SARTORIUS

Application Note

January, 2022

Keywords or phrases:
lentiviral vector, infectious titer determination, CAR-T, live-cell analysis, real-time imaging, Incucyte® Live-Cell Analysis System, virus analytics, quality control

Real-Time Live-Cell Analysis of Lentiviral Titer Determination

Kalpana Barnes[1], Jennifer J. Labisch[2,3], Philip Wiese[2,4], Karl Pflanz[2]

[1] Sartorius UK, Units 2 & 3 The Quadrant, Newark Close, Royston Hertfordshire SG8 5HL United Kingdom
[2] Sartorius Stedim Biotech GmbH, August-Spindler-Str. 11, 37079 Goettingen, Germany
[3] Institute of Technical Chemistry, Leibniz University Hannover, Callinstr. 5, 30167 Hannover, Germany
[4] RWTH Aachen University, Faculty of Mathematics, Computer Science and Natural Sciences, Templergraben 59, 52062 Aachen

Introduction

Viral vectors are efficient gene delivery vehicles playing an important role for gene therapy and gene-modified somatic cell therapy products.[1] Viral vectors are used to deliver therapeutic genes into target cells. Several viral vector platforms exist including adenoviral vectors, adeno-associated vectors, and retroviral vectors, e.g. lentiviral vectors, each having its own advantages and disadvantages for specific applications. Lentiviral vectors (LV) represent the most frequently used viral gene delivery platform for the *ex vivo* generation of chimeric antigen receptor (CAR)-T cells for cancer immunotherapies.[2] Functional infectious titer determinations of viral vector samples are of major importance with vector quality control being a significant bottleneck for viral vector process development and production. To expedite the upstream and downstream development of the viral vector production process, reliable and efficient assays for their quantification are required. A method for rapid and accurate determination of lentivirus infectious titers is greatly needed for both process development and process optimization phases, where typically a high number of samples are generated.

Established and beneficial methods for infectious viral titer measurements include qPCR and flow cytometry. However, the major disadvantage is that workflows for both lack high throughput and require significant manual handling. To address notable bottlenecks of current analytical methods, we developed a novel real-time imaged based approach to quantify the infectious titer of anti-CD19 CAR lentiviral vectors using the Incucyte® Live-Cell Analysis System.

Assay Principle

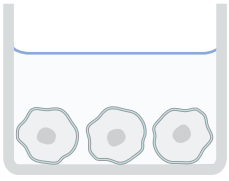
This application note describes the utility of the Incucyte® Live-Cell Analysis System in combination with Incucyte® Fabfluor-488 Antibody Labeling and Incucyte® Cytotox Dyes to enable kinetic quantification of functional lentiviral titers in live cells.

Incucyte® Fabfluor-488 Antibody Labeling Dye labels the Fc containing region of test antibodies with a green fluorophore. Once labeled, the Fabfluor-488-antibody complex is added directly to live cells in a single mix-and-read step. A suppressor of background fluorescence, Incucyte® Opti-Green, is included to minimize non-specific fluorescence from unbound Fabfluor-Antibody complexes. In the absence of expressed specific antigen, little or no signal is seen on the cells. In combination with Incucyte® Opti-Green and the Incucyte® integrated analysis software, background fluorescence is minimized, and cell surface protein expression can be quantified through an increase in green fluorescence.

Real-time measurements of cell death based on cell membrane integrity can be assessed using Incucyte® Cytotox Dyes. Dying cells are identified and quantified over time by the appearance of fluorescently labeled nuclei while viable cells remain unstained. With the Incucyte® integrated analysis software, fluorescent objects can automatically be quantified.

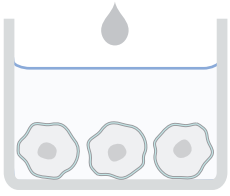
Materials and Methods

1. Seed cells (Day 1)



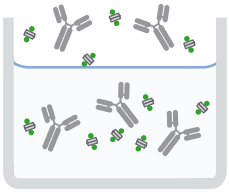
Seed adherent cells (50 µL/well) into 96-well plate. Monitor in Incucyte®.

2. Transduce cells (Day 2)



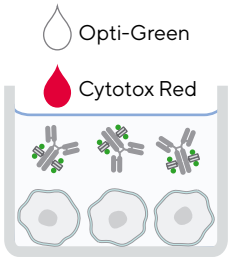
Add virus dilutions (50 µL/well) and polybrene (8 µg/mL). Monitor in Incucyte®.

3a. Label test antibody (Day 3)



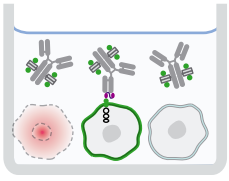
Mix antibody and Fabfluor-488 Dye at a molar ratio of 1:3 in media, 3X final concentration. Incubate for 15 minutes to allow conjugation.

3b. Add detection reagents (Day 3)



Add Opti-Green background suppressor (50 µL/well), antibody-Fabfluor mix (50 µL/well) and Cytotox Red (50 µL/well) to cell plate. Add plate to Incucyte®.

4. Live-cell fluorescent imaging (Days 3—5)



Automated imaging and quantitative analysis. Capture images in Incucyte® (10x).

