Digital PCR

Lentiviral vector characterization for cell and gene therapy manufacturing using the QuantStudio Absolute Q Digital PCR System

Highlights

- Use of ViralSEQ Lentivirus Physical and Proviral DNA Titer Kits with the QuantStudio Absolute Q Digital PCR System
- Measurement of lentiviral vector titers and provirus copies in transduced cells by digital PCR
- LTR-based quantitation enabling correlation of total (genomic) and infectious viral particles

Introduction

Recombinant lentiviral vectors are used for *ex vivo* transgene delivery in several gene-modified cell therapies, such as chimeric antigen receptor (CAR) T cell therapy. Reliable methods to characterize lentiviral vectors and the cells transduced with them are critical to helping ensure the safety and efficacy of these biologics. According to regulatory guidance, viral vectors used for gene-modified cell therapies must be characterized for identity, quality, purity, and strength (potency), including particle concentration and transducing activity [1,2]. Cells transduced with lentiviral vectors must also be tested for vector integration [3].

Here we present two real-time PCR (qPCR) assays that have been adapted to the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System for reliable characterization of lentiviral vectors. The Applied Biosystems™ ViralSEQ™ Lentivirus Physical Titer Kit quantitates the total number of genome-containing lentiviral particles by targeting the conserved long terminal repeat (LTR) region of the genome. The Applied Biosystems™ ViralSEQ™ Lentivirus Proviral DNA Titer Kit measures vector integration in transduced cells while also targeting LTR in the provirus, and is used to calculate infectious viral titer. Together, these two assays enable seamless quantitation and correlation of total and infectious titers using a transgene-independent (platform) approach.

Digital PCR (dPCR) technology is increasingly being adopted in research, diagnostics, bioproduction, and environmental testing for absolute quantification of nucleic acids. In dPCR, a bulk PCR reaction is compartmentalized into thousands of nanoscale reactions, each containing zero, one, or a few DNA copies. Absolute quantification of a sample is achieved by counting positive reactions and applying Poisson statistics. This reduces variability and improves accuracy and analytical sensitivity, even in high-background conditions. There is no need to routinely run a quantitative control or a standard curve for quantification.

The QuantStudio Absolute Q Digital PCR System enables all the necessary steps for dPCR in a simple workflow similar to qPCR, thereby improving ease of use with minimal hands-on time while maximizing consistency. Here we demonstrate the use of the ViralSEQ Lentivirus Physical and Proviral DNA Titer Kits on the QuantStudio Absolute Q Digital PCR System to quantitate copies of lentiviral vectors and transduced cells, for correlation of total and infectious viral titers.

QuantStudio Absolute Q Digital PCR System

Accuracy and consistency are vital factors in providing high confidence in the detection and quantification of nucleic acids in biotherapeutic products. While dPCR is becoming a standard in precise nucleic acid quantification, many available dPCR technologies suffer from several limitations that hinder its broader adoption, such as tedious workflows, long time-to-results, and inconsistent reagent digitization, which can result in excessive sample waste.

The QuantStudio Absolute Q Digital PCR System is a plate-based platform powered by proprietary microfluidic array plate (MAP) technology and a single instrument to enable all the dPCR steps. Reaction compartmentalization, thermal cycling, and data collection are conducted on a single instrument with a simple workflow. This workflow minimizes hands-on steps to improve time-to-results, and helps reduce the risk of variability and sample contamination—both crucial in biotherapeutic product testing.

Each Applied Biosystems™ QuantStudio™ Absolute Q™ MAP16 Plate consists of 16 individual wells, allowing analysis of up to 16 samples in a single instrument run. Each well with the MAP technology enables a dPCR reaction to be easily and reliably compartmentalized into over 20,000 microchambers (Figure 1). Over 95% of the input sample is analyzed per reaction, compared to 25–60% on other dPCR platforms, improving both sample utilization and data consistency. The unique architecture of the QuantStudio Absolute Q system and the QuantStudio Absolute Q MAP16 Plate allows flexibility, including the ability to run custom dPCR assays, which is not offered by any other dPCR platform.

Quantitation by dPCR of lentiviral vector titer using the QuantStudio Absolute Q system

Lentiviral vector titer is a critical quality attribute (CQA) in vector manufacturing and can be measured by genomic quantitation (by RT-qPCR or RT-dPCR). The ViralSEQ Lentivirus Physical Titer Kit provides a 1-step RT-qPCR assay for quantitation of genome-containing lentiviral vectors produced in cell-based systems. The assay has a broad dynamic range and provides high target specificity with Applied Biosystems™ TaqMan™ Assay–based detection. For this study, lentiviral vectors were produced, and genome-based viral titers were measured on the QuantStudio Absolute Q Digital PCR System using the ViralSEQ Lentivirus Physical Titer Kit, as outlined in Figure 2.

