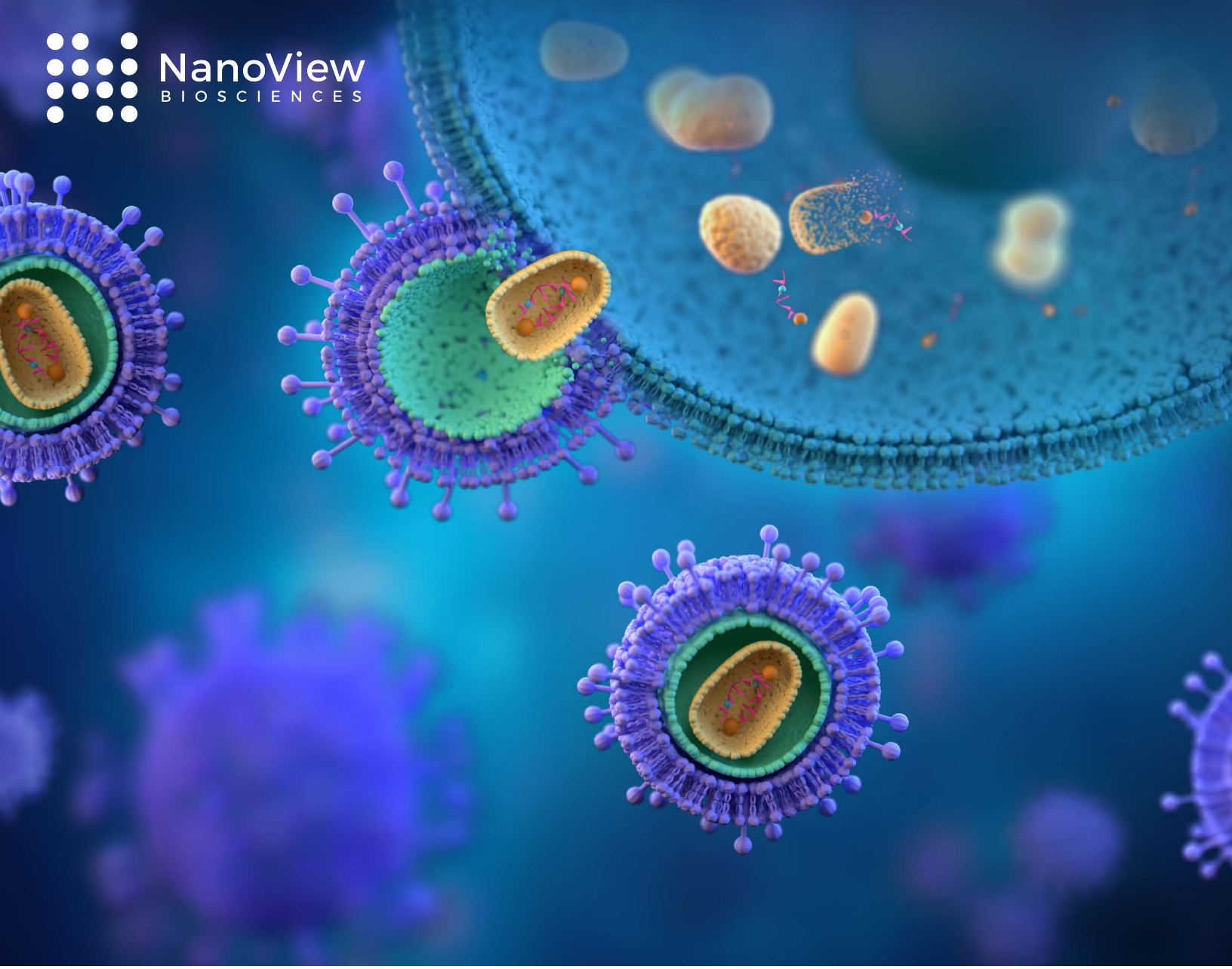
NanoView BIOSCIENCES



LentiView™ Purification-Free Lentivirus Characterization

Simultaneous measurement of full/empty titer, virus size and quantitative pseudotype profiling

WHITEPAPER THEMES:

