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Universal ddPCR-based assay for the determination of lentivirus infectious titer and lenti-modified cell vector copy number

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The translation of cell-based therapies from research to clinical setting requires robust analytical methods that successfully adhere to current good manufacturing practices and regulatory guidelines. Lentiviral vectors are commonly used for gene delivery to generate genetically modified therapeutic cell products. For some cell therapy products, standardized characterization assays for potency and safety have gained momentum. Translational applications benefit from assays that can be deployed broadly, such as for lentiviral vectors with various transgenes of interest. Development of a universal method to determine lentivirus infectious titer and vector copy number (VCN) of lenti-modified cells was performed using droplet digital PCR (ddPCR). Established methods relied on a ubiquitous lenti-specific target and a housekeeping gene that demonstrated comparability among flow cytometry-based methods. A linearized plasmid control was used to determine assay linearity/range, sensitivity, accuracy, and limits of quantification. Implementing this assay, infectious titer was assessed for various production runs that demonstrated comparability to the flow cytometry titer. The ddPCR assay described here also indicates suitability in the determination of VCN for genetically modified CAR-T cell products. Overall, the development of these universal assays supports the implementation of standardized characterization methods for quality control.

INTRODUCTION

Clinical relevance

Clinical applications of cell and gene therapies have greatly expanded in recent decades. Those applications and early clinical successes have led to the approval of the commercial products Kymriah, Yescarta, Tecartus, Breyanzi, Abecma, and Carvykti in the United States.[1] These cellular therapies have unlocked novel medical treatments for clinicians and their patients and have thus far demonstrated effective safety profiles and remarkable clinical outcomes.[2] As a part of this process, streamlined workflows for chimeric antigen receptor (CAR) T cell generation and characterization offer reduced timelines and standardized manufacturing approaches. At an estimated 80,000 new cases of non-Hodgkin's lymphomas per year,[3,4] various CAR-T cell therapies are currently in large-scale clinical trials, with two products approved by the Food and Drug Administration (FDA).[3] These cell therapies utilize the common CD19 antigen target present on normal and malignant B cells. Overall, approved CAR-T products range from targeting various aggressive hematological malignancies including acute lymphoblastic leukemia, relapsed/refractory large B cell lymphoma, and multiple myeloma.[5] Whereas small-molecule drugs or other pharmaceutical treatment assist with disease management, cell and gene therapies deliver long-lasting therapeutic outcomes via autologous or emerging allogeneic approaches.[2]

Currently, CAR-T cell generation workflows involving lentivirus (LV) necessitate characterization benchmarks for both the LV vector and the cell product following *ex vivo* or *in vivo* modification. Characterization assays such as lentivirus infectious titer and CAR-T cell safety are key to understanding downstream clinical effects and dose dependence. The assay described herein addresses both aspects and provides utility in quality control. A key advantage of this assay is the universal aspect that can be utilized in both droplet digital PCR (ddPCR) and qPCR measurement systems.

CAR-T cell production workflows require highly characterized lentivirus vectors that have been tested via infectious titer assays for subsequent control of multiplicity of infection (MOI) during transduction. Whereas total viral particle titer is often quantified directly by PCR, infectious titer identifies integrated LV proviral DNA copies in the genome of cells following transduction.[6,7] The precise and accurate measurement of these integration events into the host genome provides insight into gene transfer efficiency and potential therapeutic efficacy.

Vector copy number (VCN), an assay that quantifies the copy number of integrated viral genomes into the host cell genome, is expected by the FDA to have on average fewer than five copies per cell.[8] High or excessive copy numbers of the transgene vector post-transduction has the potential for genotoxicity and may increase the possibility of

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transgene integration near oncogenes.[7] Simultaneously, the copy number of integrated vector genomes into the cell genome is related to clinical potency of the cell therapy. A balance between product potency and safety is required for the delivered vector genome copies.

Autologous CAR-T therapy manufacturing encounters the inconsistent growth and purity of a patient's T cells that are compromised by the disease.[9] The current CAR-T cell manufacturing process encompasses approximately 24 days from leukapheresis to lentiviral vector transduction, cell expansion, cryopreservation, and product shipment.[10] Next-generation CAR-T manufacturing aims to reduce manufacturing timelines to 7 days or less.[11] Regardless of the manufacturing process, quality control testing of the final cell product is required to ensure a safe, potent, and pure product is generated. Because manufacturing methods are not standardized, robust VCN methods are further crucial to ensure consistent release testing of the functional cell therapy product.[8]

Infectious titer and VCN technology expansion

Based on the clinical impact and motivation to streamline infectious titer testing and post-transduction VCN determination, several technological advancements have been implemented.