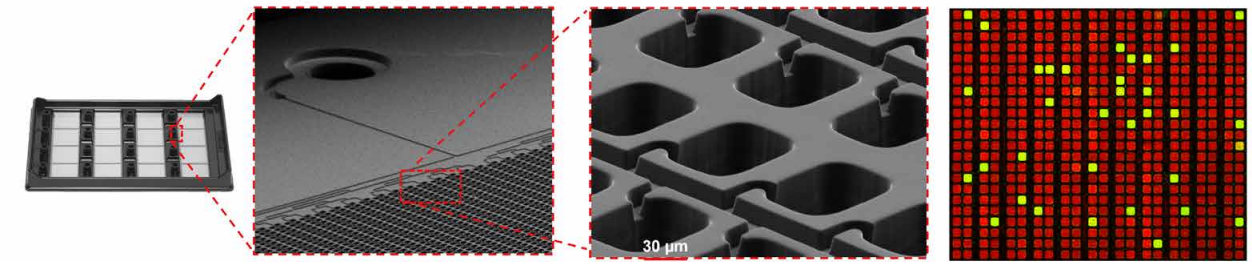


Figure 1. QuantStudio Absolute Q MAP16 Plate.

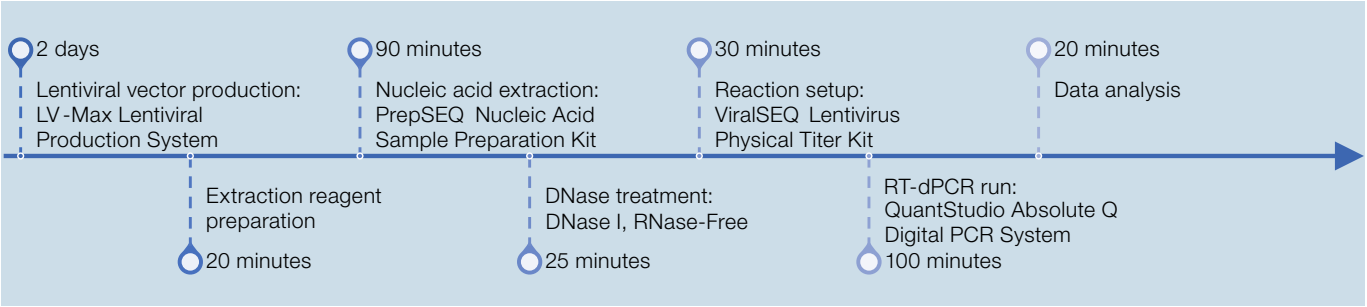


Figure 2. Sample preparation and assay workflow for physical titer quantitation.

Methods

Lentivirus production and sample prep

Two lentiviral vectors, LV-GFP (sample A) and LV-antiCD19-CAR-V5 (sample B), were generated in culture using the Gibco™ LV-MAX™ Lentiviral Production System as outlined in the manual, at the 125 mL shake flask scale. At 48 hours post-transfection, cultures were centrifuged for 15 min at 1,300 x *g*, and the supernatants were collected and divided into aliquots.

Nucleic acid was extracted from the lentiviral vectors in the supernatants using the Applied Biosystems™ PrepSEQ™ Nucleic Acid Sample Preparation Kit and quantified using an Applied Biosystems™ NanoDrop™ One^{C} Microvolume UV-Vis Spectrophotometer (Table 1).

Table 1. Quantitation of total nucleic acid extracted from viral vector samples.

Lentiviral vector	Sample	Nucleic acid concentration (ng/µL)
LV-GFP	A	7.073
LV-antiCD19-CAR-V5	B	12.809

DNase treatment and dilutions

Nucleic acid extracts were treated with RNase-free DNase I for 10 minutes at 37°C. DNase I was inactivated by adding 2 µL of 50 mM EDTA, followed by a 10-minute incubation at 75°C. The DNase-treated extracts, noted as samples A and B, were then diluted using RNA dilution buffer (RDB) per Table 2.

Table 2. Dilution series for lentiviral vector stock samples A and B.

