UNCHAINED LABS

Double down with lentivirus titer and RNA content on Leprechaun

Introduction

Getting an early read on the number of intact, RNA-containing lentivirus (LV) particles in a batch makes a real difference when optimizing cell therapy development and production. Knowing post-harvest which batches have the highest titer of structurally complete viral particles, and which have the lowest, ensures you don't waste time on preps which are poor quality to begin with. Additionally, increasing focus from the FDA and other regulators on the need for analytical development and product characterization for LV based therapies makes understanding exactly what's in your LV prep more important than ever for those wishing to avoid costly delays.

Compared to other viral vectors, the analytical techniques available for LV characterization are still underdeveloped. Widely used assays, such as the p24 ELISA and qRT-PCR, provide limited information as they are destructive, detect only one part of the viral structure, and analyze the total amount of capsid or gene of interest without checking how much is inside viral particles. On the other hand, technologies which rely on non-specific particle analysis can detect and count individual particles but make assumptions on their content without directly checking what's inside or on the surface.

Figure 1: Leprechaun is the only platform to provide complete biophysical characterization of individual LV particles.

Leprechaun is the only platform to provide complete biophysical characterization of individual LV particles, regardless of pseudotype, at every step of the production process (Figure 1).

The Lentivirus VSVG RNA Luni consumable is pre-coated with a highly specific antibody against VSVG to capture viral particles straight from crude samples, without prior purification (Figure 2). For analysis of non-VSVG pseudotyped virus the Lentivirus Flex RNA Luni allows the capture step to be customized with your own antibody for the envelope

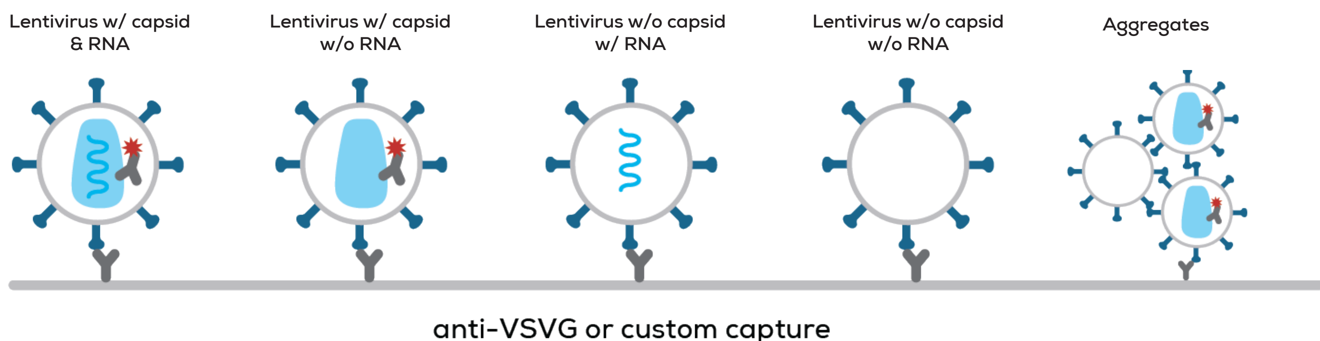

Figure 2: LV captured on the RNA Luni is sized, then stained with anti-p24-CF647 and SYTO14 to detect viral capsid and RNA. Get n≥3 technical replicates for each output from a single Luni.

protein of your choice. Once bound, Leprechaun uses single-particle interferometry to measure the size of each viral particle to check whether it is intact, aggregated or a fragment. Antibodies confirm the presence of the viral envelope and p24 capsid, while fluorescent RNA dye checks for the all-important nucleic acid. Regardless of how dirty your sample is, Leprechaun can measure the titer of RNA-containing lentivirus down to $1x10^7$ particles/mL.

This app note describes how Leprechaun's Lentivirus RNA application quantifies RNA and capsid-containing LV in crude and pure samples, and demonstrates the value of LV structural analysis for both process and analytical development.