Lentivirus titer techniques have expanded upon physical titer methods to include functional measurements of LV proviral DNA integration. Physical titer procedures include direct testing of LV preps via p24 antigen ELISA, genomic RNA concentration by RT-qPCR, and reverse transcriptase assessment.[12,13] Alternatively, an emerging method based on real-time image analysis of a reporter gene has been tested for CD19-targeting CAR lentivirus.[14]

For a post-transduction VCN assay, bulk methods involve the assessment of a heterogeneous population of the cell therapy product. For cell therapies, transduction efficiency is dependent on the donor, transduction modality (viral or non-viral), potency of viral vector, and additional processing steps. Advances in automated manufacturing methods aim to improve CAR-T cell production and therapeutic outcomes.[2] Based on transduction efficiency and cell-to-cell variability in distribution of vector copies, bulk analysis can be representative of the entire cell population. A common analysis implements vector-specific targets where primer and probe sets are customized for each vector, and measurement includes qPCR, ddPCR, and digital PCR (dPCR).[12,15] ddPCR is a third-generation PCR technology that employs water-oil emulsion droplet technology to provide absolute quantification of a DNA target without the need for a standard curve. Here, approximately 20 μL of sample is partitioned into 20,000 self-contained droplets for endpoint PCR reactions. Following thermal cycling, these droplets are separated and measured for fluorescent intensity. Then, using Poisson statistics, the distribution of PCR positive and negative droplets is calculated to determine the target copy number of an unknown sample.[16] Commonly, the human albumin gene (ALB) has been utilized as a housekeeping gene for normalization coupled with the vector-specific WPRE sequence[17] in addition to Rev response element (RRE) and ribosomal protein L32 (RPL32) reference genes.[13]

Use of standards for translational considerations

Application of lentiviral vector infectious titer and VCN assays requires release criteria to be met that satisfies industry standards and regulatory concerns.[7] The establishment of VCN validity criteria is important during assay development prior to adherence in GMP quality control (QC). For example, standard curve efficiency and linearity of qPCR reactions includes an R^2 value greater than 0.98 with mean efficiency of 100% ± 10%.[15] Precision and accuracy are also evaluated during assay qualification and assay validation.[18] Alternatively, ddPCR offers increased accuracy and precision than qPCR based on the reliance on absolute quantification thus removing standard curve requirements.[12]

Additionally, the use of reference standards is increasing as NIST developed a set of clonal Jurkat cell lines with defined copy numbers as a reference material.[19] These standards help calibrate in-house vector-specific methods to ensure consistency among laboratories.

RESULTS

Method establishment with positive control

Initial studies were completed to determine a suitable assay positive control that demonstrates accuracy and repeatability. In particular, the use of a synthetic plasmid is advantageous due to its consistency and reliability to maintain inter- and intra-assay variability to a minimum. By these means, a linearized plasmid standard (LPS) containing the lenti-specific gene and various housekeeping genes was generated and established as the assay positive control reference. First, target gene recovery was assessed to ensure alignment with the expected theoretical copy number of the synthetic plasmid. Target recovery of the LPS was determined for various dilution buffers including nuclease-free water, TE buffer, and sample dilution buffer (SDB), which contains a stabilizing reagent to prevent template loss (Figures 1 and 2). Measured target concentration was plotted against theoretical concentration, both in copies per microliter for the lenti-specific target (RRE) and the housekeeping gene (GAPDH). Results were assessed based on established validity criteria. Linearity was observed across all dilutions and targets, and recoveries of the targets were all within the acceptable validity criteria of 70%–130%. Data suggest that the designed LPS is suitable as a positive control in subsequent assay testing. Evaluation of diluent stabilizers and carrier DNA including single strand DNA and lambda DNA reduce absorption to low binding tubes, thus allowing for long-term DNA storage for ddPCR-based determination of copy number when analyzing highly diluted DNA.[20]

To further support the use of LPS as a positive control, a suitable concentration was identified within the assay and instrument operating range (Figure 3). Adequate separation among the positive (+) and negative (−) droplet populations was observed based on a lambda value of 0.7, which corresponds to the point at which there is a half positive and half negative population. Lambda equals the natural log of the quotient of the number of negative droplets and the number of total accepted droplets. An optimal linearized plasmid concentration was set to 3.5E3 copies/μL as a positive control for both assays to