Dilution	Template	Template volume (µL)	Diluent	Diluent volume (µL)	Total volume (µL)	Serial dilution factor
D1	Stock	5	RDB	245	250	50
D2	D1	5	RDB	120	125	1,250
D3	D2	100	RDB	100	200	2,500
D4	D3	100	RDB	100	200	5,000
D5	D4	100	RDB	100	200	10,000
D6	D5	100	RDB	100	200	20,000

RT-dPCR setup and run

Dilutions D2–D6 (noted in Table 2) for each sample (A and B) were used for viral titer quantification by 1-step reverse-transcription dPCR (RT-dPCR). The reaction mixture for each sample was prepared following the standard protocol shown in Table 3. The ViralSEQ Lentivirus Physical Titer Kit contains a physical titer assay mix, physical titer RNA control (positive control), and nuclease-free water (no-template control). Additional reagents required to run the dPCR assay include the QuantStudio Absolute Q MAP16 Plate Kit, Applied Biosystems™ Absolute Q™ 1-Step RT-dPCR Master Mix, and isolation buffer.

Table 3. dPCR setup.

Component	Volume per reaction with 10% overage (µL)
Absolute Q 1-Step RT-dPCR Master Mix (4X)	2.5
Physical titer assay mix	2
Nuclease-free water	3.5
Lentiviral vector dilution	2
Total volume	10

A volume of 9 µL of each reaction mixture was loaded into a single well of the QuantStudio Absolute Q MAP16 Plate, followed by an overlay of 15 µL of isolation buffer. Samples were loaded in triplicate on the QuantStudio Absolute Q MAP16 Plate, along with extraction controls (1X PBS), positive control, and no-template control. Plates were then loaded onto the QuantStudio Absolute Q Digital PCR System. The following thermal parameters were used for the dPCR run (Figure 3): 55°C for 10 min for RT, followed by a 96°C hold for 10 min, then 40 cycles of denaturation at 96°C for 5 sec and annealing and extension at 60°C for 10 sec. The channel for Applied Biosystems™ FAM™ dye was used to collect the data for the lentiviral target, and the channel for Applied Biosystems™ VIC™ dye was used to collect the data for the internal positive control (IPC).

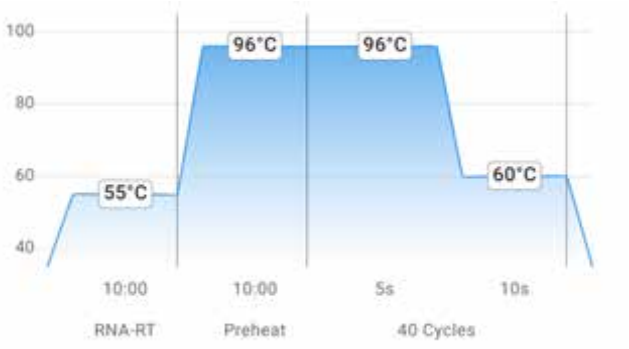


Figure 3. Thermal parameters for dPCR reactions.

Physical titer results

Concentration determined by absolute quantitation was consistent with the dilution factors used on the two starting templates (LV-GFP and LV-antiCD19-CAR-V5). The density of the positive population decreased with progressive dilution (Figure 4), and the dilution series of each target showed high linearity (Figure 5). Table 4 highlights the actual concentrations determined by the QuantStudio Absolute Q system, and the adjusted concentrations in copies/mL and viral particles/mL. Furthermore, after accounting for the serial dilution factors, the concentrations were consistent, reflecting the high precision and accuracy of the assay.

