Pair up lentiviral titer and structure with Leprechaun

Introduction

Checking on the titer, structure and contaminant levels of a lentiviral vector (LV) sample throughout the development and manufacturing process is a major challenge for gene therapy production. The methods most commonly in use are p24 ELISAs and qPCR, however both require relatively pure samples and neither provides a complete picture of what's going on in your sample.

Capsid ELISAs fail to separate soluble p24 from virally encapsulated protein resulting in an overestimation of LV titer in all but the cleanest samples. qPCR offers direct detection of the gene of interest, but results can suffer from substantial variability making qPCR unreliable for determining LV titer[1]. Both techniques focus on only one LV component, and their requirement to lyse the sample prior to analysis means they can't be multiplexed to provide information on viral structure. Both techniques are also highly specific to their targets, so any contaminants present will not be detected.

Leprechaun is the first platform to deliver LV titer, structural characterization and contaminant analysis in a single run of any sample – crude or pure (Figure 1). Leprechaun determines whether individual LV particles are damaged or have been incorrectly assembled by analyzing particle size while simultaneously checking for the presence of a viral envelope and capsid. Aggregates can be distinguished from single particles based on size, while viral and non-viral particles are counted up separately by surface protein expression. Leprechaun quantifies 5 key components of any LV prep on a single particle basis, so you know if each individual particle has a lentivirus capsid, envelope proteins, is aggregated, or is just an extracellular vesicle (EV).

Figure 1: Leprechaun: The unmatched lentivirus titer and characterization tool.

Leprechaun's Lentivirus Luni consumable is pre-coated with highly specific antibodies against VSVG, p24 and tetraspanins to capture LV particles and non-viral contaminants straight from crude samples (Figure 2). A combination of single particle

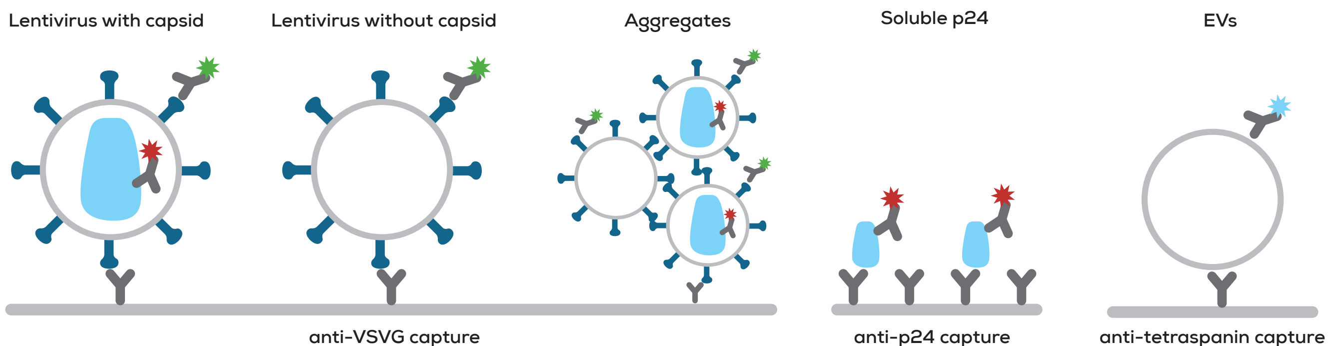


Figure 2: The Lentivirus Luni contains separate antibody capture spots for LV, soluble p24 and EVs. Get n≥3 technical replicates for each output from a single Luni.

interferometry and fluorescence microscopy with immunofluorescent probes against VSVG, p24 and tetraspanins accurately measures LV titers down to $5x10^6$ vp/mL, while simultaneously checking whether a virus has all the right structural parts and is the correct size. Soluble p24 and EVs are also quantified, all in the same run.

EVs are released from cells alongside lentivirus during production, and their similarity in size and tendency to incorporate genomic content and viral proteins means EVs are a tricky contaminant to discriminate from viral particles. As LVs become increasingly used for in vivo therapies the demand from regulatory bodies to control more critical quality attributes (CQAs), including EV contaminant concentration, will only grow. Leprechaun lets you easily monitor lentiviral titer and EV contaminant concentration at every stage of your production process, helping to speed up process development by allowing you to figure out the best method for generating high viral yields with few exosome contaminants.