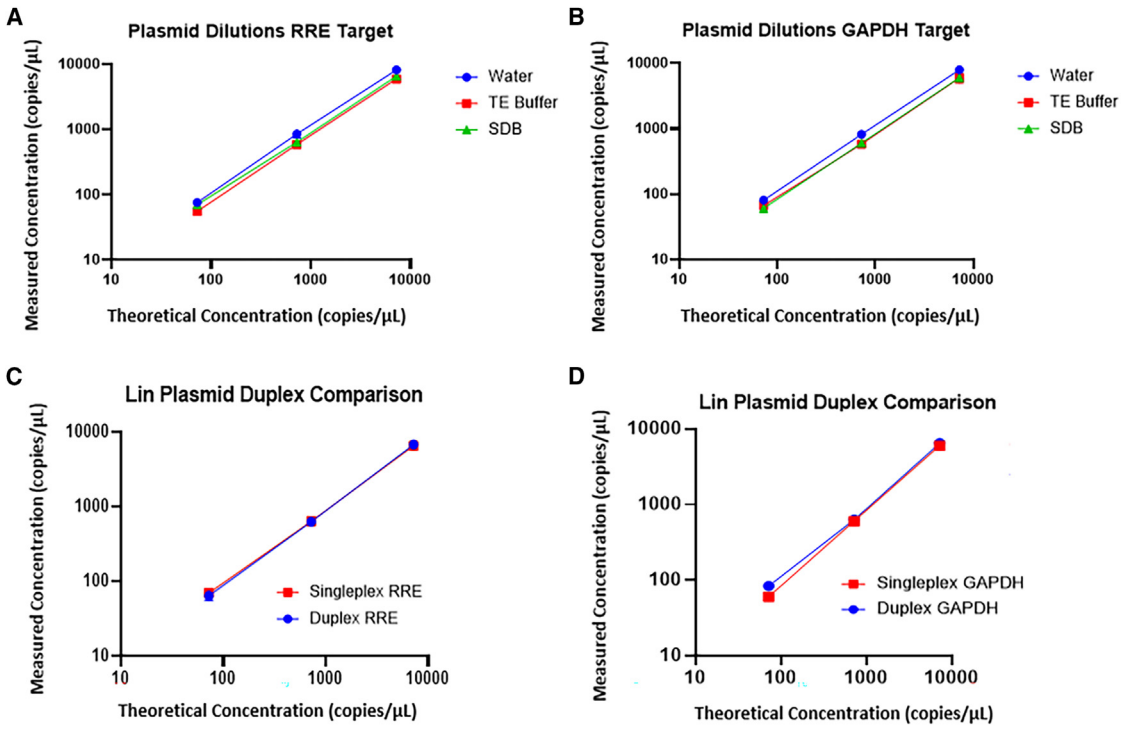

Figure 1. Linearized plasmid testing for positive control establishment

(A) RRE Target (B) GAPDH Target measured concentrations (copies/µL) across water, TE buffer and sample dilution buffer (SDB) diluents. No significant difference (p value >0.05) among diluents for either target based on one-way ANOVA. Singleplex and duplex reactions demonstrate linearity for (C) RRE target comparison and (D) GAPDH target in which R^2 was 0.99. No significant difference (p value >0.05) among singleplex or duplex for either target based on t test comparison. Standard error bars from the mean (n = 3) are smaller than the symbol.

achieve an equal distribution of positive and negative droplet populations within the assay validity range. This separation among droplet populations is critical to ensure robust calculation of sample concentration using the Poisson distribution.[21]

Determination of assay dynamic range and limit of quantification of RRE

An integral component of assay development and qualification, when evaluating lentivirus and CAR-T product, is the determination of limits of quantification/detection and range.[22] For the ddPCR instrument, the range spans 1 to 100,000 target gene copies per reaction[16]; however, the measurement assay range is dependent on target-specific variables. To assess this, the dynamic range was determined based on the lenti-specific target quantification in the presence of genomic DNA, matrix effect components. HT1080 genomic DNA (250 ng) was spiked into various dilutions of the established LSP containing one copy of the lentiviral vector-specific transgene and one copy of the housekeeping gene. A correction factor was applied to raw concentration values to account for initial DNA concentration per well. Acceptance criteria were applied that include recovery within 70%–130%, which includes a CV less than 30% (Table 1). Results indicate that the linear range for the assay is 1.16 to 1.11E3 copies/µL, which encompasses at least three logarithmic viral dilutions evaluated by the infectious titer assay (Figure 4). Lower limit of quantification was determined to be approximately 1.16 copies/µL, which translates to 12 copies/µg genomic DNA based on the concentration loaded per well. Representative 2D amplitude plots of RRE and GAPDH droplets demonstrate the decrease in RRE(+) GAPDH(+) population with the increase of LPS dilution. The RRE(+) cluster is absent based on the input, considering the LPS contains equal copies of both targets resulting in the RRE(+) GAPDH(+) population. By these means, the lower limit of quantification (LLOQ) is determined to be 1.16 copies/µL.