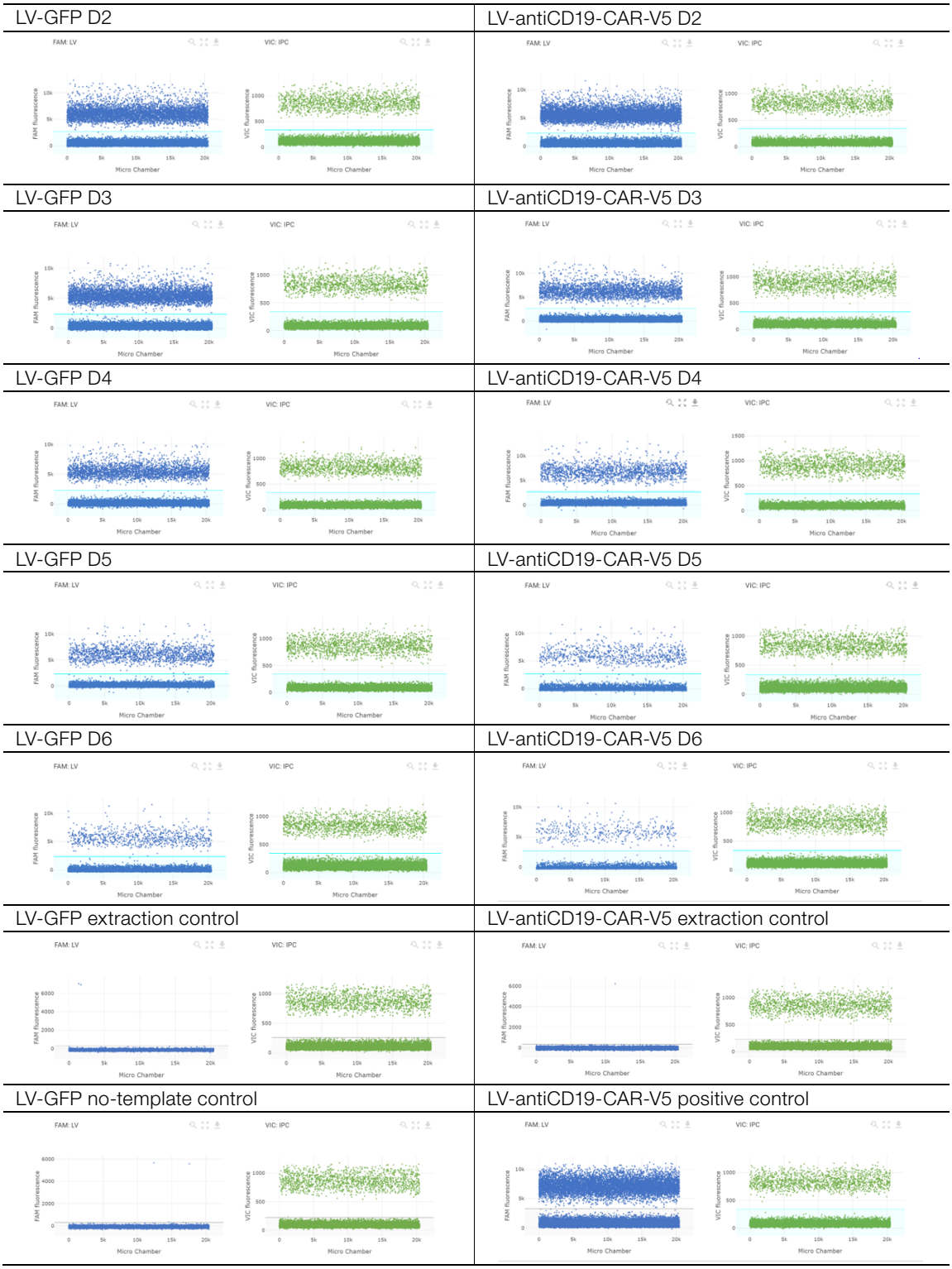
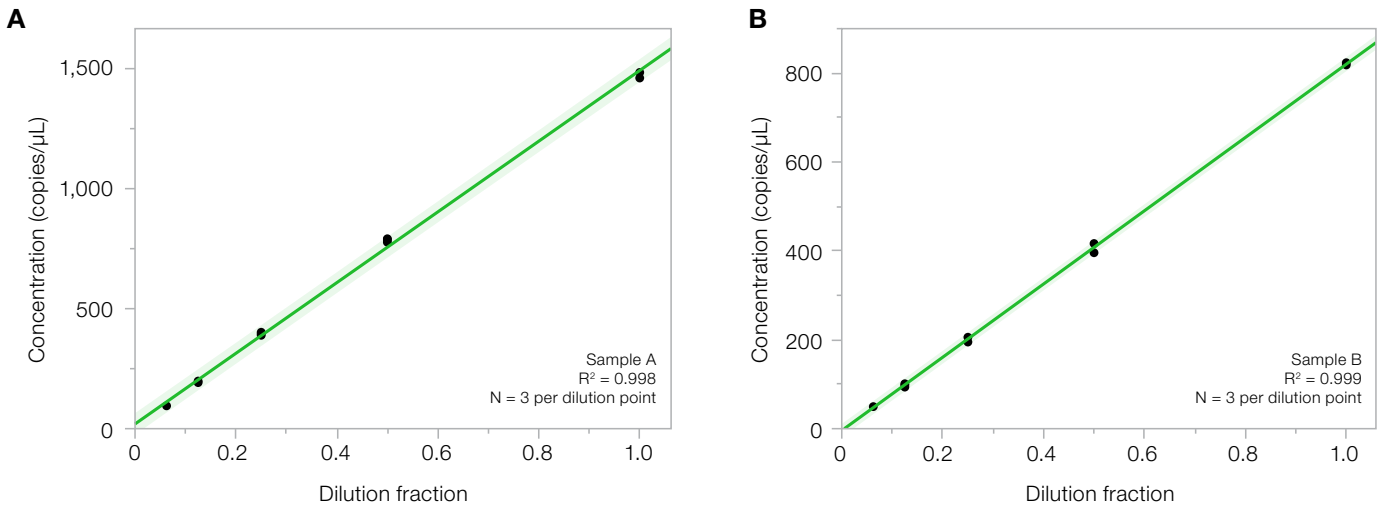


Figure 4. One-dimensional dPCR fluorescent plots for lentiviral dilution series generated according to Table 2. Fluorescence intensity of all microchambers for a given sample is plotted. All plots show a threshold line; signals above the threshold line are counted as positives.