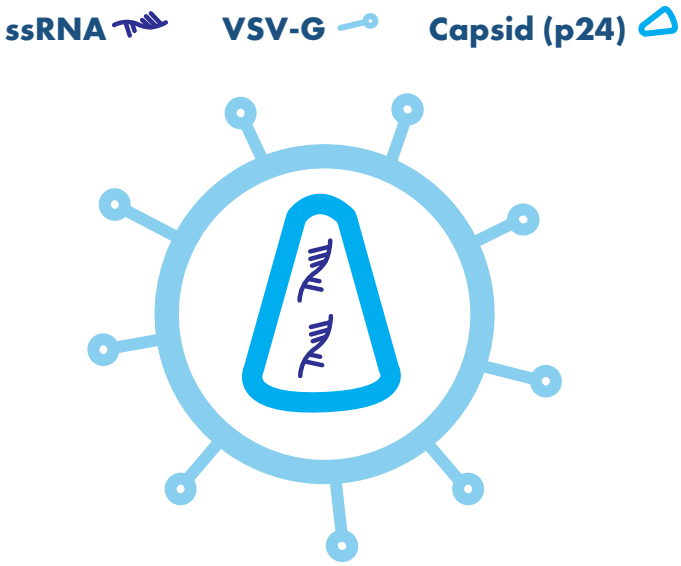
- Purification-free lentivirus titer
- Sensitive detection of lentivirus in complex matrices
- Discrimination of full versus empty lentivirus via capsid detection
- Measurement of virus size and sample aggregation
- Sensitive 4-color fluorescence
- Pseudotype profiling and quantification

Lentiviral vectors in gene therapy

Gene therapy is based on the ability to modify the genome of a target cell, by replacing or inactivating disease-causing genes, or inserting new genes. Lentiviruses are slow replicating retroviruses derived from HIV-1 that are commonly used as a delivery vector in gene therapy. They are capable of infecting replicating and non-replicating cells and can stably integrate into the host genome with a high transduction efficiency, even in cells that are traditionally challenging to transduce. Lentiviral infection rarely generates immunogenic proteins and can deliver large transgene payloads (approx. 5-10 kbp), making them suitable for a broad range of gene delivery applications. In addition, lentiviral pseudotyping allows foreign proteins (e.g. immune cell or other tissue-specific receptors) to be engineered into the viral envelope, enabling targeting of recombinant lentiviruses to specific cells or tissues.

Lentiviral vectors have developed from first generation to second and third generation vectors, aimed at improving the safety of the vector. While first generation vectors are no longer used over safety concerns, second and third generation vectors are routinely used for gene delivery. Second generation vectors utilize three delivery plasmids allowing the essential components of the lentivirus to be delivered via independent plasmids, preventing accidental reconstruction of wild-type viral particles. The three delivery plasmids are known as the transfer, packing and envelope plasmids, and importantly, these do not contain any genes involved in virus propagation. Third generation vectors add an additional delivery plasmid, to further reduce safety concerns.

FIGURE 1 Example image of a lentivirus



Measuring Lentivirus Titer

It is important to measure the viral titer of a preparation as it indicates the quality of the preparation and estimates the quantity of vector that is required for transduction. Many of the methods used to determine lentiviral titer are limited in their scope and sensitivity

and provide little information about the vector quality. For example, many techniques are limited to purified samples and are therefore unable to assess the quality of the vector in upstream non-purified sample preparations. In these instances, vector quality is only known once the sample has been fully purified which requires time and money to be invested. Most techniques also fail to link physical parameters such as size (linked to vector aggregation) to proteomic analysis. These techniques are therefore unable to identify cases where viral titer is affected by sample aggregation or virus fragmentation/disintegration, and hence fail to distinguish monodisperse preparations from aggregated samples.

In general, there are two ways of measuring lentivirus titer – physical titer and functional titer. The most common method for measuring physical lentivirus titer is a p24 ELISA assay. These commonly used assays measure the bulk concentration of p24 capsid protein present within a sample. The viruses within the sample are first lysed and the resulting p24 concentration measured against a known standard. The major limiting factor of this assay is the assumption that the concentration of p24 protein in a preparation is proportional to the quantity of virus. p24 protein is relatively abundant in free solution, resulting in innacurate virus titers. These bulk assays also fail to discriminate full, intact virus from viral fragments or other particles containing p24 (e.g., extracellular vesicles). p24 assays are an indirect, bulk method to measure viral titer; as such, they are prone to error and biases, and provide no meaningful information about the functionality and transduction efficiency of the virus sample. It is worthwhile mentioning that the described challenges of bulk protein concentration measurements not only apply for lentivirus but also any other application that requires a reliable and highly sensitive measurement of functional transducing particles (adeno-associated virus (AAV), adenovirus, measles virus or even lipid nanoparticles). Functional titer assays measure viral transduction into cells. This is commonly done by incorporating a GFP reporter transgene into the lentiviral vector genome, which upon infection of the target cells, can be detected via flow cytometry. This type of testing is unable to differentiate between cells that are infected by single or multiple viruses and hence typically provides an underestimate of viral titer. In addition, this assay does not provide any information about virus fragments or contaminants which may impede the transduction efficiency of a virus preparation, and which could pose significant harm to patients. Another commonly used technology to indirectly measure infectious viral titer is qPCR. This method accurately measures the amount of viral DNA which carries the gene(s)-of-interest. Similar to functional titer assays, qPCR analysis does not provide information about virus impurities and aggregates.