Lentivirus infectious titer

The established ddPCR assay was used to determine the infectious titer of five different lentivirus preparations. Genomic DNA extracted from LV transduced HT1080 cells was analyzed for RRE and GAPDH gene copies 4 days post-transduction. As an orthogonal method, flow cytometry was assessed for each transduced cell population using antibody-specific stain for the expressed transgene, as described in materials and methods. The flow cytometry results indicate comparability with ddPCR targeting the transgene for each lentiviral vector, with a higher sensitivity exhibited for ddPCR (Figure 5). ddPCR infectious titer results are generally within 30% difference compared with the titer obtained by flow cytometry (Table 2) across four various targets used during flow cytometry assessment.

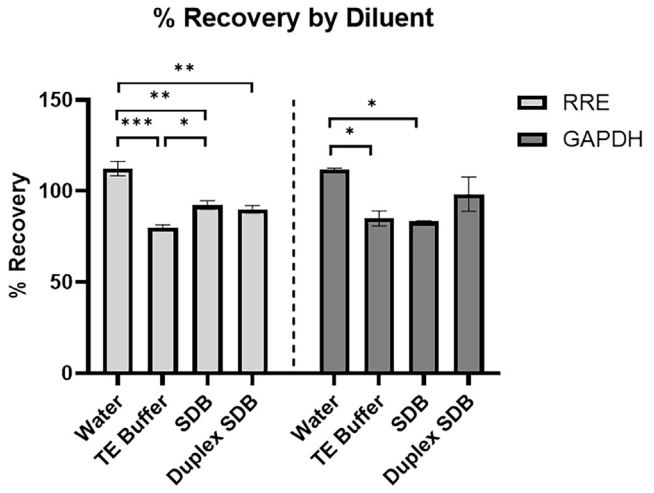

Figure 2. Recovery of linearized plasmid for positive control establishment
Acceptance criteria included recovery between 70% and 130%. Data represent the mean ± standard error where n = 6 for each diluent and target. One-way ANOVA followed by post hoc analysis. *p < 0.05, **p < 0.005, and ***p < 0.0005.

The difference in titer between ddPCR and flow cytometry for CAR-1a could be attributed to genome resolution compared with cell resolution for the specific flow cytometry reagent, anti-human EGFRt (epidermal growth factor receptor, truncated) conjugated antibody that targets the EGFRt-transgene-fused cell surface protein. Biological processes associated with the conversion of DNA to surface expressed proteins involve transcription and chromatin access, translation regulation, and trafficking to the cell membrane.[23,24] These steps are suggested to contribute to the measured titer while other factors including the number of viable and non-viable cells used for assay performance have not demonstrated titer differences by flow cytometry and an image-based titer.[14]

Next, accurate performance of the universal ddPCR-based infectious titer assay was demonstrated by close alignment of the LV titer determined by the transgene-specific target GFP as well as the lenti-specific RRE target (Figure 5) thus supporting the utilization of this universal assay. Results of this study suggest comparability among our established ddPCR and flow cytometry assays for the determination of lentivirus infectious titer.

Vector copy number

Our universal assay was also implemented to characterize CAR-T cells based on VCN (Figure 6). Initially, data were assessed based on target amplitude and location of droplet populations, key parameters that inform data analysis procedures. Results indicate the presence of four distinct populations based on RRE and GAPDH fluorescent amplitude. The presence of four distinct populations supports assay establishment and guides target baseline determination.

Generally, an increase in VCN was observed with increasing MOI for the same LV production. ddPCR methods were confirmed using an established reference standard provided by NIST. This standard contains a known VCN of one (NIST, unpublished data).[19] ddPCR of the reference standard across three independent runs produced an average value of 0.89 copies per cell with 3.6 %CV (n = 3). Results demonstrate accuracy of 11% difference to the known VCN result of one copy per cell (Figure 6).