Figure 1

Incucyte® Lentivirus Titer Assay Workflow

HEK293T cells were seeded (6 x 10^3 cells, 50 µL per well) into black walled 96-well poly-L-lysine coated tissue culture treated plates (Corning). On day 2, cells were transduced with a dilution series of lentiviral vector in the presence of polybrene (8 µg/mL). 24 h post-transduction, transduction mix was removed and replaced with a cocktail of detection reagents (50 µL, 2.5 µg/mL FAC Incucyte® Fabfluor-488 labeled anti-FMC63 scFv antibody 1:200 dilution, 50 µL, 0.5 mM FAC Incucyte® Opti-Green background suppressor, 50 µL, 250 nM FAC Incucyte® Cytotox Red).

Incucyte® Titer Assay Workflow

1. HEK293T cells (6 x 10^3 cells, 50 µL/well) are seeded into 96-well poly-L-lysine coated tissue culture treated plates (Corning) and cell proliferation monitored in an Incucyte® Live-Cell Analysis System(Standard scan type, 10x, 4 images per well, 2 h repeat scanning, phase, red and green channel, 1 day).

2. 24 h post cell seeding, cell culture media is removed and replaced with lentiviral vector (LV) samples (50 µL/well) diluted in culture media containing the transduction enhancer polybrene (8 µg/mL).

3. 24 h post transduction, the transduction mix is removed and replaced with a cocktail of detection reagents (50 µL Incucyte® Fabfluor-488 labeled anti-FMC63 scFv antibody, 50 µL Incucyte® Opti-Green background suppressor, 50 µL Incucyte® Cytotox Red).
   - Labeling of anti-FMC63 scFv mouse IgG1 antibody (AcroBiosystems, 1:200 final assay dilution) was performed in accordance with the following protocol:

     https://www.essenbioscience.com/en/applications/cell-monitoring-workflows/immunocytochemistry/

     Incucyte® Fabfluor-488 Dye was prepared at a final concentration of 2.5 µg/mL.
   - Incucyte® Cytotox Red Dye (250 nM) was prepared in accordance with the following protocol:

     https://www.essenbioscience.com/en/applications/cell-health-viability/cytotoxicity/

4. The plate was placed within the Incucyte® Live-Cell Analysis System and cell transduction and death monitored (Standard scan type, 10x, 4 images per well, 2 h repeat scanning, phase, red and green channel, up to 3 days).

HEK293T cells (ACC 635, DSMZ) were cultured in DMEM + 10% FCS (Sigma Aldrich). 96-well black walled clear bottom micro-titer plates (Corning) were coated with poly-L-lysine (Sigma Aldrich, 0.1 % solution, 30 µL/well) for 5 minutes followed by 3x wash steps (deionized water, 250 µL/well). All media removals and reagent additions to the 96-well assay plate were performed using a Picus® NxT 5-120 µL, 8-channel electronic pipette (Sartorius).

Third generation lentiviral vectors were produced within an Ambr® 250 Modular bioreactor system (Sartorius) via transient transfection of HEK293T/17 SF suspensions cells (ACS-4500, ATCC). Cell were transduced with four plasmids.[3]

Validation Data

Quantification of Positive Virus Transduced Cells and Non-viable Cells

For quantification of infectious titer, adherent HEK293T cells were transduced with serially diluted LV samples. The mouse monoclonal antibody FMC63 specific for CD19 was labeled with Incucyte® Fabfluor-488 Antibody Labeling Dye for detection of positively transduced cells. In combination with Incucyte® Opti-Green and the Incucyte® integrated analysis software, background green fluorescence from unbound Fab/antibody complexes was minimized.

Incucyte® Cytotox Red Dye stained the nuclei of non-viable cells either expressing or not expressing the CD19-CAR construct. Non-viable cells could therefore be excluded from infectious lentivirus titer measurements. Viable cells either non-transduced or transduced remain unstained with the Incucyte® Cytotox Red Dye.

Incucyte® high definition (HD) phase-contrast images were captured and segmented to return a value of total cell area (Figure 2A). Segmented green and red fluorescent images generated area metrics for positively transduced HEK293T cells expressing the CAR construct (Figure 2B) and non-viable, dead cells (Figure 2E) respectively.