This app note describes how Leprechaun's Lentivirus application quantifies capsid containing lentivirus, lentivirus without capsid, soluble p24 contamination, exosome contamination and measures aggregation.

Methods

Lentivirus samples were generously provided by the Birnbaum Lab (MIT). For titration experiments Lentivirus was diluted in PBS to between $1.23x10^6$ - $3.15x10^8$ vp/mL as determined by p24 ELISA. Analysis was performed using the Leprechaun Lentivirus Kit (Unchained Labs). Samples were diluted 1:2 in manufacturer supplied Incubation Solution and incubated for 1 hour at RT on Lentivirus Lunis. After washing in Solution A, Lunis were incubated for 1 hour at RT with kit supplied antibodies; anti-VSVG-CF555 (1:500), anti-p24-CF647 (1:250) and anti-tetraspanin-CF488 (1:500). Lunis were then washed, dried and run on Leprechaun using the Lentivirus application. Lentiviral titer, aggregate titer, EV concentration, soluble and viral p24 concentration were determined by Leprechaun Analysis.

Results

After immuno-capture of viral particles by the Lentivirus Luni, Leprechaun combines fluorescence microscopy with interferometry to determine the titer of capsid-containing lentivirus. The combination of these techniques allows protein expression and size to be analyzed at the individual virus level for captured particles.

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 lentiviral particles with all the parts.

Size alone however is not enough to identify a particle as lentiviral. Adding fluorescent staining for VSVG and p24 provides critical structural information (Table 1). CF555-tagged anti-VSVG confirms the presence

Particle type	Luni capture spot	Particle definition	
		Size (nm)	Proteins detected
LV with capsid	anti-VSVG	35 – 130	$VSVG^+ p24^+$
LV without capsid	anti-VSVG	35 – 130	$VSVG^+ p24^-$
Aggregates	anti-VSVG	>130 – 200	$VSVG^+ p24^\pm$
Viral p24	anti-VSVG	N/A	$VSVG^+ p24^+$
EVs	anti-tetraspanin	35 – 200	$Tetra^+ VSVG^\pm p24^\pm$
Soluble p24	anti-p24	N/A	$p24^+ VSVG^- Tetra^-$

Table 1: Summary of definitions for LV particle types.

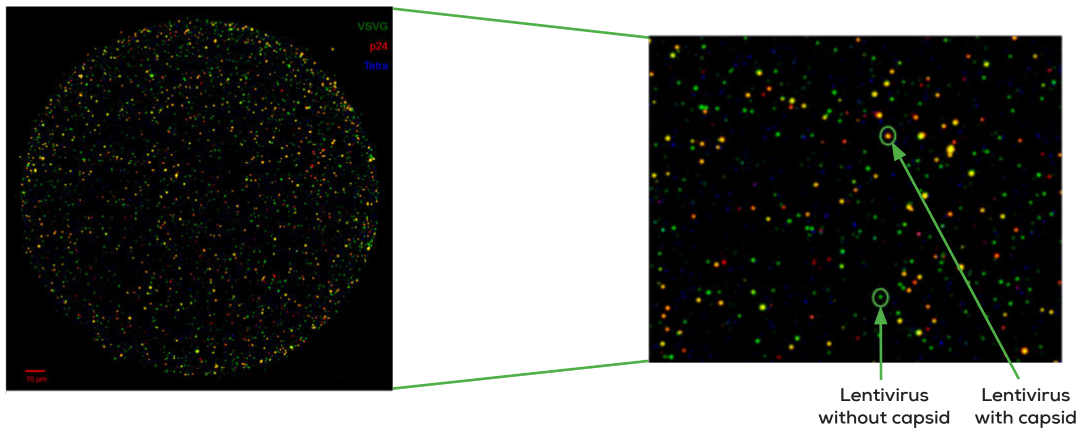


Figure 3: VSVG capture spot on Lentivirus Luni. Sample was fixed and permeabilized to allow probing for encapsulated p24. Lentivirus with both the VSVG envelope protein and p24 capsid is yellow, lentivirus that does not contain a capsid is green.

of the viral envelope and VSVG pseudotype, and after a mild fixation and permeabilization anti-p24-CF647 detects encapsulated capsid (Figure 3).