Methods

Leprechaun Analysis

LV samples were acquired from five vendors and Leprechaun analysis performed using the Lentivirus VSVG RNA Kit (Unchained Labs). LV was diluted between 1:12 and 1:2000 in manufacturer supplied Incubation Solution with added 1% FBS. Kit supplied SYTO14 was diluted to 10 µM in Incubation Solution and added to the diluted LV sample at a dye to sample ratio of 1:10. The dye-sample mixture was incubated for 16 hours at 37 °C. Post dye incubation, 50 µL SYTO14 labelled sample was incubated for 1 hour at RT on Lentivirus VSVG RNA Lunis. After fixation and permeabilization by Solution C and D, Lunis were incubated for 1 hour at RT with kit supplied anti-p24-CF647 (1:250). Lunis were washed, dried and run on Leprechaun using the Lentivirus RNA application.

Benzonase Treatment

25 U/µL benzonase stock (Sigma-Aldrich) was diluted 1:8 in benzonase incubation buffer (20 mM Tris-HCl, 4 mM MgCl2, 20 mM NaCl), 1 µL LV sample added per 2 µL, and the mixture incubated at 37 °C for 1 hour. For Leprechaun analysis, SYTO14 (or an equal volume of PBS for non-dye samples) was added to benzonase treated samples and Luni preparation performed as per above method.

Benzonase functionality was verified by adding 1 mg/mL calf liver RNA (Sigma-Aldrich) to 25 U/µL benzonase (in benzonase incubation buffer) and 10 µM SYTO14. For the non-benzonase treated control, benzonase was replaced with an equivalent volume of buffer (20 mM Tris-HCl, 2 mM MgCl2, 20 mM NaCl and 50% glycerol). Samples were incubated at 37 °C for 1 hour using the Isothermal application on Uncle (Unchained Labs) and fluorescence emission monitored between 500 – 630 nm.

qRT-PCR Analysis

Analysis was performed by SydLabs using the Lenti-X qRT-PCR Titration Kit (Takara Bio), according to the kit protocol. Three dilutions of each sample were run in duplicate and results averaged to obtain genomic titer.

Particle type	Capture antibody	Particle definition	
		Size (nm)	Component detected
LV w/ capsid & RNA	anti-VSVG/custom	35 – 130	$VSVG^+$ $p24^+$ RNA^+
LV w/ capsid w/o RNA	anti-VSVG/custom	35 – 130	$VSVG^+$ $p24^+$ RNA^-
LV w/o capsid w/ RNA	anti-VSVG/custom	35 – 130	$VSVG^+$ $p24^-$ RNA^+
LV w/o capsid w/o RNA	anti-VSVG/custom	35 – 130	$VSVG^+$ $p24^-$ RNA^-
Aggregates	anti-VSVG/custom	>130 – 200	$VSVG^+$ $p24^\pm$ $RNA^\pm$
Viral p24	anti-VSVG/custom	N/A	$VSVG^+$ $p24^+$ $RNA^\pm$
Soluble p24	anti-p24	N/A	$p24^+$ $VSVG^-$

Table 1: Definitions for LV particle types analyzed on the VSVG and Flex RNA Lunis. Default size ranges can be customized during analysis. In addition to the viral capture spots, p24 is also present on the Luni surface providing quantification of soluble p24 alongside viral titer.