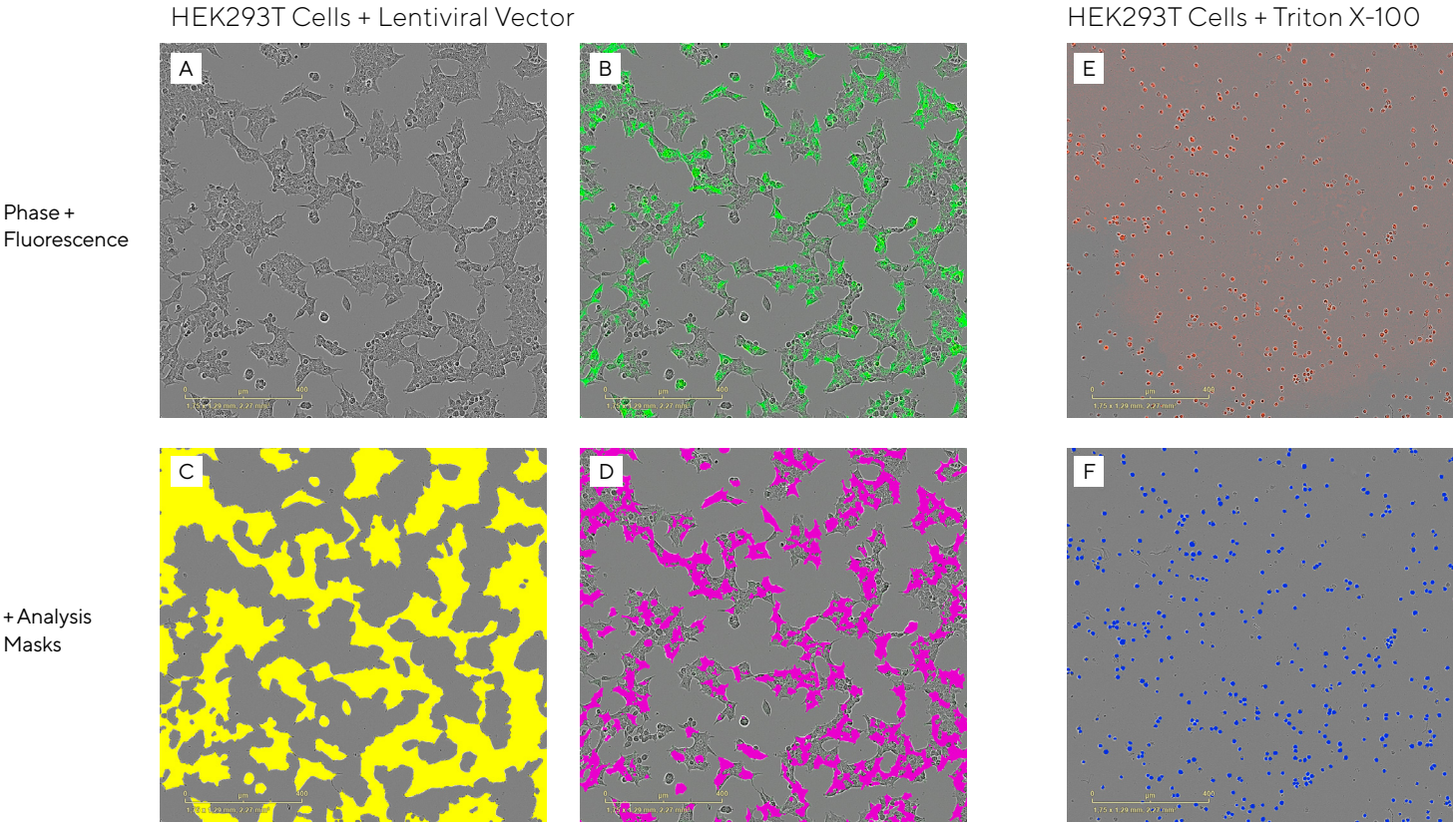


Figure 2

Real-Time Visualization and Quantification of Expressed CAR-Construct Using the Incucyte® Live-Cell Analysis System

HEK293T cells (6,000 cells/well) were transduced with lentiviral vector (1:128 dilution). Incucyte® Fabfluor-488 labeled anti-FMC63 scFv antibody was used to detect cells expressing the CAR construct. (A) Representative phase-contrast and (B) blended green fluorescence images with corresponding segmentation masks generated using Incucyte® integrated image analysis tools are shown. (C) Yellow and (D) magenta masks quantify total cell area and area of cells expressing the CAR construct, respectively. For live/dead cell quantification, cells were treated with Triton X-100 (0.005 %) in the presence of Incucyte® Cytotox Red Dye. (E) Phase-contrast and blended fluorescence image with (F) segmentation mask (blue) corresponding to area of dead cells are shown. All images at 10x magnification.

Calculating Infectious Titers

Infectious titers were calculated using the following equation:

$$\text{Titer (TU/mL)} = \frac{\mathbf{P}\ (\%) \times \mathbf{N} \times \mathbf{D}}{\mathbf{V} \times 100\ (\%)} = \frac{\mathbf{P}\text{ositive Cells (\%)} \times\ \mathbf{N}\text{umber Transduced Cells} \times \text{Virus } \mathbf{D}\text{ilution Factor}}{\text{Transduction Volume (mL)} \times 100\ (\%)}$$

Where **P** equates to the percentage of viable cells expressing the CAR construct (i.e. positive viable cells), **N** the number of cells transduced, **D** the virus dilution factor and **V** the transduction volume.

Non-viable cells were excluded from titer determinations. Non-viable cells included (1) both non-transduced dead cells stained red with the Incucyte® Cytotox Red Dye alone and (2) positively transduced but dead cells stained both green with the Incucyte® Fabfluor-488 labeled anti-FMC63 scFv antibody and red with the Incucyte® Cytotox Red Dye.