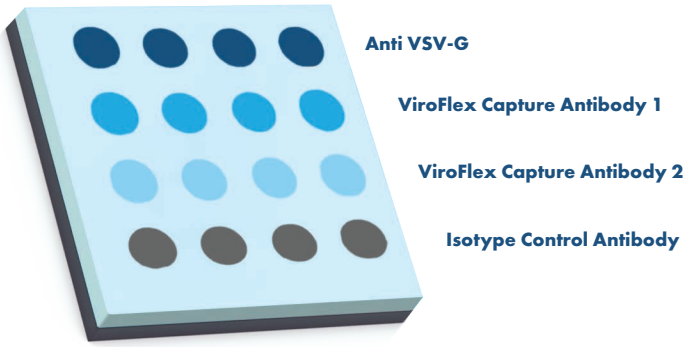
Introducing LentiView™

NanoView has developed a technology that allows lentiviruses to be measured at all stages during the purification process and which links proteomic information to physical viral titer without the need for sample purification. Key features of the technology include:

- Measurement of physical lentivirus titer (i.e., particles/ml) at all stages of the purification process with no sample pre-treatment required.
- Sensitive detection of virus in complex matrices down to 10E6 virus particles (VP)/ml.
- Discrimination of full versus empty lentivirus via capsid detection.
- Simultaneous measurement of virus size and of viral protein and capsid markers.
- Sensitive 4 color fluorescence detection of any virus.
- Pseudotype-profiling: Quantitative analysis of targeting molecules engineered into the vector.
- Measurement of contaminating particles (e.g., extracellular vesicles), fragments and aggregates that co-purify with many purification technologies.

NanoView's LentiView™ assay immuno-captures lentiviral particles using highly specific antibodies immobilized on a chip surface. As little as 25 µl of unpurified or purified sample is required with a concentration as low as 10E6 VP/ml (with much lower corresponding functional titers possible). Once the virus has been captured on the chip, surface and internal viral proteins can be probed, virus subpopulations can be characterized, and viral titers of specific populations can be measured. Viruses can be sized on a virus-by-virus basis providing a measure of sample aggregation which in turn can be linked to the transduction efficiency of a vector. These measurements can be performed at all stages of the purification process without additional processing, providing a robust and simple measure of functionality and purity throughout the downstream purification process.

FIGURE 2 Image of a LentiView™ chip and capture antibody configuration.



LentiView™ allows lentiviruses to be detected and characterized without any sample preparation. Viral titer, size and pseudotype can be readily identified in an automated analysis workflow.

Full vs Empty Quantification

One of the most common tools to assess viral particle titer is a p24 ELISA assay. This assay measures the concentration of p24 capsid protein and converts it into a viral particle titer. The presence of soluble p24, variability in the amount of p24 per virus and, sample aggregation pose significant issues with this methodology. In addition, the p24 ELISA is unable to detect particles that lack the viral capsid (termed empty). These empty particles are associated with poor transfection efficiency. In this technical note we highlight how NanoView's LentiView™ technology can overcome these limitations to provide a quantitative, highly sensitive tool to determine titer, distinguish empty from full viral particles and identify sample impurities.

Figure 3 shows the basic configuration of lentivirus capture and labeling on a LentiView™ chip. Viruses are captured on the LentiView™ chip via surface immobilized anti-VSV-G antibodies. Once immobilized the viruses are fixed and probed for surface and internal protein markers. The presence of VSV-G (green) and p24 capsid protein (red) is reported using immunofluorescence staining. **Figure 4** shows a representative dual-color image acquired with the LentiView technology. Green puncta represent VSV-G-positive viral particles devoid of capsid and yellow puncta represent full capsid-containing viral particles.