Sample	A (LV-GFP)	B (LV-antiCD19-CAR-V5)
R^2	0.998	0.999

Figure 5. Linearity of dilutions. The observed concentrations corresponded correctly to dilution factors for both **(A)** LV-GFP and **(B)** LV-antiCD19-CAR-V5. The dilution series was tested across 5 concentrations, with three replicates per concentration. The R^2 values demonstrate the precision of absolute quantification across this range.

Calculation of physical titer

1. Total dilution factor = extraction dilution x DNase dilution x serial dilution x reaction dilution
   E.g., for sample A, dilution D2 → total dilution factor = 2 x 2 x 1,250 x 5 = 2.5×10^4
2. LV stock in copies/mL = average dPCR read x total dilution factor x 1,000 µL/mL
3. LV physical titer in VP/mL = LV stock ÷ 2

Table 4. Quantitative results of dPCR physical titer assay across the dilution series. The total dilution factor was calculated using equation 1, then leveraged in equations 2 and 3 using the average dPCR concentration determined by QuantStudio Absolute Q dPCR software, to determine the lentiviral stock concentration and the physical titer. NA: not applicable.

LV sample	Dilution	Average dPCR read (copies/µL)	CV of replicates	Total dilution factor	LV stock (copies/mL)	LV physical titer (VP/mL)
A (LV-GFP)	D2	1,479.29	0.97%	2.5×10^4	3.70×10^{10}	1.85×10^{10}
	D3	786.21	0.97%	5.0×10^4	3.93×10^{10}	1.97×10^{10}
	D4	395.99	1.60%	1.0×10^5	3.96×10^{10}	1.98×10^{10}
	D5	196.73	2.05%	2.0×10^5	3.93×10^{10}	1.97×10^{10}
	D6	96.57	1.76%	4.0×10^5	3.86×10^{10}	1.93×10^{10}
B (LV-anti CD19-CAR-V5)	D2	822.21	0.43%	2.5×10^4	2.06×10^{10}	1.03×10^{10}
	D3	403.06	2.91%	5.0×10^4	2.02×10^{10}	1.01×10^{10}
	D4	202.61	3.33%	1.0×10^5	2.03×10^{10}	1.01×10^{10}
	D5	97.50	3.91%	2.0×10^5	1.95×10^9	9.75×10^9
	D6	50.56	1.82%	4.0×10^5	2.02×10^{10}	1.01×10^{10}
Positive control	NA	1,368.66	4.47%	NA	NA	NA
Extraction control	NA	0	NA	NA	NA	NA
No-template control	NA	0	NA	NA	NA	NA

Proviral DNA titer

The titers of infectious lentiviruses and integrated vectors (proviruses) in transduced cells are critical quality attributes for gene-modified cell therapy products. Infectious viral titer can be calculated from numbers of proviruses measured by genomic assays (qPCR or dPCR). The ViralSEQ Lentivirus Proviral DNA Titer Kit quantitates proviruses in transduced cells. The assay provides excellent sensitivity and high specificity for proviral DNA using TaqMan Assay–based detection. For this study, cells were transduced with lentiviral vectors, and proviruses were counted on the QuantStudio Absolute Q Digital PCR System using the ViralSEQ Lentivirus Proviral DNA Titer Kit, as outlined in Figure 6. Subsequently, infectious viral titers for the two lentiviral vector samples were calculated.

Methods

Lentiviral transduction of cells and sample prep

Gibco™ Viral Production Cells derived from the HEK293 cell line were seeded at 5 x 10^5 cells/well in a 6-well plate in a 2 mL volume. Cells were transduced with serially diluted lentivirus sample A (LV-GFP) at MOIs of 2.00, 0.20, and 0.02, and sample B (LV-antiCD19-CAR-V5) at MOIs of 1.00, 0.10, and 0.01. Infected cells and untransduced cells (negative control) were harvested 48 hours post-infection and pelleted by centrifugation.