The percentage of viable cells expressing the CAR construct (i.e. positive viable cells), **P** was calculated using the following equation:

$$\% \text{ Positive viable cells } (\mathbf{P}) = \frac{\text{Positive Viable Cell Population}(\mathbf{g}\text{-}\mathbf{gr})}{\text{Total Viable Cell Population } (\mathbf{pc}\text{-}\mathbf{r})} \times 100$$

Where total viable cell population was calculated by removal of dead cells (red fluorescent area only; **r**) from total cell population (phase confluence area; **pc**).

Positive viable cell population was calculated by removal of positively transduced dead cells (green and red overlap fluorescent area; **gr**) from total positively transduced cells (green fluorescent area; **g**).

The number of transduced cells (**N**) was derived by correlating cell area (Incucyte® phase confluence metric) with the number of cells seeded per well ascertained via an offline cell count using a CEDEX HiRes® analyzer (Figure 5).

Linear Range of Detection and Assay Precision

For accurate titer determinations, it is important to ensure virus dilutions used for numerations and hence the signal from positive cells fall within the Incucyte® linear range of detection. HEK293T cells were transduced with serially diluted LV and positively transduced cells detected and quantified over time. Time-courses show positive area measurements (green fluorescent area) displayed both a time and concentration-dependency (Figure 3). For all experiments, a readout time was defined for each curve, which corresponded to the maximum signal (peak response) over the measured period.

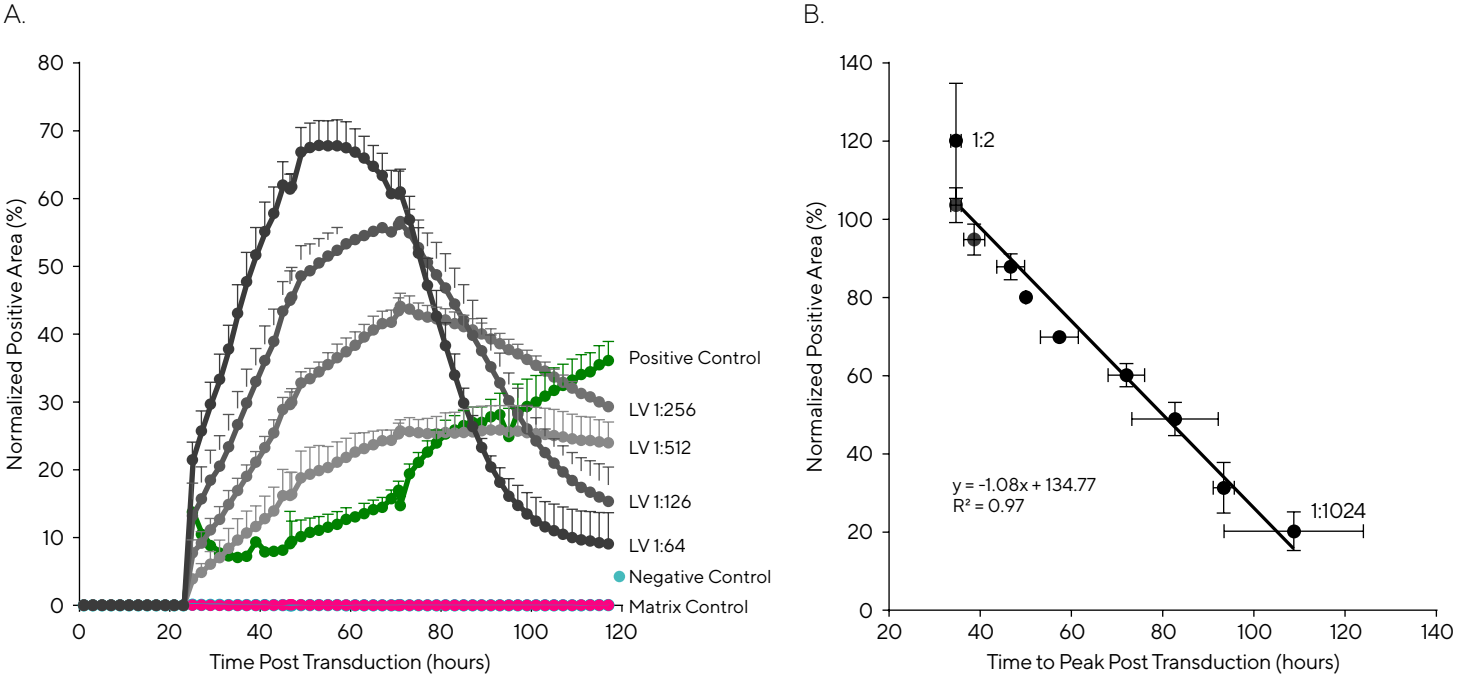


Figure 3

Time Course Series of Normalized Positive Cells

(A) Positive cell area (green fluorescent area) normalized to total cell area (phase confluence) over time after transduction. The IgG1 isotype negative control and the lentiviral vector (LV) negative control (matrix) showed no green fluorescent signal. The anti-transferrin-receptor IgG1 positive control antibody confirmed successful Incucyte® Fabfluor-488 labeling of the test antibodies. Matrix control overlaps with the negative control. Data represent the mean ± S.D. of three technical replicates. (B) Normalized Incucyte® Fabfluor-488 peak value vs. time at which this value is reached. The datapoint in the upper left with the highest normalized positive area represents the 1:2 LV dilution. The other datapoints represent two-fold serially diluted LV up to a dilution of 1:1024 with the lowest normalized positive area and the longest time after which the peak value is reached. Data represent the mean ± S.D. of three biological replicates.