FIGURE 3 LentiView™ assay for detecting full vs empty lentiviruses.

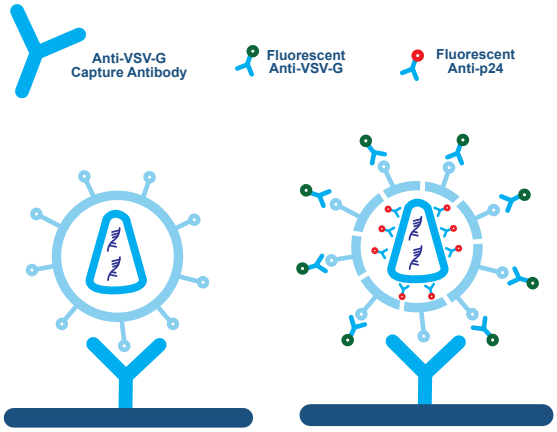
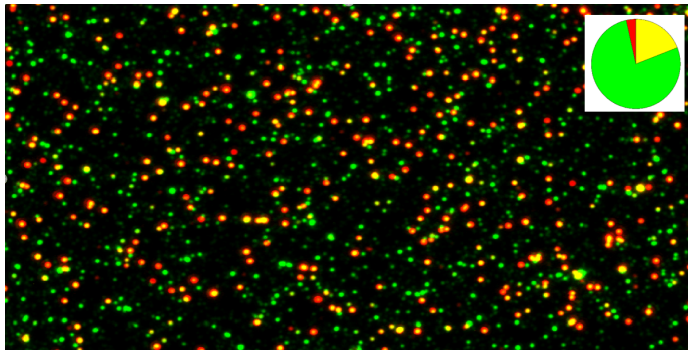


FIGURE 4 LentiView™ assay for detecting full vs empty lentiviruses.



LentiView™ can rapidly quantitate the number of lentiviruses that contain or do not contain capsid proteins (full versus empty).

Linking Viral Vector Profile to Transduction Efficiency

Viral pseudotyping is the process whereby viral vectors are engineered with foreign viral envelope proteins in order to restrict or broaden host cell tropism. An industry-wide challenge is an inability to predicatively link results from current methods of characterization to transduction efficiency of viral pseudotypes. A such, three different lentivirus samples were characterized using the LentiView™ assay to detect viral particle sub-populations, providing critical insight into the transduction efficiency of these preparations. A wildtype lentivirus vector was modified so that it encodes for the surface expression of a non-native protein complex in addition to VSV-G. Two different plasmid concentrations were used to generate vectors alongside the VSV-G wildtype.

- Wild-Type Lenti (VSV wt)
- Engineered targeting ligand with high concentration of plasmid (ETL-HC)
- Engineered targeting ligand with a low concentration of plasmid (ETL-LC)

These vectors were tested using p24 ELISA and functional titer assays (**Figure 5** and **Table 1**). **Figure 5** shows the transduction efficiency for each of the three vectors (VSV-G – wildtype, ETL-HC and ETL-LC). **Table 1** shows the corresponding physical p24 titer determined by ELISA and functional titer deduced from the data plotted in **Figure 5**. **Table 2** shows a summary of this data.

Table 1 p24 and functional titers of three lentiviral preparations.

	VSV wt	ETL- High Conc	ETL- Low Conc
p24 ELISA	3.07E9	6.57E7	2.28E9
Functional Titer	1.05E7	4.19E4	1.20E6

Figure 5 Transduction efficiency of three lentiviral preparations.

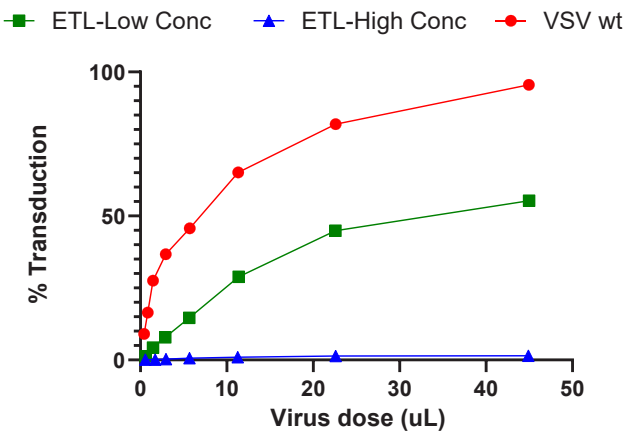


Table 2 Summary table of transduction efficiency versus physical titer (p24 assay).