Additionally, the VCN of CAR-T cells demonstrates a dose response based on the known LV MOIs used during transduction. MOI range

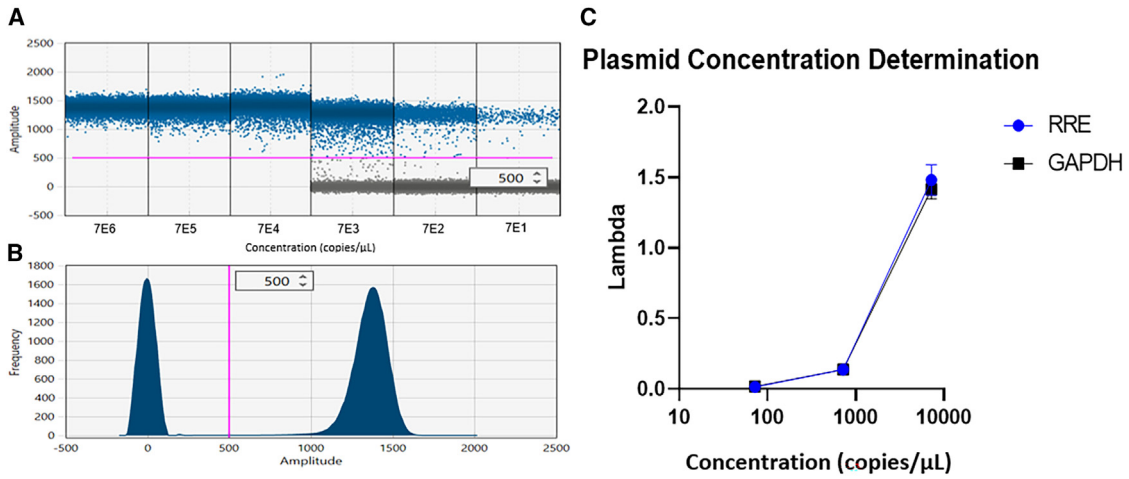

Figure 3. Determination of linearized plasmid concentration for positive control establishment
(A) Representative RRE target ddPCR 1D amplitude plot that indicates the establishment of the baseline at 500 relative units (singleplex reaction) in addition to the positive population (blue droplets). (B) Histogram of the frequency of the positive and negative droplets. (C) Plot of lambda versus the theoretical target concentration (copies/µL). A plasmid concentration in which lambda equals 0.7 indicates a half positive and half negative population and is supportive for use as the assay positive control. Data represent the mean ± standard error (n = 3 for each concentration and target).

Table 1. Assay dynamic range results for LLOQ determination, as indicated by percent recovery of RRE linearized plasmid DNA target

Plasmid dilution series	Theoretical RRE concentration (copies/μL)	Average measured RRE concentration (copies/μL)	Average measured RRE concentration (copies/μL) with correction factor	% CV	Average % recovery
1	7.22E4	5.56E3	3.89E4	OOR	OOR
2	7.22E3	1.11E3	7.77E3	5%	108%
3	7.22E2	9.88E1	6.92E2	13%	96%
4	7.22E1	1.09E1	7.63E1	9%	106%
5	7.22	1.16	8.12	17%	112%
6	0.722	0.28	1.96	OOR	OOR

Out of range (OOR) indicates values outside of the 70%–130% recovery range.
An instrument correction factor of 7 was applied to the measured RRE concentration (copies/μL) based on the initial reaction concentration and volume analyzed by the ddPCR instrument. Data represented as average of three independent runs.

with a distribution of VCN spanning 0.2 to 2.0 copies per cell was identified for LV CAR productions. This information is crucial for CAR-T generation using automated systems in which a single MOI is targeted/programmed during transduction[25] where high transduction efficiency and low VCN are key concerns. In subsequent CAR-T productions using an automated system (CliniMACS Prodigy, Miltenyi Biotec), VCN increased linearly with increasing MOI from 1 to 7.3 copies/cell. The tested parameters for transduced CAR-T cells satisfy current FDA guidance and release criteria with the VCN below five copies per genome.[26]

DISCUSSION

ddPCR is a rapidly expanding technology and industry standard that utilizes absolute quantification to determine sample concentration without standard curve generation. In this way, droplet generation by random partitioning allows for independent target molecules of identical volume, approximately 1 nL, to be analyzed as separate reactions. Using the Poisson distribution, sample concentration is calculated using the fraction of droplets that contain the target DNA and the total number of measured droplets.[21] Based on these advantages, the developed assays leverage ddPCR technology to accurately determine key characteristics.