A logarithmic trend (Figure 4A) and broad linear range (Figure 4B) was observed across tested dilutions (1:64 to 1:512) with an R^2 of 0.96 The lower limit of detection (LLOD), and the upper limit of detection corresponded to a positive area of 31% and 70% respectively.

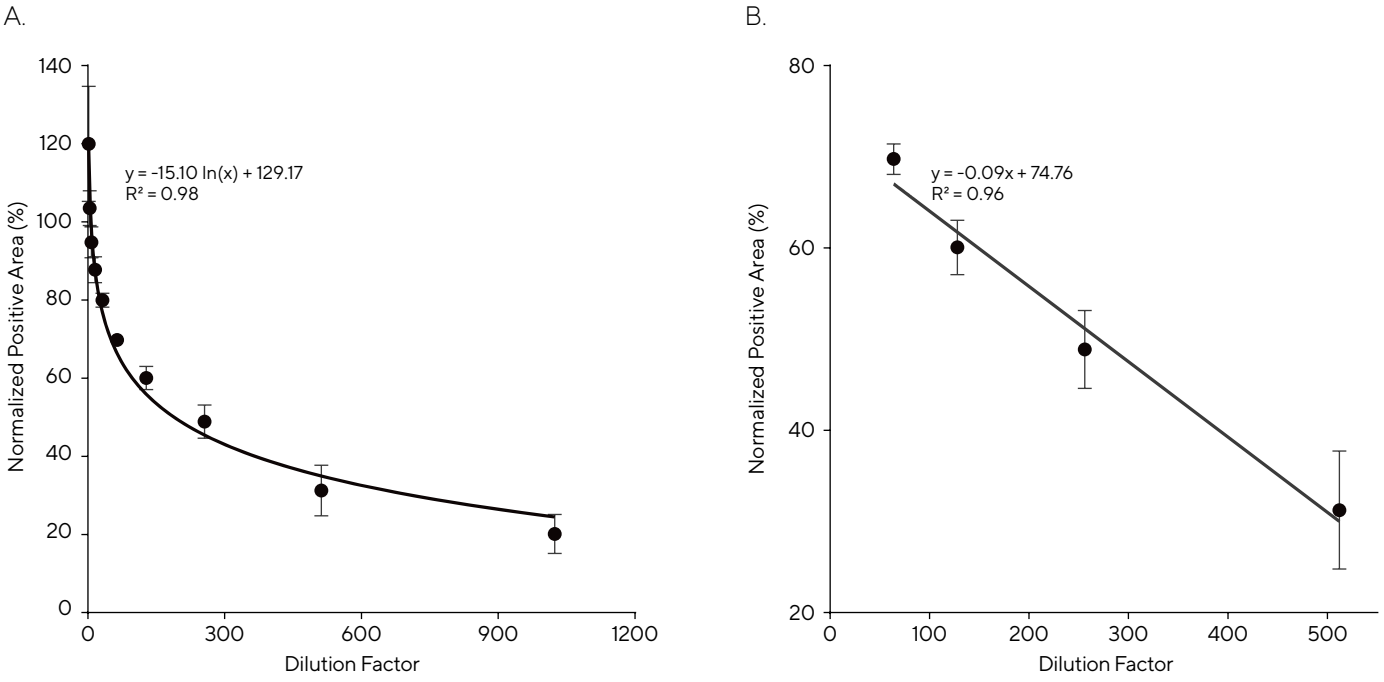


Figure 4

Linear Range of the Infectious Titer Assay

(A) Positively transduced cell area (green fluorescent area) plotted against dilution factors from a serially diluted LV. A logarithmic trend (R^2 = 0.98) was observed across the entire range of dilutions. (B) A linear dependence (R^2 = 0.96) was determined for 1:64 to 1:512 dilutions. Data represent the mean ± S.D. of three biological replicates.

Inter-assay precision between three independently performed assays was assessed using standard deviation and coefficient of variation determinations (Table 1). Day-to-day, technician-to-technician and batch-to-batch variation of the working cell bank were evaluated. The Incucyte® Titer Assay displayed good inter-assay precision (CV < 15 %) with a marginally higher (CV = 21 %) than the accepted 20 % at the LLOD (1:512 dilution).[4] Indicative of a robust assay platform amenable to screening, inter-and intra-assay Z′-factors of ≥ 0.5 were obtained for the range of tested LV dilutions (Table 1).

