Rapid and Reliable Quantification of Lentiviral Vector Particles Using the Virus Counter® 3100 Technology

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Abstract

Lentiviral vectors (LVs) are effective gene delivery vehicles that have been successfully used in cell and gene therapies. They are most commonly produced via transient transfection of several plasmid constructs in adherent or suspension culture of HEK293 cells. A reliable, fast and precise enumeration of the total particle count of gene therapy vectors is critical in minimizing the risk of adverse immune responses in patients as well as to better understand and optimize production processes. Currently, quantification of LV particles is reliant on error-prone, time-consuming, and costly methods such as ELISA and qRT-PCR. The Virus Counter® instrument in combination with the antibody-based ViroTag® VSVG reagent offers an alternative to these methods, allowing for the rapid and precise quantitation of LV particles. The reagent utilizes serotype-specific fluorescently labeled antibodies with high affinity for intact LV particles expressing the VSV-G epitope.

Here we demonstrate via our collaboration with Yposkesi that the Virus Counter® instrument coupled with the ViroTag® VSVG reagent provides a rapid, biologically-relevant method for quantifying VSV-G pseudotyped LVs for both adherent and suspension production processes. Utilizing a patented, no-wash assay, LV samples are stained in 30 minutes and then counted in 3 minutes per sample. This speed allows for in-process monitoring and production optimization of VSV-G pseudotyped LV products, making the Virus Counter® instrument and ViroTag® reagents a valuable addition to bioprocessing applications utilizing LV particles.

Keywords or phrases:
Virus quantification, Lentiviral vectors, Virus Counter, Vector production

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Introduction

The remarkable commercial approvals of CAR T-cell immunotherapies Kymriah™ (Novartis) and Yescarta™ (Gilead) and gene therapy Luxturna™ (Spark Therapeutics) have started a new era of biological treatments that heavily rely on viral vector production. These vectors may be used ex-vivo, as in the case of the newest cell-based immunotherapies, or in-vivo, as in the case of gene therapies. Currently, LVs are primarily used ex-vivo to transduce T-cells to express new chimeric antigen receptors (CARs; there are also in-vivo applications of LVs that are in on-going clinical studies).

LVs are traditionally produced by transient transfection of 3–4 plasmids into adherent HEK-293 cells. Scaling up cell cultivation and LV production is pushing the industry towards adapting their HEK-293 cells to suspension cultivation and the creation of stably transduced, inducibly expressing clones. This allows manufacturers to improve their vector yields and to ease overall the complete production process.

As of today, the two main challenges faced by the industry in the production of LVs are minimizing the loss of viral particle during purification and accessing more suitable and reliable assays for viral particle quantification in terms of both total and infectious particles. In this application note we are proposing a simple, rapid and reliable solution for this second challenge.

Thanks to an important collaboration between Yposkesi and Sartorius, LVs produced via both adherent and suspension HEK-293 processes were sampled at every process step. The particles were subsequently stained and quantified with the Virus Counter® platform.

Materials and Methods

Materials

Virus Counter® 3100 platform
The Virus Counter® platform is purpose-built for the enumeration of viral particles. The instrument utilizes the principles of flow cytometry to detect viral particles in a fluid stream. Briefly, fluorescently labeled particles flow past a laser that excites the fluor associated with the viral particles. The fluorophores emit light which is detected by the instrument. The sample flow rate in the instrument is tightly controlled and measured which allows the Virus Counter® software application to precisely determine the concentration of the virus sample in virus particles/mL (vp/mL).

ViroTag® VSVG reagent – Highly specific, antibody-based detection of viruses
The ViroTag® reagent family utilizes fluorescently labeled, high-affinity antibodies that bind to a unique viral epitope. The ViroTag® VSVG reagent is highly specific for VSV-G, the most commonly protein used in pseudotyping LVs.

P24 ELISA assay
(ELISA kit NEK 050B, PerkinElmer Life Science | Synergy H1 reader, BioTek) After denaturation of the sample to release the lentivirus-associated p24 proteins, the concentration of p24 proteins is quantified by ELISA.

Infectious qPCR assay
(DNA extraction kit, Macherey-Nagel/Reagents and QuantStudio™ 7 Flex Real-Time PCR system, Thermofisher)
After cell (HCT16 cells) infection by LVs, the cell DNA is extracted and the copy number of a specific viral sequence is quantified by qPCR. The concentration of infectious virus from the initial sample is then obtained.

Flow cytometry assay
(CytoFlex S, Beckman Coulter)
The eGFP-expressing lentivirus concentration is measured by determination of the percentage of HEK293 cell expressing eGFP after infection by flow cytometry.