	Transduction Efficiency	Physical Titer
VSV wt	High	High
ETL – Low Concentration	Medium	High
ETL – High Concentration	Low	Low

Predictive lentivirus characterization using NanoView's LentiView™

As a first step in testing NanoView's predictive capabilities, particle size analysis for each of the three preparations was performed using the LentiView technology. The assay configuration is described in **Figure 6**. Each of the 3 samples was then assessed for full viruses and virus fragments, with smaller virus fragments likely to have a size smaller than the known size for a lentivirus. As can be seen from **Table 3**, there is a strong correlation between the ratio of viruses above 50 nm, the number of virus fragments below 50 nm and the transduction efficiency of the vector.

Figure 6 Sizing assay for Lentivirus and fragment detection.

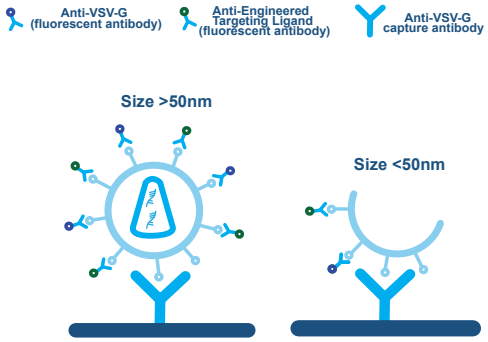


Table 3 Summary table of vector transduction performance versus ratio of sub-50 nm virus fragments to larger

	Transduction Efficiency	Ratio of >50nm viruses to<50nm fragments
VSV wt	High	63% / 37%
ETL – Low Concentration	Medium	54% / 46%
ETL – High Concentration	Low	41% / 58%

FIGURE 7.1 Sizing results from LentiView™ assay comparing ratio VSV-G positive lentivirus and smaller sub 50 nm fragments with vector transduction efficiency.

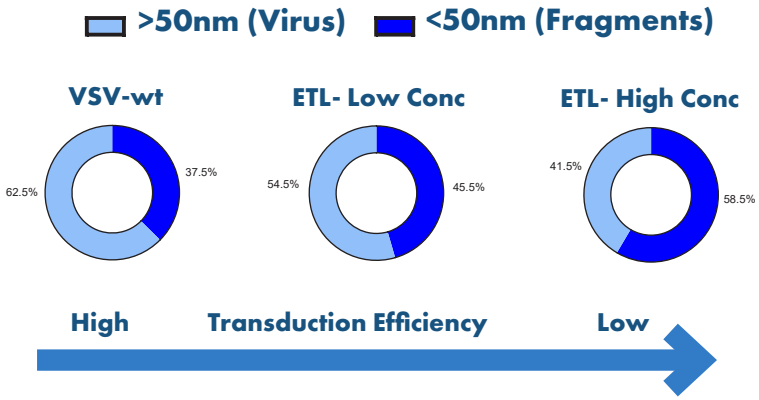
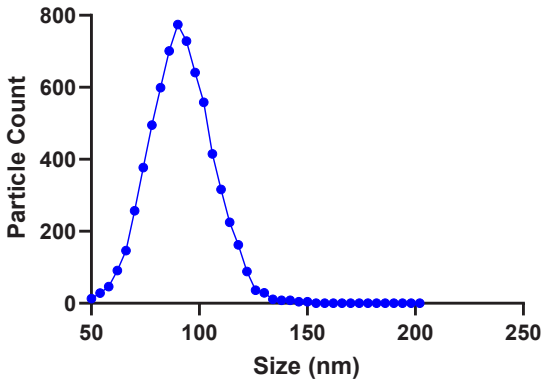


FIGURE 7.2 Lentivirus size distribution as generated by LentiView™.



Transduction efficiency correlates strongly with the ratio of VSV-G positive viral particles to fragments as measured by LentiView™. LentiView™ can measure the size distribution of lentivirus in purified and unpurified samples.