	Sample Dilution Factor	Mean	SD	CV (%)	Z′ Factor
Inter-assay precision[a]	2	119.97	14.74	12.29	0.58
	4	103.53	4.46	4.30	0.81
	8	94.75	3.97	4.19	0.81
	16	87.76	3.29	3.75	0.81
	32	79.94	1.77	2.21	0.85
	64	69.76	1.67	2.39	0.84
	128	60.06	3.00	4.99	0.74
	256	48.86	4.28	8.76	0.60
	512	31.23	6.47	20.70	0.15
Intra-assay precision[b]	16	75.45	2.53	3.35	0.89
	128	40.98	3.91	9.53	0.71
	512	20.79	1.85	8.91	0.72

Table 1

Inter- and Intra-Assay Precision

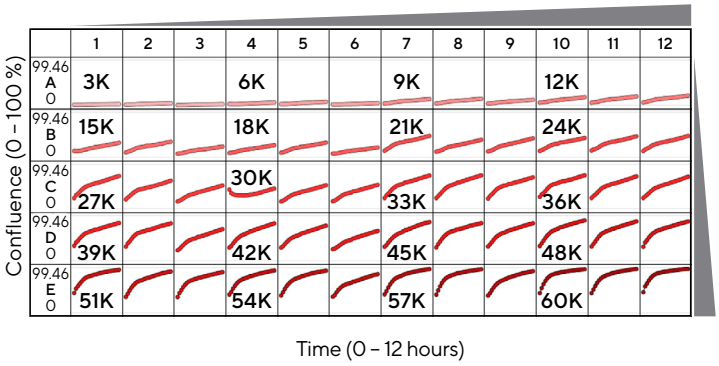
Mean of normalized positive area is given in %, standard deviation (SD), coefficient of variation (CV), and Z′-factor. [a]n = 3, [b]n = 10.

Quantification of Number of Transduced Cells

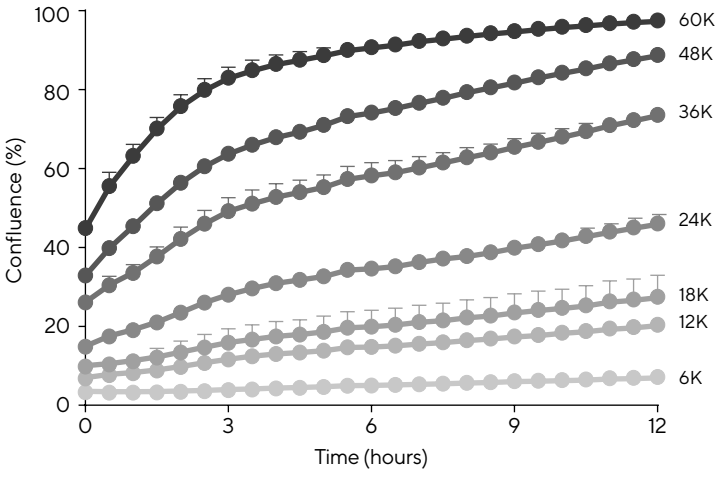
Accurate titer measurements rely on defining the number of cells being transduced. For cell types with a clear and high cell boundary contrast, the Incucyte® Cell-by-Cell Analysis Software Module allows for segmentation and classification of individual cells using area and fluorescence intensity of labeled cells. This enables identification of all populations (e.g., viable, non-viable, labeled, and non-labeled cells); the determination of cell counts at the time of transduction and the number of positive cells. Since HEK293T cells have a low cell boundary contrast, an alternative method was used. This was to correlate known cell seeding densities as determined by an off-line cell count (CEDEX HiRes®) with cell area (% confluence) measurements quantified using the Incucyte® Live-Cell Analysis System. Figure 5C shows a linear correlation between the number of cells seeded per well and the Incucyte® phase confluence metric. This standard curve could therefore be used to calculate the number of cells at time of transduction.

The inclusion of the Incucyte® Cytotox Red Dye allowed identification and exclusion of non-viable cells from titer calculations. Using the Incucyte® live-cell analysis workflow, comparable titers were derived when considering all cells (viable and non-viable) or only viable cells (Figure 5D). In the absence of procedures associated with traditional antigen/antibody detection approaches (e.g., cell centrifugation, washing and fixation steps), high cell viability (99.9 ± 0.1 %) was maintained over the whole experiment time course of the Incucyte® workflow.

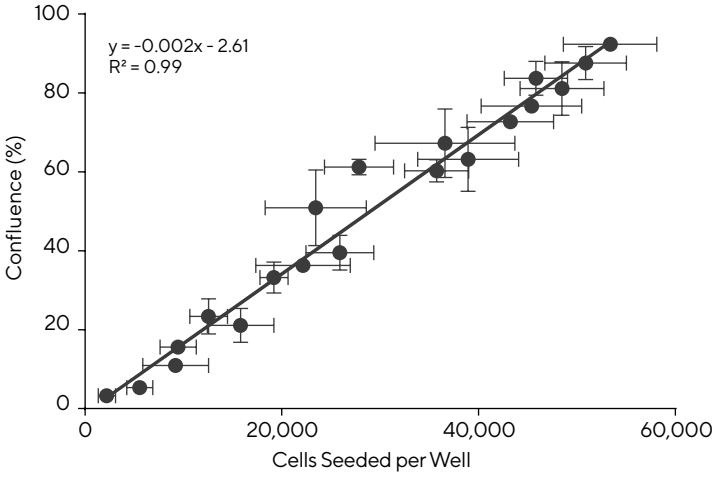
A.



B.



C.



D.