Nucleic acid was extracted from cells using the PrepSEQ Nucleic Acid Sample Preparation Kit and quantified using the NanoDrop One^C Spectrophotometer and an Invitrogen™ Qubit™ DNA assay (Table 5).

dPCR setup and run

Proviral copies were measured by dPCR for cells transduced with lentivirus samples A and B at the specified MOI. The reaction mixture for each sample was prepared following the standard protocol as shown in Table 6. The ViralSEQ Lentivirus Proviral DNA Titer Kit contains a proviral titer DNA assay mix, proviral titer DNA control (positive control), and nuclease-free water (no-template control). Additional reagents required to run the dPCR assay include the QuantStudio Absolute Q MAP16 Plate Kit, Applied Biosystems™ Absolute™ Q DNA Digital PCR Master Mix, and isolation buffer.

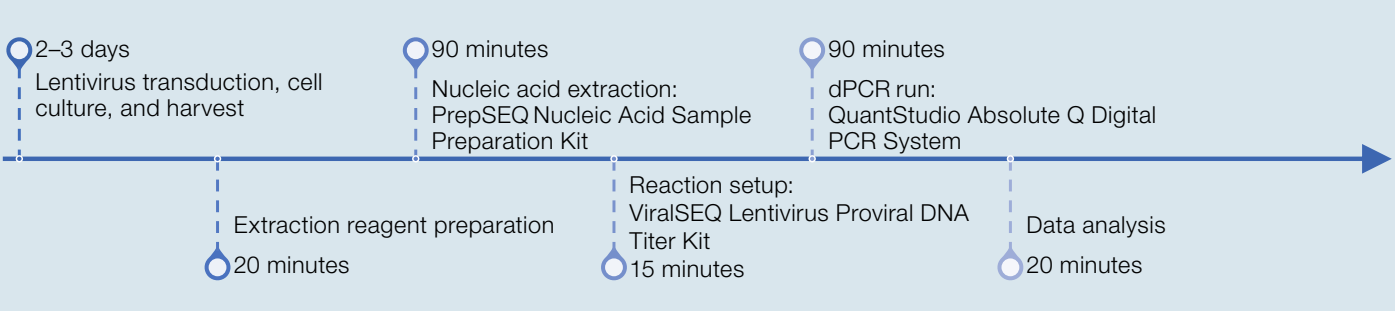


Figure 6. Sample preparation and assay workflow for proviral DNA titer quantitation.

Table 5. Quantitation of total nucleic acid extracted from transduced cells.

Cell line	LV sample	MOI	Sample name	Nucleic acid concentration from NanoDrop Spectrophotometer (ng/µL)	DNA concentration via Qubit assay (ng/mL)
Viral Production Cells	A	2.0	A_2.0	11.931	252
		0.2	A_0.2	10.593	116
		0.02	A_0.02	18.690	466
	None	Untransduced	A_X	13.272	60.8
Viral Production Cells	B	1.0	B_1.0	18.596	288
		0.1	B_0.1	23.058	353
		0.01	B_0.01	22.377	525
	None	Untransduced	B_X	19.907	27.9

A volume of 9 µL of each reaction mixture was loaded into a single well of the QuantStudio Absolute Q MAP16 Plate, followed by an overlay of 15 µL of isolation buffer. Samples were loaded in triplicate on the QuantStudio Absolute Q MAP16 Plate, along with extraction controls (1X PBS), positive control, and no-template control. Plates were then loaded onto the QuantStudio Absolute Q Digital PCR System. The following thermal parameters were used for the dPCR run (Figure 7): 96°C hold for 10 min, followed by 40 cycles of denaturation at 96°C for 5 sec and annealing and extension at 60°C for 15 sec. The channel for FAM dye was used to collect the data for the lentiviral target, and the channel for VIC dye was used to collect the data for the internal positive control (IPC).

Table 6. dPCR setup.

Component	Volume per reaction with overage (µL)
Digital PCR Master Mix (5X)	2
Proviral DNA titer assay mix	2
Cell extract	6
Total volume	10

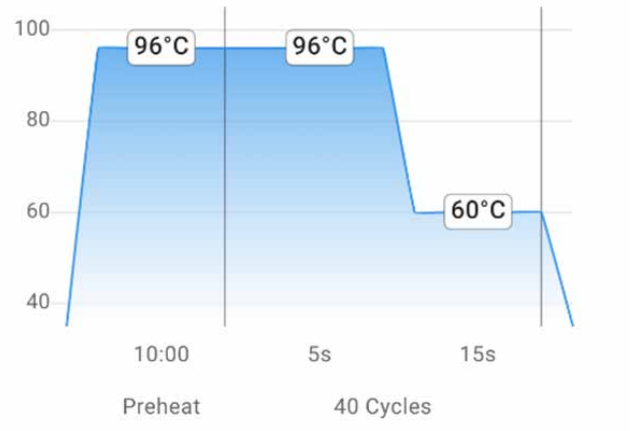


Figure 7. Thermal parameters for dPCR reactions.