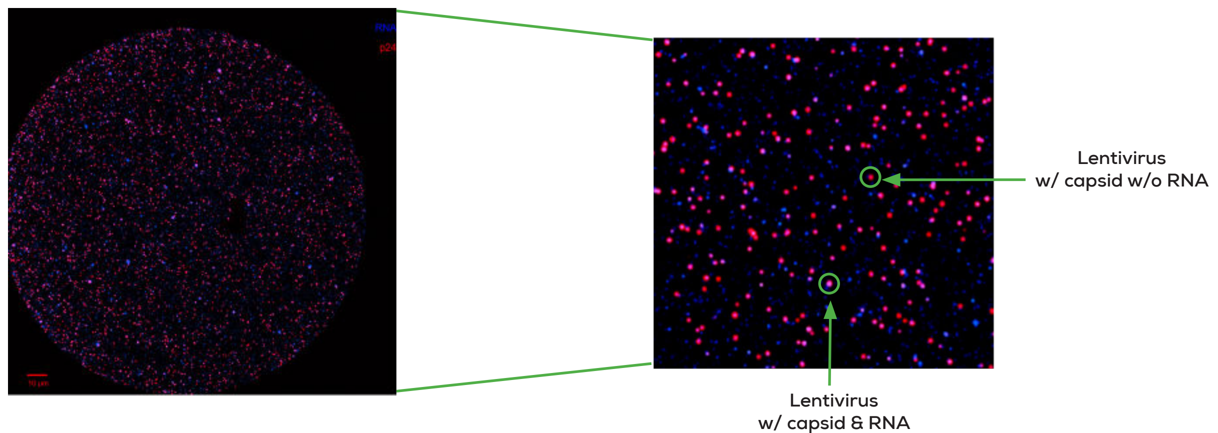

Figure 3: VSVG capture spot on the VSVG RNA Luni. Sample was incubated with SYTO14 (blue), then fixed and permeabilized to allow probing for encapsulated p24 (red). LV containing both a p24 capsid and RNA is purple, LV with capsid but without RNA is red and virus with RNA but no capsid is blue.

Results

How it all comes together

Viral particles are immuno-captured on the surface of the RNA Luni via their envelope protein, then their structure is analyzed using a combination of interferometry and fluorescence microscopy. Single particle interferometry uses the signal enhancing silicon dioxide surface of the Luni to maximize interference of light reflected from the Luni surface and scattered by the captured virus. This results in high resolution sizing of single particles, down to 35 nm in diameter. Establishing viral size allows Leprechaun to separate viral fragments and aggregates from single viruses.

Size alone is not enough to identify a particle as an intact LV. Fluorescent staining for p24 and RNA checks for the key structural components (Table 1). Samples are incubated with the membrane permeable RNA dye SYTO14 prior to immobilization on the Luni, labelling viral RNA. Once bound and after a mild fixation and permeabilization, CF647-labelled anti-p24 detects virally encased capsid (Figure 3).

Validated RNA Detection

SYTO14 emission peaks at 530 nm upon binding to RNA, which is detected by Leprechaun's blue channel. To confirm that blue channel fluorescence is specific to the binding of SYTO14 to LV particles, Lunis were incubated with four conditions: with/without virus and with/without dye (Figure 4).

Counts are only detectable in the blue channel when both a LV sample and the SYTO14 dye are present. When dye is removed, and the viral sample analyzed alone there is no detectable colocalization of blue signal with VSVG positive particles. This results in all viral particles being classified as 'without RNA' and confirms that signal is specific to SYTO14 binding to RNA, not a result of autofluorescence from the sample or bleed through from other fluorescence channels. Furthermore, no blue signal is detectable when Lunis are incubated with dye alone, verifying that SYTO14 does not stick non-specifically to the Luni surface.

SYTO14 was selected for LV RNA detection since it is cell permeable and able to cross the viral envelope without compromising the viral structure. To confirm SYTO14 is binding to RNA inside the

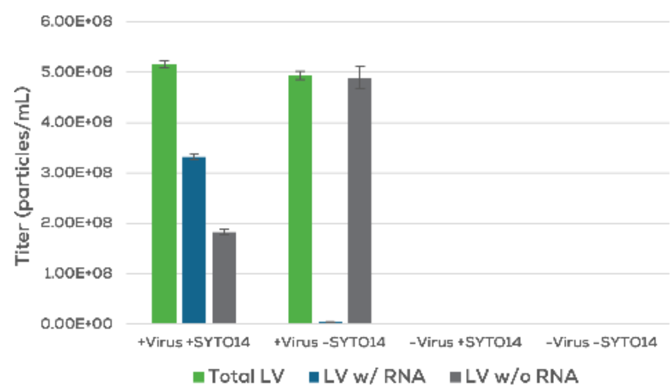

Figure 4: Negative controls confirm that blue signal is specific to binding of SYTO14 with LV. Error bars are standard deviation, n = 3 for each condition.