Assay establishment with positive control

An established positive control standard is a critical component of assay development that drives this ddPCR-based universal assay for lentivirus infectious titer and lenti-modified VCN. Validated controls support ongoing efforts toward gene therapy standardization[26] while also fueling reproducibility and comparability of results across institutions.

By these means, results support the implementation of the established LPS for use as a ddPCR assay development substrate and as an assay positive/validity control for both the lentivirus infectious titer and post-transduction VCN assays. This linearized plasmid substrate offers a consistent and reliable control within the assay range[27] that reduces potential variability of cell-based standards. Validity criteria were determined and applied in subsequent testing.

In addition to the LPS positive control, the field has investigated alternative controls that demonstrate effective mode of action and represent suitable controls. For example, transduced cell standards with a known range of lentiviral copies can be utilized. Specifically, LV transduced Jurkat cell lines were characterized for copy number and genomic integrity, which were further tested by qPCR.[19] Similarly, lentiviral standards generated from LV transduced HCT116 colorectal human cells at various MOI were magnetically enriched with CD271 beads prior to clonal expansion and gDNA extraction.[28] Overall, these transduced cell standards are used to calibrate VCN assays used for QC purposes.

Outcomes of infectious titer and VCN

Following assay development, the established LPS positive control was employed across further assay performance to identify lentivirus infectious titer, a key metric informing upstream and downstream viral vector processes. The alignment of infectious titer between ddPCR and flow cytometry methods across four lentivirus productions supports the consideration of this assay into routine workflows.

There are several distinct advantages of ddPCR compared with the commonly used flow cytometry-based methods including its sensitivity, specificity, versatility, readily available reagents, and short development time. Whereas flow cytometry is dependent on expression of the transgene specific to the construct, this ddPCR assay can rely on lenti-specific or common targets (e.g., RRE) appropriate for lentivirus. Flow cytometry also entirely depends on the generation of transgene product-specific antibodies, which may have long development/production lead times coupled with challenges with reagent quality and consistency.[29] The developed ddPCR assay is universal, which demonstrates utility across lentivirus vectors containing various transfer genes of interest.

Further, the established assay employed for VCN determination exhibits accurate quantification of lenti-modified CAR-T cells. This is supported by the evaluation of the positive control sample containing a known VCN of one copy per cell provided by NIST.[19] Overall, these assays provide robust determination of lentivirus functional titer and CAR-T cell safety.