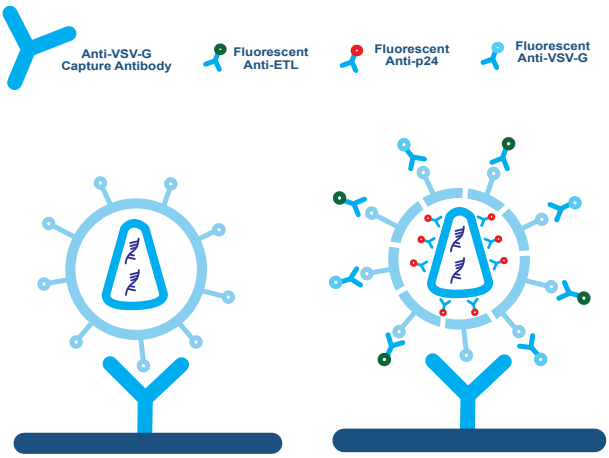
Pseudotype and Full/Empty Characterization

In addition to measuring virus size, the LentiView™ technology can probe for internal and surface proteins with single molecule sensitivity. The colocalization of proteins on individual viruses can be achieved with up to 5 proteins detectable on a single virus particle. The three different lentivirus vectors were characterized for surface modification expression as per **Figure 8** and experimental plan described below:

- Capture of lentivirus to chip via VSV-G lentivirus envelope protein.
- Detection of VSV-G via a fluorescent VSV-G specific antibody probe in the **blue** channel.
- Detection of capsid protein (p24) via fluorescent p24-specific antibody probe in the **red** channel.
- Detection of engineered targeting ligands to detect lentivirus modifications via a fluorescent anti ETL-specific antibody probe in the **green** channel.

The three preparations were analyzed for surface protein expression as well as the presence of p24 capsid protein. The results were linked to transduction efficiency to determine what links

FIGURE 8 Pseudotype characterization of engineered lentivirus expressing custom targeting ligands.



might be present between pseudotype and vector performance, the results are shown in **Figure 9.1** and **Table 4**. The definition of full/empty is described below:

- Full virus – any viral particle expressing either (or both) VSV-G and ETL as well as p24 capsid protein.
- Empty virus – any viral particles expressing either (or both) VSV-G and ETL but no p24 capsid protein.

FIGURE 9.1 Ratio of full versus empty viral particles versus vector transduction efficiency.

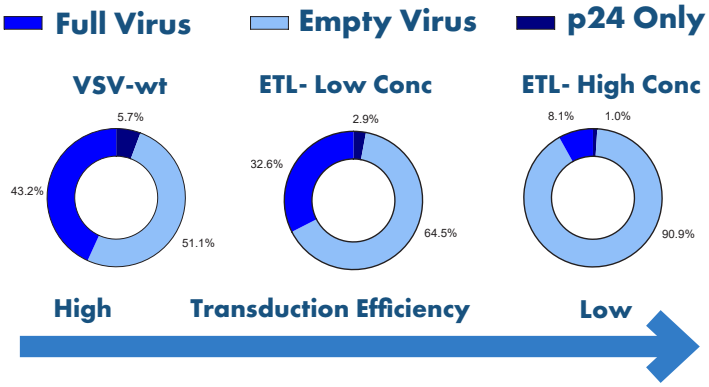


FIGURE 9.2 Size distribution of full and empty lentivirus as measured by LentiView™. Full viruses size ~ 13 nm larger than empty viruses (mode of 94 nm vs 81 nm).

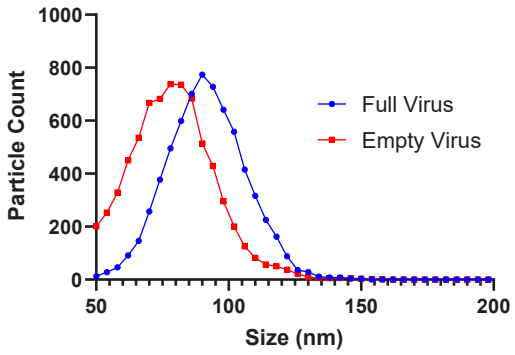


Table 4 Summary table of vector transduction performance versus ratio of full versus empty viral particles

	Transduction Efficiency	p24 Concentration	Ratio of full / empty viruses by LentiView™
VSV wt	High	3.1E9	43% / 51%
ETL – Low Concentration	Medium	2.3E9	33% / 65%
ETL – High Concentration	Low	6.6E7	8% / 91%