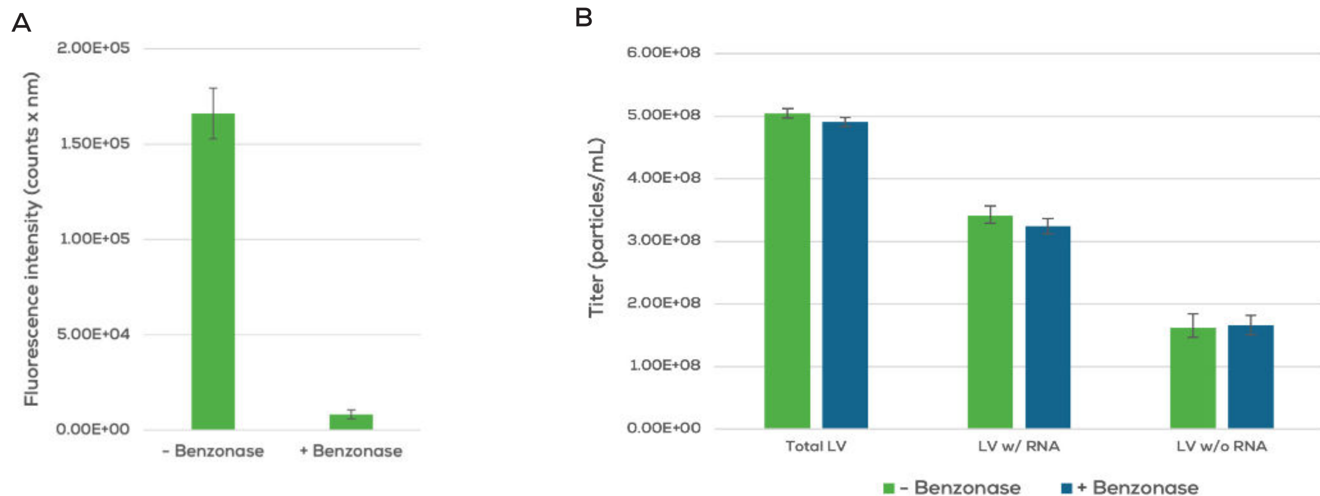

Figure 5: (**A**) Confirmation of nuclease activity. SYTO14 and RNA were incubated in the presence and absence of benzonase and the resulting fluorescence measured on Uncle. (**B**) Impact of nuclease treatment on the titer of LV which contains RNA as analyzed on Leprechaun. For both graphs error bars are standard deviation, n = 3 for each condition.

virus, rather than nucleic acid stuck to the surface or free in solution, LV samples were treated with the nuclease benzonase prior to incubation on the Luni. Benzonase activity was first tested by incubating RNA and SYTO14 in the presence and absence of the enzyme, and the resulting fluorescence signal measured on Uncle (Figure 5A). Addition of benzonase led to a 95% reduction in fluorescence, verifying nuclease functionality. Benzonase treatment had no impact on the number of LV particles identified as containing RNA by Leprechaun (Figure 5B), confirming that SYTO14 is binding to RNA inside viral particles.

Uptake of small molecule dyes, such as SYTO14, across viral protein capsids is known to be time and temperature dependent[1,2]. To ensure a high efficiency of RNA staining SYTO14 was incubated with LV for increasing lengths of time, and at different temperatures. The percentage of virus found to contain RNA increases with time when incubated at 37 °C, before levelling off after 16 hours (Figure 6A). Lowering the incubation temperature to 25 °C or 4 °C significantly reduces SYTO14 staining of LV particles (Figure 6B). Taken together this data confirms that a high temperature and overnight incubation period aids efficient uptake of SYTO14 by the virus and access to the encapsulated RNA.