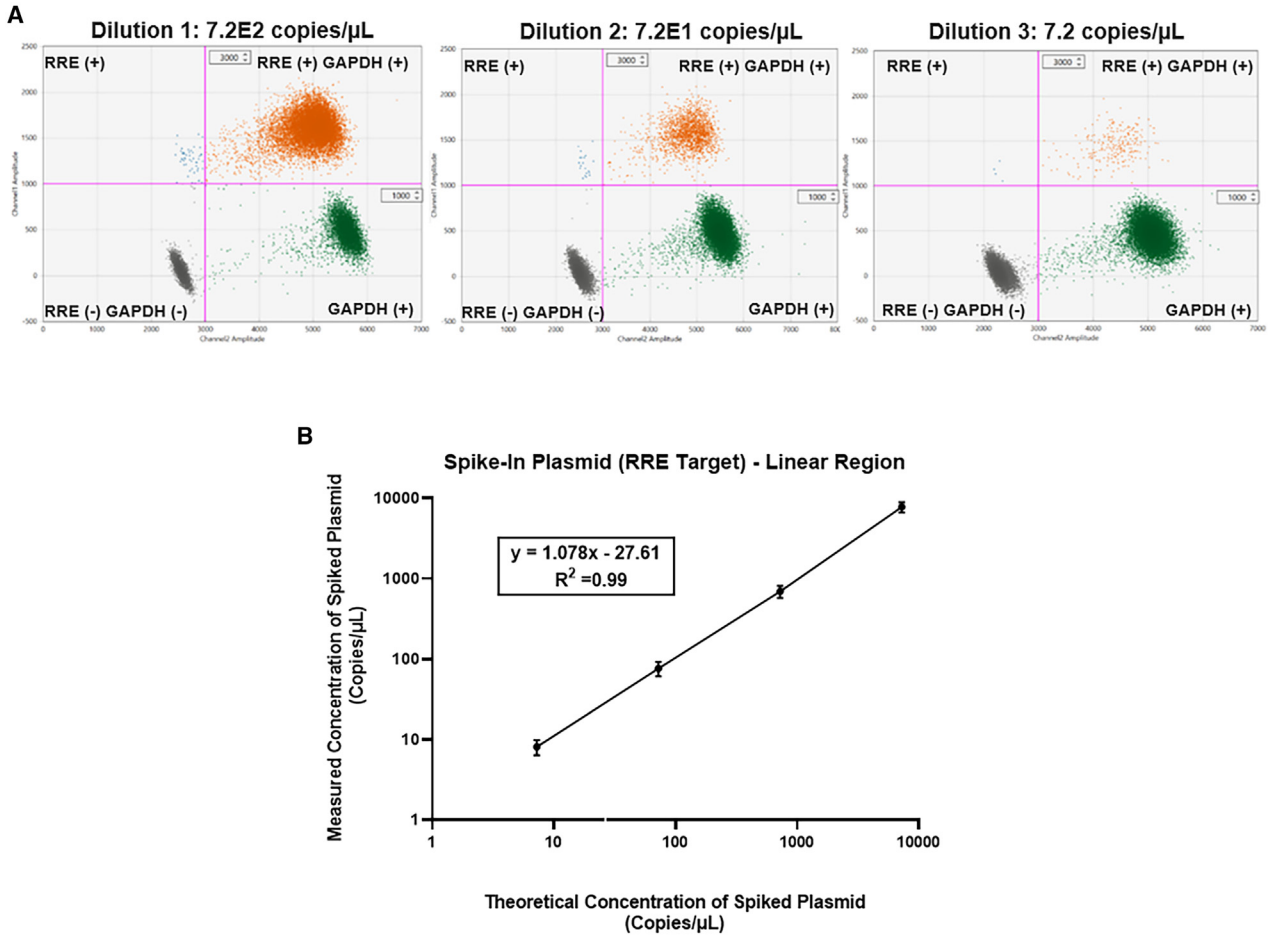

Figure 4. Determination of assay dynamic range and LLOQ

(A) Representative 2D amplitude plots for plasmid spike-in assay results for reactions containing 7.2E3, 7.2E2, and 7.2E1, and 7.2 RRE copies/µL of plasmid spiked into 250 ng of HT1080 gDNA. There is an observed reduction in the RRE(+) GAPDH(+) population with increased LPS dilution. Clear separation among positive and negative populations was observed for both targets with consistent thresholds. (B) Graph of measured RRE copies of spiked plasmid versus theoretical copies for the dilutions within the linear range. LLOQ was determined to be approximately 1.16 copies/µL ddPCR output based on the lower linearity dilution value. Data represent the mean ± standard error (n = 3 for each concentration).

Applicability to cell and gene therapy

Transgene delivery using lentiviral vectors enables the generation of gene-modified cell therapy products for patient use. Key quality metrics for both the lentiviral vector and cell therapy product are critical characterization parameters that guide transduction MOI and safety release aspects, respectively. Due to quality standardization concerns that utilize various custom assays, a universal assay can provide accurate quantification of VCN post-transduction to meet regulatory expectations intended for patient protection.[26]

As a further advancement in this field, the developed universal assay can be performed using orthogonal methods including qPCR, ddPCR, and dPCR in which the established LPS provides utility across measurement systems. Since the traditional qPCR method relies on standard curve generation for sample reference, the well-characterized LSP could serve as a synthetic plasmid with established gene copy number obtained during standard calibration by ddPCR or dPCR. In this way, ddPCR and dPCR methods are cross-functional, which can be implemented for both calibration during assay development and as a control for routine assay performance. By these means, the universal assay can provide a comparison when developing custom gene-specific assays by introduction of the GOI to the LPS. Results from the comparison of the universal target to GFP further support this key development opportunity.

In conclusion, this study supports the use of ddPCR as a method for lentivirus infectious titer and VCN determination in the development and manufacture of cell therapy products. As part of assay development, the linear dynamic range, LLOQ, assay validity criteria, accuracy, and positive control suitability were determined. Singleplex and duplex reactions for lentiviral RRE and host cell GAPDH demonstrated valid recoveries of 70%–130% for an LPS.