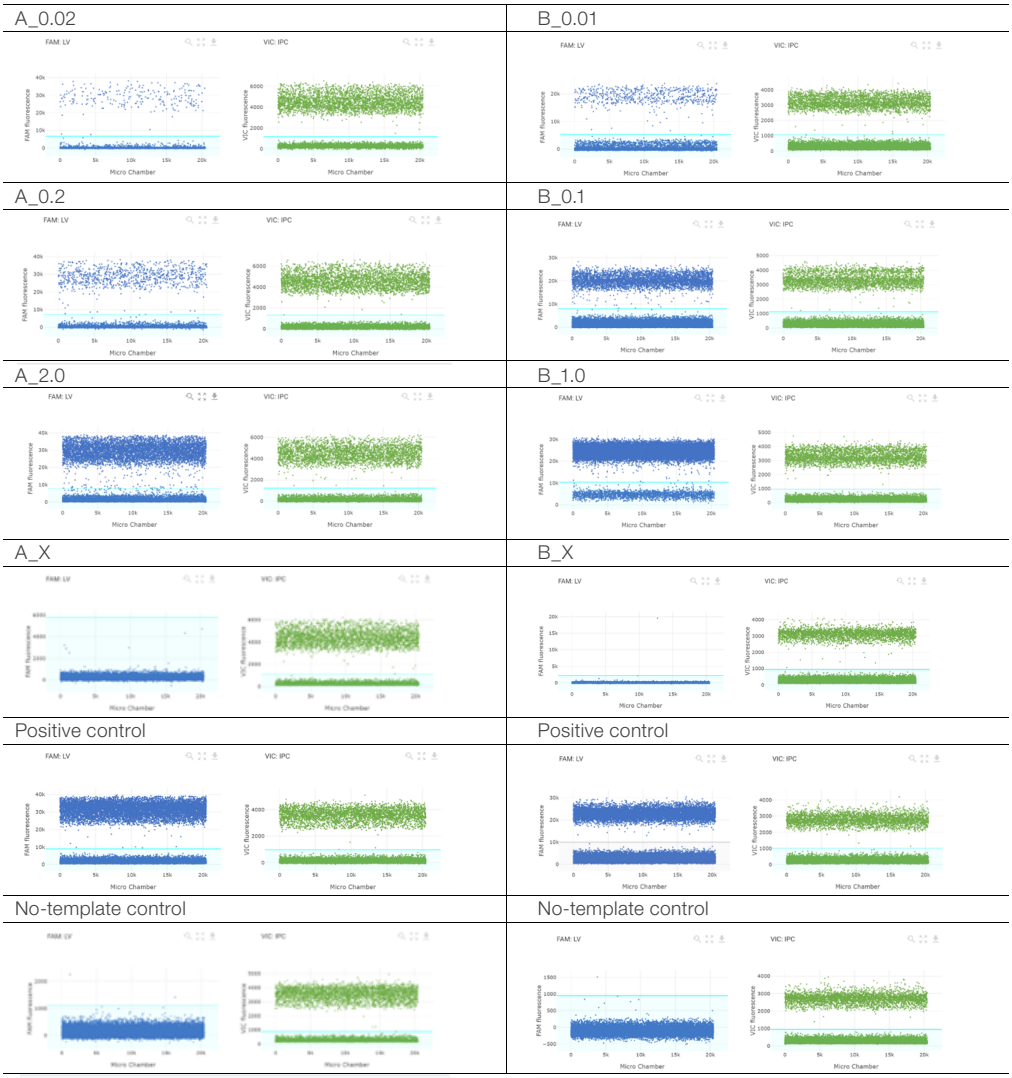


Figure 8. One-dimensional dPCR fluorescent plots for lentiviral dilution series generated according to Table 5. Fluorescence intensity of all microchambers for a given sample is plotted. All plots show a threshold line; signals above the threshold line are counted as positives.

Proviral titer results

The concentrations (Figure 8 and tables below) are consistent with the dilution factors leveraged for this experimental setup. Both the positive and negative controls for the proviral target (FAM) show signal and lack of signal, as anticipated. Based on the dPCR quantitation, the concentration of the proviral copies in the sample can be derived (Table 7). The proviral copies per cell, the multiplicity of infection (MOI; Table 8) and infectious viral titer can be calculated accordingly (Table 9). Information of the physical viral titer and the infectious viral titer provides insights to the efficiency of the viral packaging and infection process.