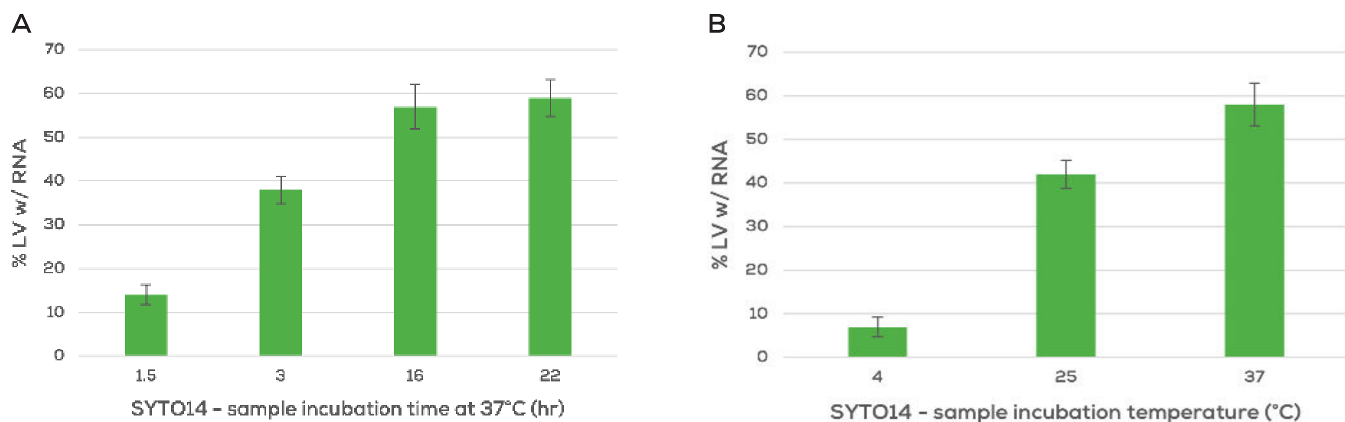

Figure 6: (**A**) Time dependence of SYTO14 staining of LV RNA. Sample was incubated with 10 μM dye for between 1.5 and 22 hours at 37 °C. (**B**) Temperature dependence of SYTO14 staining. Sample was incubated with 10 μM dye for 16 hours at 4 °C, 25 °C and 37 °C. For both graphs error bars are standard deviation, n = 3 for each condition.

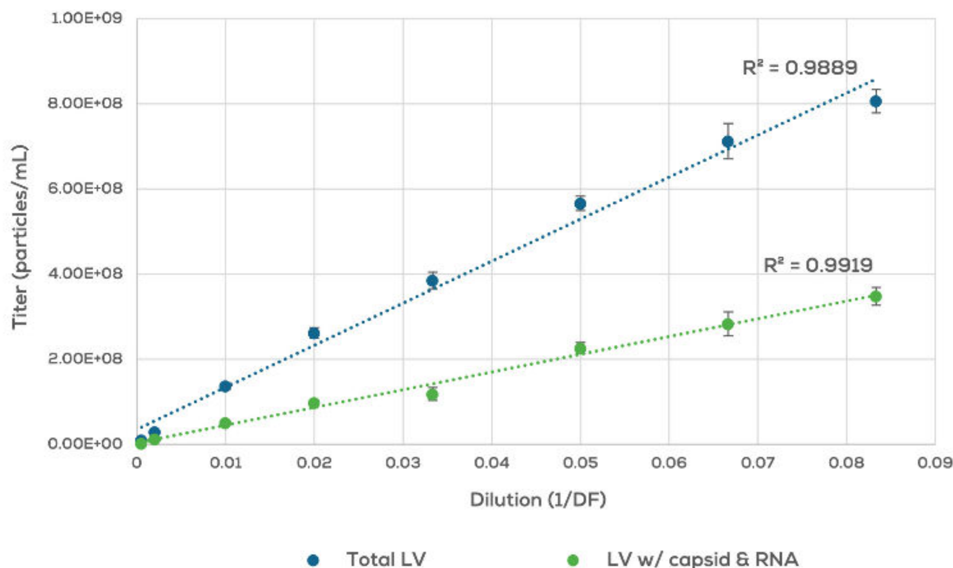

Figure 7: Linearity of LV titer as measured by Leprechaun. Error bars are SD.

Particle type	Dynamic range
LV w/ capsid & RNA	$1x10^7$ – $5x10^8$ particles/mL
LV w/ capsid w/o RNA	
LV w/o capsid w/ RNA	
LV w/o capsid w/o RNA	
Aggregates	
Soluble p24	5 – 10,000 pg/mL
Viral p24	1,000 - 50,000 pg/mL

Table 2: Dynamic range for all outputs provided by the Leprechaun Lentivirus VSVG/Flex RNA Kits.

Linear and Precise

Envelope specific capture of LV particles on the Luni surface stops non-viral particles from binding, preventing contaminants from interfering with viral titer. It is this step that means Leprechaun can maintain a linear range for lentiviral titer down to $1x10^7$ particles/mL in both crude and purified samples (Figure 7, Table 2), with a typical % CV of 8%.

