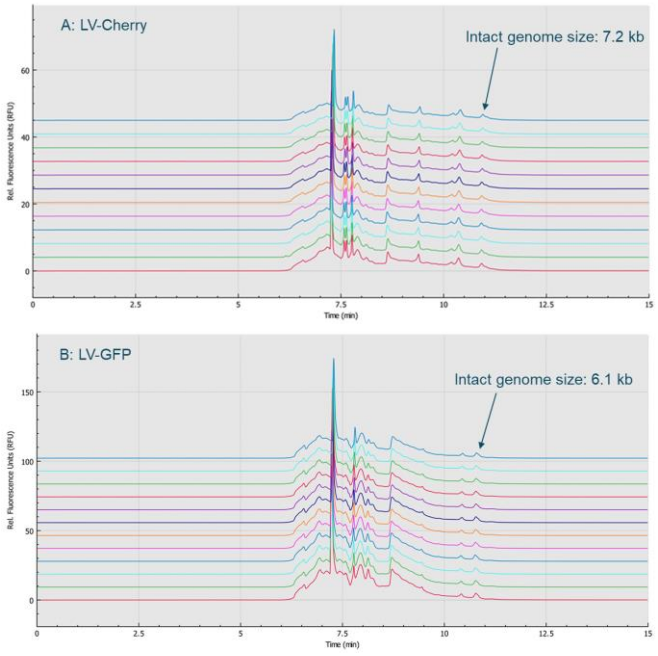

Figure 10. Repeatability test for sample separation.

Conclusions

- A simple, fast, amplification-free workflow for LV genome integrity analysis by CE-LIF, with excellent repeatability, has been developed
- Excellent resolution was demonstrated on the BioPhase 8800 system in separating intact LV RNA genome from various impurities
- Multiple LVs with different genome sizes were analyzed in parallel, with 15 minutes sample separation time and 1 hour total analysis time for 8 samples
- The multiplexing capability of the BioPhase 8800 system enables faster assessment of genome integrity of multiple LVs which is valuable in accelerating screening and process development of LV products

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