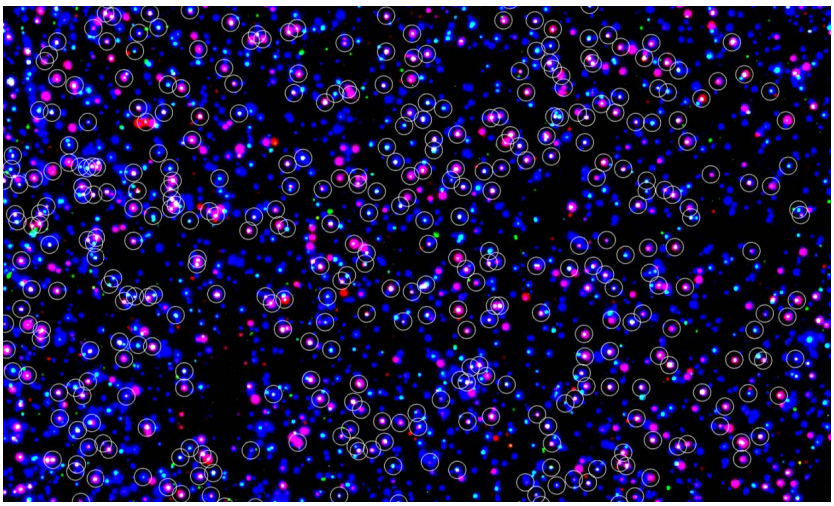


Figure 10
NanoView image of ETL-Low Conc. VSV-G in blue, p24 in red and ETL in green. Circled spots indicate a >50nm virus particle.

The data demonstrates that the LentiView™ technology is not only able to detect viruses and measure their size and the presence or absence of capsids, but also detects protein markers that have been engineered into the vector. Importantly, vector performance can be assessed relative to all of these variables. From the data, there is an adverse correlation between ETL expression and transduction efficiency of the vector. ETL high concentration showed a high percentage of events that expressed ETL but an overall low ratio of filled to empty virus particles. The ratio of filled versus empty capsids appears to be the dominant variable in determining transduction efficiency in these samples. It is unclear if the ETL expression causes the formation of viruses that do not contain capsids or if ETL expression is increased in vectors that inefficiently form. Either way ETL expression seems to be adversely linked to the amount of filled viruses and resultant transduction efficiency.

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The LentiView™ technology is highly predictive of vector performance in the samples tested. LentiView™ can be used at all stages within the purification as it requires no purification.

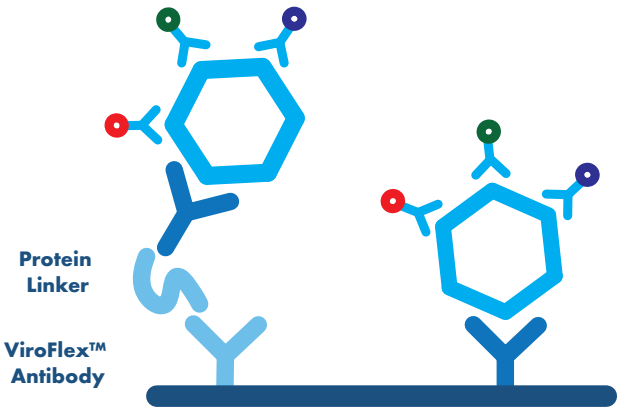
Future Assays and User Customization via ViroFlex

NanoView customers can customize capture antibodies easily in their own lab via the NanoView ViroFlex technology. In these experiments a protein linker can be used to functionalize a ViroFlex chip with antibodies against any virus. Therefore, highly customized assays can be routinely and easily created in your own lab.

Figure 10 shows how the ViroFlex technology works versus standard capture onto a NanoView chip. Antibodies can be directly printed by NanoView (right) onto the chip allowing capture of any virus via an appropriate surface protein on the virus. Alternatively, customers can functionalize a ViroFlex chip in their own lab:

- A protein linker is conjugated to any custom antibody that is required for capture to a ViroFlex chip.
- The linker and antibody conjugate is then bound to the chip via an interaction of the protein linker with the ViroFlex capture antibody.
- The ViroFlex chip is therefore functionalized with custom capture antibody.
- The sample is incubated and viruses are captured via the custom capture antibody.
- Each chip can be functionalized with up to two custom capture antibodies.
- The user can now measure full/empty, biomarker colocalization, viral particle titer and virus size.

FIGURE 10 ViroFlex™ capture (left) and standard capture (right) of any virus to NanoView chip



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