Correlation with Genomic Titer

Crude and purified LV samples from a range of suppliers, with different viral genomes, were analyzed by both Leprechaun and qRT-PCR. Comparison of genomic titer with the overall titer of LV w/ RNA as measured on Leprechaun revealed a positive correlation (R^2 = 0.95) for purified samples (Figure 8A).

Interestingly, the same correlation was not observed in crude samples (Figure 8B). This strongly suggests there are a significant number of particles released from the producer cell which are the same size as LV, express VSVG, and contain RNA other than the intended viral genome. Most likely these particles are vesicles released alongside LV particles, and which contain cytoplasmic RNA. Leprechaun handles this population by probing for capsid protein at the same time as RNA to help ensure you're only counting LV which have undergone the correct assembly process. It is therefore vital that any technique relying on generic RNA detection for analysis of early-stage LV samples also checks for the presence of p24 capsid.

Track Purification

Leprechaun allows you to track the quality of your LV batch throughout the production process, revealing if you're enriching for that crucial capsid & RNA-containing LV and removing incorrectly assembled viral-like particles. Samples from the same LV batch, but from different steps of the purification process – crude harvest, post PEG precipitation and final product (PEG, UC and UF purified) – were analyzed on Leprechaun (Figure 9A).

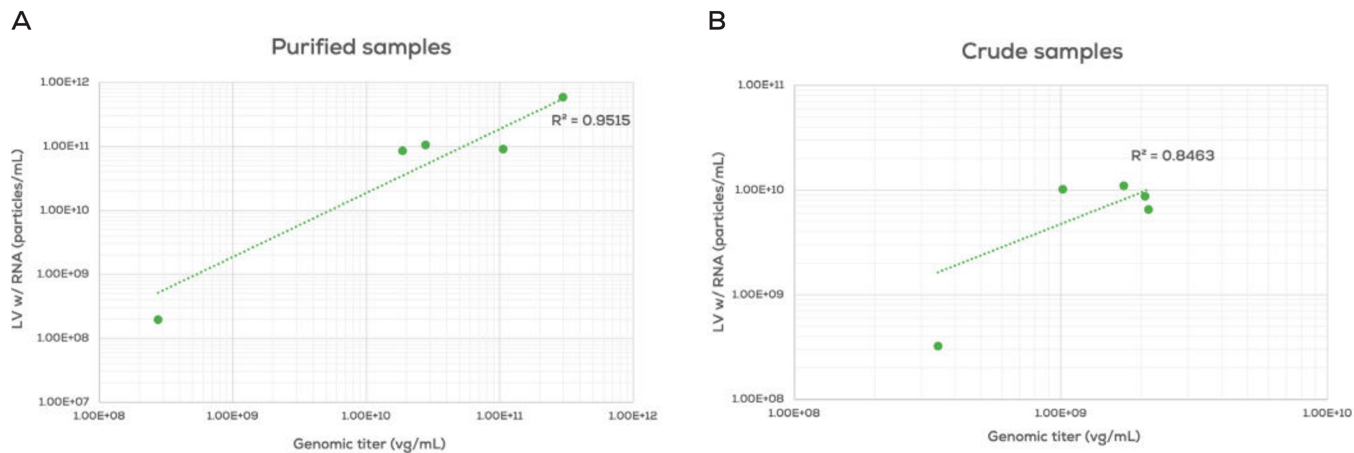

Figure 8: Relationship between the titer of LV w/ RNA as measured by Leprechaun and genomic titer from qRT-PCR in pure (**A**) and crude (**B**) samples.

Checking the structure of individual particles on Leprechaun reveals that although total LV titer increases with each purification step, the proportion of structurally complete viral particles is no higher in the final product than in the crude harvest (Figure 9A – blue line). This indicates that while standard LV purification techniques successfully concentrate particles which have the size and density of LV, they are not necessarily effective at enriching for structurally complete LV.

The total p24 content of the three samples was analyzed on Leprechaun and titer calculated using the conversion of 1x10^4 physical LV particles per pg of p24 to produce a comparable output to a p24 ELISA[3]. p24 concentration increases with each step, implying an increase in viral titer (Figure 9B). However, this output is misleading without the context of how many p24 negative viral particles are still present (Figure 9A – gray, yellow bars) – information not provided by a traditional ELISA. These particles express the same envelope protein, may contain RNA and have the potential to compete with intact LV for transduction of target cells. When p24 negative particles are taken into consideration the final product has a similar proportion of capsid-containing LV to the crude harvest.