Considered Cells	Titer (TU/ mL)
All cells	$1.61 \times 10^7 \pm 0.53 \times 10^7$
Viable cells only	$1.65 \times 10^7 \pm 0.58 \times 10^7$

Figure 5

Correlation of Cell Count and Confluence

HEK293T cells were seeded (3,00 – 60,000 cells/well) in a single 96-well plate. (A) Integrated Incucyte® software allows all wells to be imaged and analyzed automatically in a microplate view of over time. Microplate view shows kinetic increases in total cell area (% confluence) across 24 cell densities seeded across triplicate wells. (B) Time-course plot reveals seeding-density dependent effect on total cell area (% confluence). (C) Total cell area (% confluence) was measured 7 h post cell seeding with the Incucyte® Live-Cell Analysis System and plotted against the number of cells seeded per well as determined by the Cedex HiRes® Analyzer. Linear regression fit applied with $R^2 = 0.99$. (D) Infectious titer numerations considering all cells (viable and non-viable) or only viable cells. All data are mean ± standard deviation of three biological replicates.

Viral Vector Stability Study

Current viral vector production methodologies often result in low and varying functional titers and depending on the production system used, often contain varying levels of impurities.[5] For use in cell therapy and clinical settings, down streaming processing (DSP) of viral vectors is often required. DSP exposes viral vectors to a range of physical conditions such as temperature, pH, conductivity, and shear stress.[5] Investigating which process parameters affect infectious titers is important to ensuring the infectivity potential of viral vectors is maintained.

The utility of the Incucyte® Titer Assay was illustrated in a stability study examining the effect of a range of physical conditions virus infectivity. LV titer was maintained over four freeze-thaw cycles enabling storage of LV between DSP steps at -80 °C for future analytical testing (Figure 6A). Figure 6B shows the temperature and time effect on titers. For ease and cost efficiencies, LV purification steps at higher temperature are preferred. In this study, 4 °C storage over time had no significant effects on the LV titer whereas at 22 °C (room temperature) and 37 °C a decline of infectious titer was observed with a half-life of 75 ± 12 h and 37 ± 12 h respectively (Figure 6B). Virus purification via ion exchange chromatography often involves the use of sodium chloride in the range of 0.5 – 1.0 M as the virus elution buffer.[5] As shown in Figure 6C, a decrease in infectious titer was attained over a NaCl concentration range of 0.6 M to 1 M with a 17 % decrease at 1 M. Virus suspensions undergo sheer stress during various DSP steps. Here we induced sheer stress by continuously pumping LV through a peristaltic pump. At a flow rate of 78.2 mL/min for a duration of 30 minutes a significant decline (17 %) in infectious titer was observed. Shorter durations or flow rates had little or no impact on infectious titer values (Figure 6D).

Our stability study assessing the effect of temperature, pH and sheer stress required titer determinations for 78 samples which using the Incucyte® workflow, were determined within one week. This rapid assessment of DSP parameters on functional infectious titers could not be achieved with an alternative flow cytometry workflow which would have required approximately 4 – 7 weeks to complete.