The specificity of the anti-VSVG capture step stops non-viral particles from binding, preventing contaminants from interfering with viral titer. It is this step that means Leprechaun maintains a linear range down to $5x10^6$ vp/mL in both crude and purified samples (Figure 4, Table 2), with a typical %CV for LV titer of 6%. Soluble p24 measurements have a typical CV of 8%.

Leprechaun breaks down viral titer into two categories - LV with p24 capsid and virus without capsid (Figure 5A). In this instance a sample with a total LV titer of $6.2x10^8$ vp/mL is shown to contain $2.7x10^8$ vp/mL capsid positive LV – 43%. The size profile of all components in the LV sample confirms a low level of aggregates (>130 nm) in this example (Figure 5B).

To measure the amount of EV contaminants, the Luni specifically captures EVs on an anti-tetraspanin surface. For the same sample with a total LV titer of $6.2x10^8$ vp/mL, $5.7x10^8$ particles/mL EVs were present (Figure 5A). Determining the concentration of EVs in your LV prep also helps you to assess whether EVs hinder or help the performance of your vector.

Leprechaun also quantifies soluble p24 contamination (Figure 5C). As sample integrity is maintained throughout the assay, soluble and virally-associated p24 can be quantified separately.

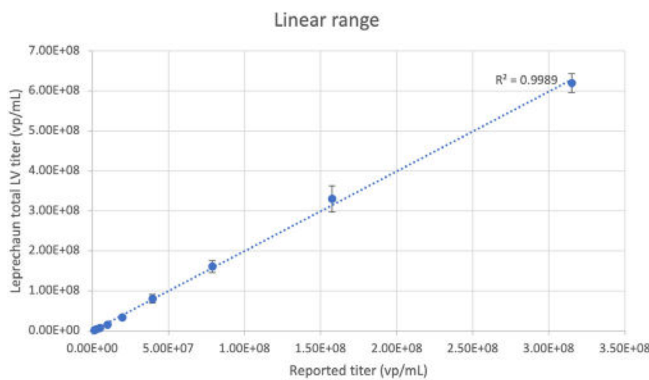
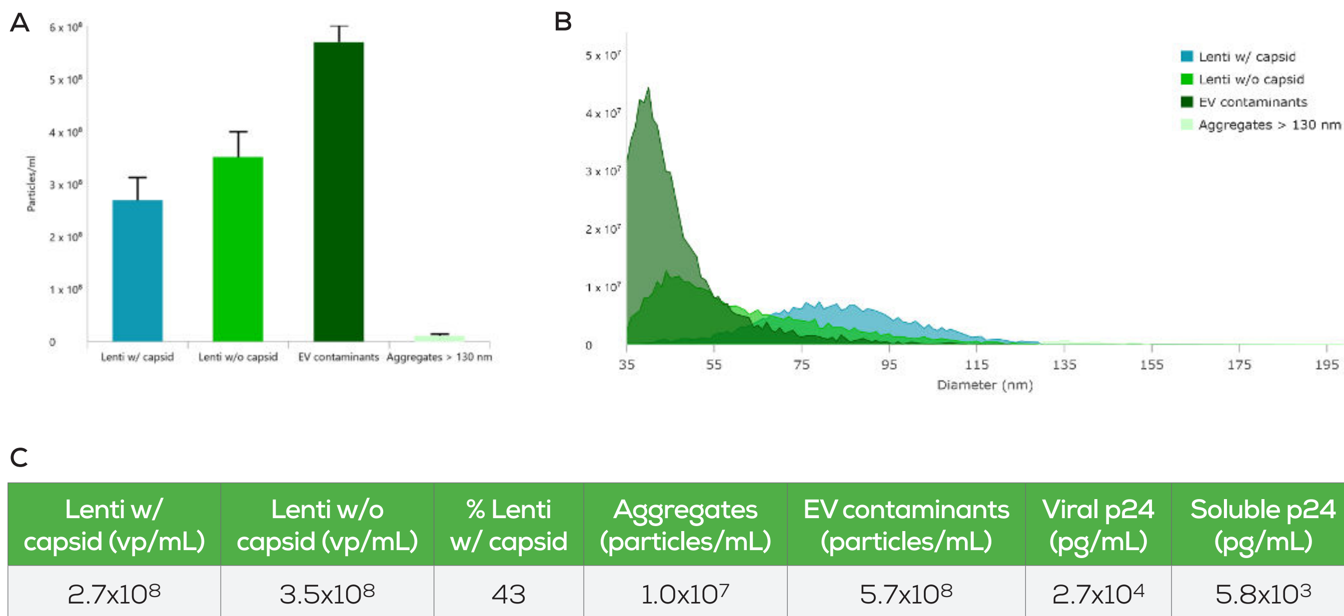