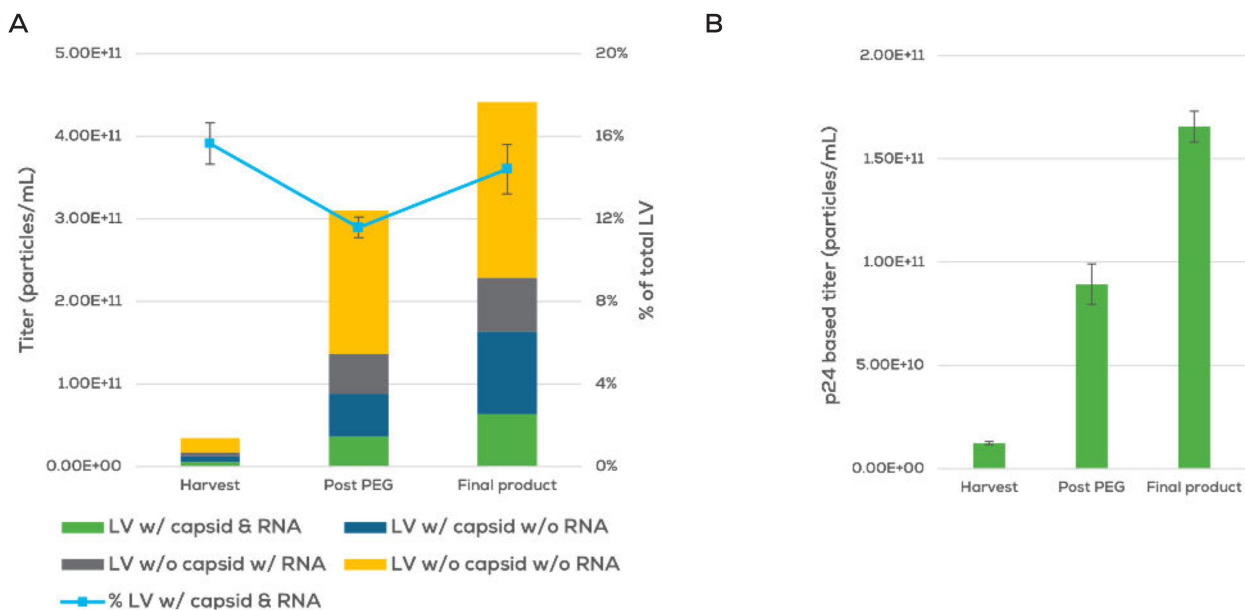

Figure 9: (**A**) Leprechaun analysis of a LV batch at different stages of purification. (**B**) p24 based titer as measured on Leprechaun. Titer is calculated from the sum of the concentration of viral and soluble p24. Error bars = SD, n = 3.