Method

1. Screening assay
The detection range of the Virus Counter® instrument is between 5 × 10⁵ – 1 × 10⁸ vp/mL with the most precise results obtained between 1 to 5 × 10⁷ vp/mL. For this reason, it is best to quantify samples at a dilution that produces counts close to this sweet spot. The optimal dilution can be found by screening the sample. To perform a screening assay, serial 10-fold dilutions of sample are prepared in Sample Dilution Buffer (figure 1). Typically, a dilution series of the virus sample at 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ is sufficient to determine an approximation of the virus concentration, the linear range, and matrix signal. As an optimal negative control, sample matrix, the solution in which the viruses are suspended without the virus (i.e. conditioned medium, elution buffer, formulation buffer, etc.) is diluted in an equivalent manner resulting in paired sample and matrix dilutions. In the absence of sample matrix material, a single negative control can be prepared using Sample Dilution Buffer. This control will reflect the background from the kit reagents but does not account for background due to sample matrix.

![Figure 1: Sample 10-fold dilution series](image)

2. Viral particle titer determination
Using the optimal dilution factor determined in the screening assay, stained sample and matrix dilutions are analyzed. It is recommended that a minimum of 3 replicates of sample and matrix be analyzed so that a reliable standard deviation may be calculated. Samples are stained and measured in the same manner as for the screening assay. An average titer corrected for background, a standard deviation, and a % C.V. (coefficient of variation) is calculated and reported by the application.
Results and Discussion

1. Adherent process
A transiently transfected adherent HEK-293 LV production process was followed at every process step with Virus Counter®, p24 ELISA and qPCR assays. For the Virus Counter®, the vector particles were stained with ViroTag® VSVG reagent.

Result analysis
Throughout the downstream process the quantification results of the ELISA and the Virus Counter® platform track together. Titers determined with the ELISA were around ten times higher than the titer results for the Virus Counter® platform. This discrepancy can be attributed to the different metrics these two methods quantify. P24 ELISA assays measure the amount of p24 proteins within the sample after denaturation and then calculate titers from this viral building block. In contrast, the Virus Counter® platform quantifies total virus particles in a fluidic stream by staining the particles with an antibody specific for VSV-G. It is therefore possible that the results diverge due to residual free p24 proteins in the virus sample or inconsistent amounts of target proteins in the viral envelopes.

The function of the viral particles through the adherent cell process was monitored by infectious qPCR. These numbers tracked with both ELISA and Virus Counter® titers. However, the infectious qPCR results were consistently approximately 100-fold below the p24 ELISA and 10-fold below the Virus Counter® data. This is to be expected as only a subset of LV particles will be capable of infecting a cell. What is notable here is that the ratio of non-infectious to infectious particles does not undergo much fluctuation through the process, indicating that the procedures utilized here are not affecting the functionality of the virus preparation.

2. Suspension process
A transiently transfected suspension HEK-293 LV production process of LVs was followed at every process step with Virus Counter®, p24 ELISA and qPCR assays. For the Virus Counter® assay, the samples were stained with ViroTag® VSVG reagent.

Result analysis
In this process, suspension HEK-293 were used. Interestingly in this case ELISA assay and Virus Counter® results are much more similar as seen for the adherent HEK-293 process. But the difference measured by the two assays is still relatively constant throughout the complete production process. The variation from the adherent to the suspension process may be explained by the inherent changed process parameters which is impacting the sample composition and possibly reduced the occurrence of free p24 proteins.

For this process, vector function was monitored through flow cytometry (GFP marker monitored). Here again, the infectivity results consistently track with p24 ELISA and the Virus Counter® assay. However, the functional titers seen for this process are 1000-fold below the physical titers. This indicates that the suspension cell process is generating a lower ratio of infectious LV particles vs total LV particles produced than the adherent process. However, it should be noted that the functional titer is evaluated by different methodologies for the suspension and adherent cell process.

![Figure 2: Titer determination for every unit operation of the adherent LVs production process (ELISA & ViroTag® VSVG in vp/mL, Infectious qPCR in transducing unit/mL)]
Conclusion

The Virus Counter® 3100 platform can successfully quantify total LV particles at every step for both adherent and suspension LV processes. The ViroTag® VSVG reagent provides a rapid and highly specific method to quantify total LV particle concentrations and aligns well with results obtained with standard p24 ELISA assays. The initial screen of the optimal dilution of a sample is only recommended the first time an unknown sample is analyzed. Thereafter, the quantification of LV particle, including sample preparation, read and analysis times, takes less than 1h.

The results generated in collaboration with Yposkesi demonstrate that the Sartorius Virus Counter® platform, utilizing the ViroTag® VSVG reagent, provides a reliable method for LV particle quantification, which is faster (less than 1h for Virus Counter® results vs 8 hours for the ELISA assay) and more precise than alternative methods, while delivering comparable quantification results.

These results also illustrate discrepancies between adherent and suspension processes and highlight differences between functional and non-functional viral particle titers within a production process. This information is of high value for patient safety considerations and optimization of the manufacturing process.