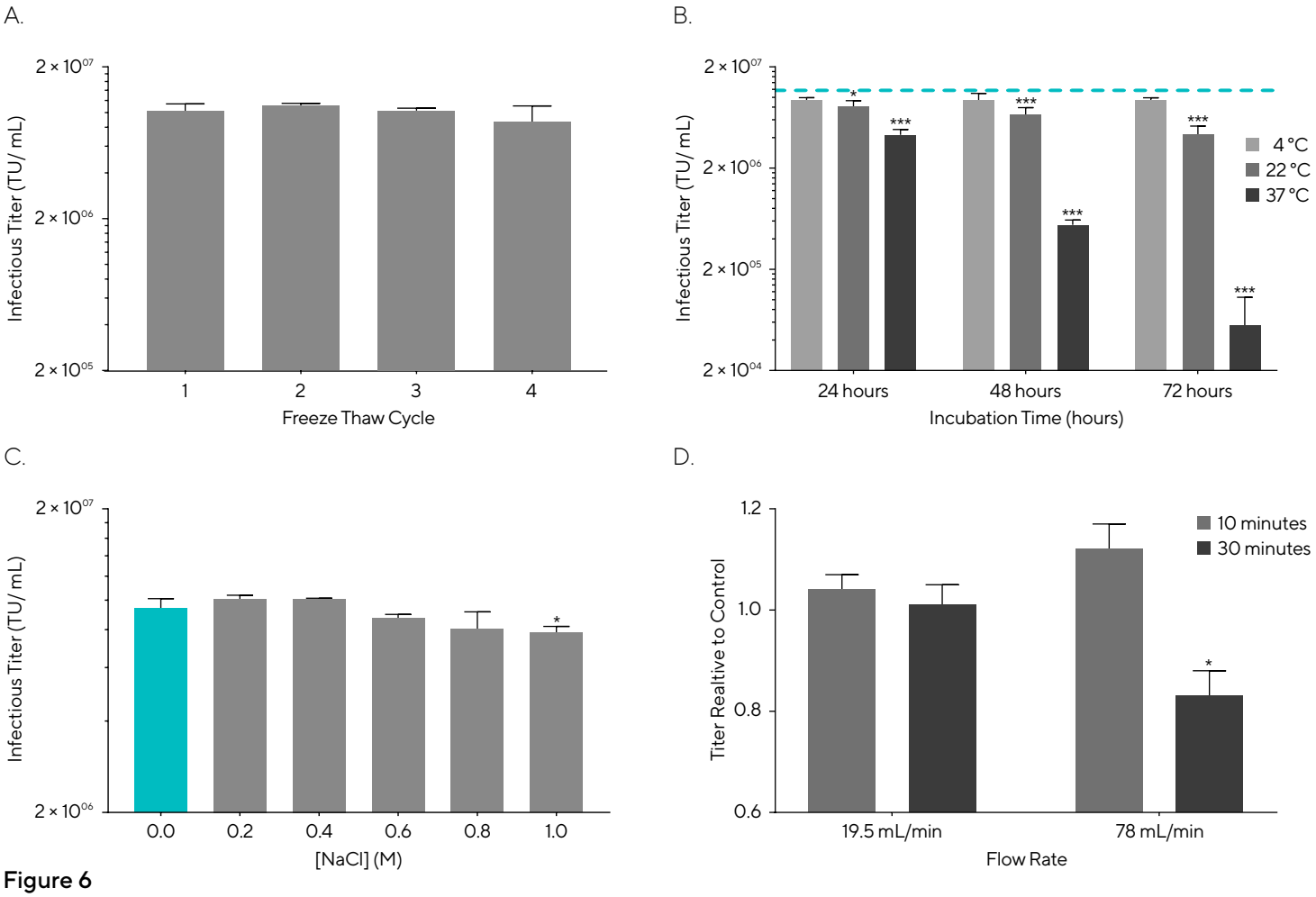


Figure 6

Assessment of External Factors on Lentiviral Vector (LV) Stability

(A) Influence of repeated freeze and thaw cycles and (B) storage at different temperatures over time. Dotted control line represents non-treated LV sample. (C) Impact of sodium chloride (NaCl) concentrations and (D) shear stress induced by a peristaltic pump. Experiments were performed in biological triplicates. Data represent the mean ± S.D. P-values indicated as: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.

Summary and Outlook

In this application note, we developed a real-time image-based method to quantify the infectious titer of anti-CD19 CAR lentiviral vectors (LV) using the Incucyte® Live-Cell Analysis System. We have demonstrated:

- A rapid, non-invasive, and simplified approach for high-throughput functional quantification of infectious titers.
- Real-time, kinetic readouts and integrated software enable tracking of viable transduced cells over-time and allow for determination of optimal readout timepoints.
- A broad linear detection range applicable for analyzing other viral vectors that transfer a gene of interest coding for an extracellular protein or a cell surface receptor.
- Real-time titer insights, in-process monitoring and optimization of lentiviral vector production.
- Use of this approach to rapidly assess viral vector stability through investigating the effect of different process parameters.
- Flexibility/applicability of assay: to determine optimal processing conditions for improved viral vector development.

Comment

Data within this application note was extracted from Labisch JJ, Wiese GP, Barnes K, Bollmann F, Pflanz K (2021). Infectious titer determination of lentiviral vectors using a temporal immunological real-time imaging approach. PLoS ONE 16(7): e0254739.
https://doi.org/10.1371/journal.pone.0254739

References

1. Hanna, E., Rémuzat, C., Auquier, P., and Toumi, M. 2016. Advanced therapy medicinal products: current and future perspectives. Journal of market access & health policy 4. https://doi.org/10.3402/jmahp.v4.31036
2. Rininger, J., Fennell, A., Schoukroun-Barnes, L., Peterson, C., and Speidel, J. 2019. Capacity analysis for viral vector manufacturing: Is there enough? *BioProcess international* 2019.
3. Labisch, J. J., Bollmann, F., Wolff, M. W., and Pflanz, K. 2021. A new simplified clarification approach for lentiviral vectors using diatomaceous earth improves throughput and safe handling. *Journal of Biotechnology*, 326, 11–20. https://doi.org/10.1016/j.jbiotec.2020.12.004
4. FDA/CDER/CVM. 2018. Bioanalytical method validation Guidance for industry. *Guidance for industry.* Accessed 11 January 2021. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry
5. Moreira AS., Cavaco DG., Faria TQ., Alves PM., Carrondo MJT., Peixoto C. Advances in Lentivirus Purification. Biotechnol J. 2020:e2000019. https://doi.org/10.1002/biot.202000019

North America
Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
USA
Phone +1 734 769 1600
Email: orders.US07@sartorius.com

Europe
Sartorius UK Ltd.
Longmead Business Centre
Blenheim Road
Epsom
Surrey, KT19 9QQ
United Kingdom
Phone +44 1763 227400
Email: euorders.UK03@sartorius.com

Asia Pacific
Sartorius Japan K.K.
4th Floor, Daiwa Shinagawa North Bldg.
1-8-11, Kita-Shinagawa 1-chome
Shinagawa-Ku
Tokyo 140-0001
Japan
Phone +81 3 6478 5202
Email: orders.US07@sartorius.com

Find out more: www.sartorius.com/incucyte

For questions, email: AskAScientist@sartorius.com

Incucyte-Titer-Assay-App-Note-en-L-Sartorius Status: 03 | 2022