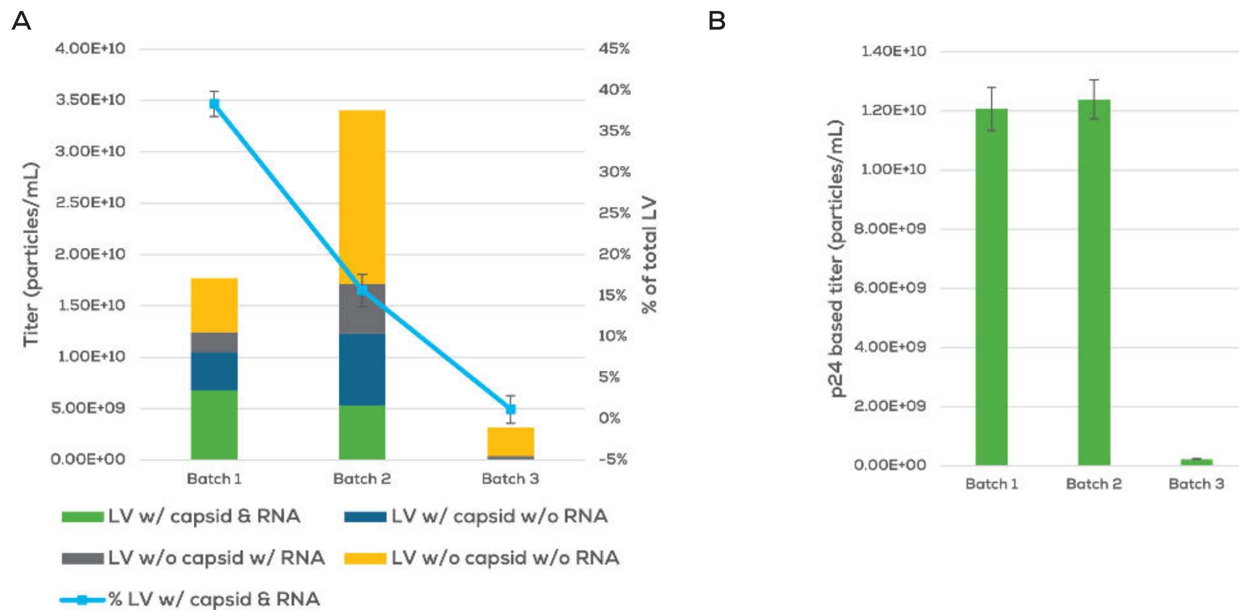

Figure 10: (**A**) Leprechaun analysis of the structural composition of three crude LV samples. (**B**) p24 based LV titer as determined by Leprechaun. Error bars = SD, n = 3.

ID a Good Batch

Leprechaun also provides the capability to screen LV samples at harvest to check if a batch has a high titer of structurally complete LV, and is worth continuing with, or whether you should cut your losses as there is a low titer of intact LV indicating that the batch is mostly junk. Crude samples from three LV batches were analyzed on Leprechaun and their compositions compared (Figure 10A).

At first glance batch 2 has the highest overall titer of LV particles, however a closer look at the structural composition of these particles shows that a significant proportion of batch 2 are empty viral particles containing neither capsid nor RNA. Batch 1 and 2 have comparable titers of LV w/ capsid & RNA, but batch 1 contains far fewer structurally incomplete LV contaminants, meaning it is significantly more enriched for LV w/ capsid & RNA (39% v 14% of total LV particles). As viral particles missing capsid and/or RNA are difficult to remove during processing, selecting a starting material with a low proportion of these particles is critical, in this case batch 1.

As with the previous set of samples, the p24 content was measured by Leprechaun and converted to a physical titer comparable to that provided by ELISA (Figure 10B). While p24 content alone is successful in identifying batch 3 as the lowest titer sample, it cannot differentiate between the quality of batch 1 and 2 due to the lack of information on both capsid negative LV and the RNA content of the capsid positive particles.

Conclusion

Traditional purification methods are effective at concentrating LV but can struggle to remove LV particles which are the correct size and density but lack the key structural components – specifically a p24 capsid & RNA. These particles have the same envelope protein as intact LV and can compete for transduction of target cells but are not capable of delivering the desired payload and may indeed carry RNA other than the viral genome. Delivery of unknown RNA sequences to cells intended for treatment of patients needs to be avoided to ensure safe and effective cell therapies.

Current LV analytical techniques only look for either capsid or RNA, and therefore fail to provide any information on how many structurally incomplete viral particles are present. Even cell based functional assays focus exclusively on particles with the correct viral genome, and do not measure the impact of particles carrying other RNA or cargo proteins. Take your process development to the next level with the help of Leprechaun, the only platform to show you the complete picture of your LV sample, including

how many viruses are missing p24 capsid, RNA or both. For the first time you can truly know what's in your sample and get a detailed insight into the effectiveness of each step of your LV production process to make fully informed decisions about your process development.

Leprechaun puts you in the best position to produce a high-quality final product by showing you which upstream material is highest in LV w/ capsid & RNA, and low in structurally incomplete LV which may alter your target cells in unknown ways. Identifying the top samples early on is critical to overcoming the limitations in LV purification techniques that are present in even the best production processes. As regulatory demand for LV characterization grows, revolutionize your LV analytics with Leprechaun and ensure your LV preps always meet the gold standard.

References

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