Figure 4: Linear correlation between the lentiviral titer measured by Leprechaun and reported viral titer from p24 ELISA. Error bars are SD.

Particle type	Dynamic range
LV with capsid	$5x10^6$ – $5x10^8$ particles/mL
LV without capsid	
Aggregates	
EVs	
Soluble p24	5 – 10,000 pg/mL
Viral p24	500 - 50,000 pg/mL

Table 2: Dynamic range for each LV related particle type on Leprechaun.



Lenti w/ capsid (vp/mL)	Lenti w/o capsid (vp/mL)	% Lenti w/ capsid	Aggregates (particles/mL)	EV contaminants (particles/mL)	Viral p24 (pg/mL)	Soluble p24 (pg/mL)
$2.7x10^8$	$3.5x10^8$	43	$1.0x10^7$	$5.7x10^8$	$2.7x10^4$	$5.8x10^3$

Figure 5: (**A**) Leprechaun reports lentivirus with capsid titer, lentivirus without capsid titer, aggregate titer and concentration of EV contaminants. (**B**) Size profile of LV, aggregates and exosome populations. (**C**) Comprehensive quantification of the different components within a LV sample.

Soluble p24 is measured directly on the Luni's anti-p24 spots which captures p24 that is free in solution. The fluorescence intensity of bound p24 is converted to pg/mL via an internal standard curve. This enables Leprechaun to provide an accurate measurement of the concentration of contaminating p24, so the amount can be tracked throughout purification. To provide context for soluble p24 concentration, Leprechaun's software quantifies viral p24 concentration as well, using the conversion of $1x10^4$ physical lentiviral particles per pg of p24[2].

Conclusion

Tracking capsid containing LV titer provides a unique insight into the proportion of your LV which has the potential to carry payload and lets you spot workflow steps which are damaging your vector. The ability to do this is hampered by existing analytical methods that are ineffective in crude samples and focus on only one lentivirus characteristic, with no structural characterization.

Leprechaun gives the full picture of your LV sample, from cell culture to final formulation, without the need for multiple analytical techniques or assay optimization. By analyzing structural information like surface and capsid protein expression, Leprechaun serves up greater detail on size, titer and contaminant detection in one go. Leprechaun lets you handle crude samples with ease by specifically immuno-capturing viral particles and contaminants separately. This helps you assess the impact of every step on viral titer and integrity, and provides rapid feedback on any changes to your development process. Whatever the stage of your LV development, Leprechaun leads you straight to the viral titer you've been looking for.

References

1. Quantification of reverse transcriptase activity by real-time PCR as a fast and accurate method for titration of HIV, lenti- and retroviral vectors. Vermeire, J. et al. PLoS One, 2012. 7(12):e50859.

2. Production of high-titer lentiviral vectors. Zufferey, R. and D. Trono. Curr Protocols in Neuroscience, 2001. 12: 4.21.1-4.21.12.

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