Calculation of number of proviral copies

1. Total dilution factor = extraction dilution x reaction dilution
   E.g., for sample A_0.02 → total dilution factor = 2 x 1.67 = 3.34
2. Number of cells = DNA concentration (by Qubit assay) ÷ 6.6 pg/cell
3. Proviral copies per cell = (proviral copies per µL x total dilution factor) ÷ number of cells harvested
4. Infectious titer = (proviral copies per cell x number of cells seeded) ÷ volume of LV used

Table 7. Concentration of proviral copies (in copies/µL) measured by dPCR and calculated proviral copy concentration in sample. The total dilution factor was calculated using equation 1, then leveraged in equations 2–4 using the average dPCR concentration determined by QuantStudio Absolute Q dPCR software, to determine the concentration of proviral copies.

Sample	Description	Average dPCR read (copies/µL)	CV of replicates	Total dilution factor	Proviral copies per µL
A (LV-GFP)	MOI 0.02	25.6	6.4%	3.34	85.3
	MOI 0.2	102.5	2.9%	3.34	341.7
	MOI 2.0	948.1	0.8%	3.34	3,160.3
Negative control	Untransduced cells	0.0	NA	NA	NA
Positive control	DNA control	1,352.0	0.0%	NA	NA
Extraction control	1X PBS	0.1	NA	NA	NA
No-template control	Nuclease-free water	0.2	NA	NA	NA
B (LV-antiCD19-CAR-V5)	MOI 0.01	74.0	1.6%	3.34	246.7
	MOI 0.1	625.4	2.1%	3.34	2,084.6
	MOI 1.0	5,193.4	0.5%	3.34	17,311.5
Negative control	Untransduced cells	0.1	NA	NA	NA
Positive control	DNA control	1,387.3	1.5%	NA	NA
Extraction control	1X PBS	0.1	NA	NA	NA
No-template control	Nuclease-free water	0.1	NA	NA	NA

Table 8. Infectious titer measurements across the dilution series.

Sample	Proviral copies per µL	Number of cells per µL	Proviral copies per cell	Number of cells seeded	LV used for transduction (mL)	Infectious titer (TU/mL)
A_0.02	85.3	70.61	1.21	5×10^5	0.2	3.03×10^6
A_0.2	341.7	17.58	19.47	5×10^5	0.2	4.87×10^7
A_2.0	3,160.3	38.18	82.94	5×10^5	0.2	2.07×10^8
B_0.01	246.7	79.55	3.11	5×10^5	0.2	7.77×10^6
B_0.1	2,084.6	53.48	39.06	5×10^5	0.2	9.76×10^7
B_1.0	17,311.5	43.64	397.48	5×10^5	0.2	9.94×10^8

Table 9. Dilution-adjusted infectious titers.

Sample	Infectious titer (TU/mL)	Dilution factor	Infectious titer (TU/mL)
A_0.02	3.03×10^6	1,000	3.03×10^9
A_0.2	4.87×10^7	100	4.87×10^9
A_2.0	2.07×10^8	10	2.07×10^9
B_0.01	7.77×10^6	1,000	7.77×10^9
B_0.1	9.76×10^7	100	9.76×10^9
B_1.0	9.94×10^8	10	9.94×10^9

Table 10. Comparing physical and proviral DNA titers.

Sample	Physical titer (VP/mL)	Proviral DNA titer (TU/mL)
A	1.94×10^{10}	3.32×10^9
B	1.01×10^{10}	9.16×10^9

Conclusions

ViralSEQ Lentivirus Titer Kits are robust assays for characterization of lentiviral vectors and enable seamless quantitation and correlation of total and infectious titers using a transgene-independent (platform) approach. Here we demonstrated the feasibility of adopting these assays to the QuantStudio Absolute Q Digital PCR System with relevant Absolute Q master mixes.

Quantitation of both lentiviral vector titer and proviral copy numbers were performed without the need for a standard curve. For all PCR runs, there was clear separation between positive and negative signals, suggesting no PCR inhibition and good specificity of the assay.

The ViralSEQ Lentivirus Physical Titer Kit showed excellent linearity across serial dilutions of the two lentiviral vector samples as measured on the QuantStudio Absolute Q system. Measured replicates had high precision (CV <5%) for all dilution points tested.

The ViralSEQ Lentivirus Proviral DNA Titer Kit demonstrated a good correlation of number of proviral copies per cell with the level of MOI in two different lentiviral vectors. Replicates showed low variation (CV <7%) as measured on the QuantStudio Absolute Q system.

Comparison between physical titer (VP/mL) and proviral DNA titer (TU/mL) data provides the necessary readout for optimizing lentivirus preparation and transduction.

In summary, we have shown that the easy-to-use ViralSEQ Lentivirus Titer Kits, combined with the sensitivity and accuracy of the QuantStudio Absolute Q Digital PCR System, further enable regulatory compliance without sacrificing time and resources.

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