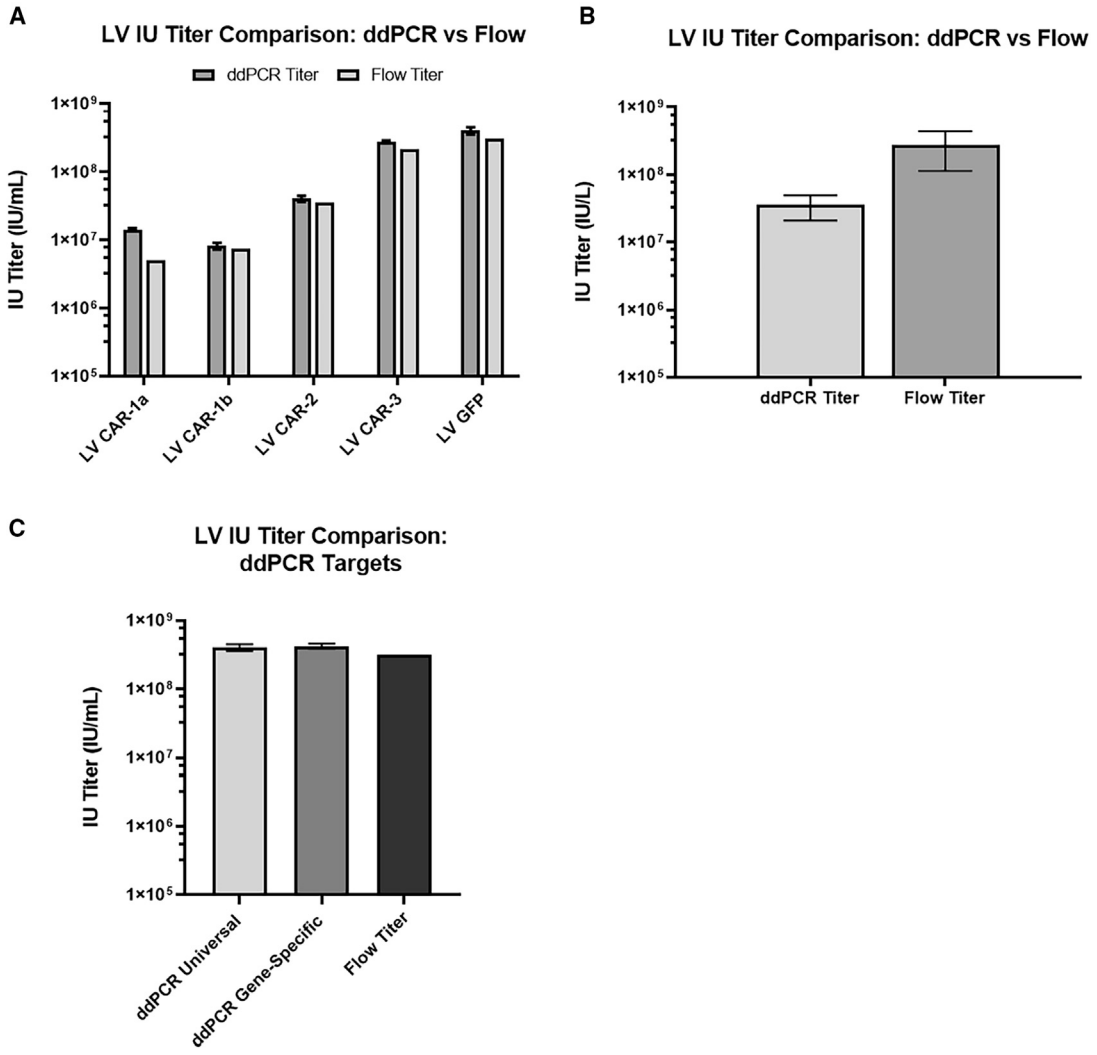

Figure 5. Lentivirus infectious titer across productions and titer methods

(A) Lentivirus infectious titer determined by ddPCR and flow cytometry for five lentiviral vectors. Various CAR constructs as indicated by CAR-1, CAR-2, and CAR-3 in addition to the GFP construct. Each LV preparation CAR-1a, CAR-1b, CAR-2, CAR-3, and GFP was titered by flow cytometry using stains for EGFRt, V5 tag, FMC63, anti-CD34, and GFP, respectively. ddPCR data represent the mean ± standard error (n = 3 for ddPCR titer). (B) Lentivirus infectious titer for separate LV CAR productions normalized to production scale (L). No significance (p value >0.05) between titer methods for average values among productions where n = 2 biological replicates. (C) Lentivirus infectious titer for GFP-LV production titered using the universal ddPCR targets and the gene-specific GFP target. Data represent the mean ± standard error (n = 3 for ddPCR titer).

The assay workflow presented in this study allows for the universal determination of key quality parameters, and thus eliminates the dependence on antibody-specific staining and user defined gates associated with flow cytometry. Considering quality and regulatory guidelines, this assay supports the use in cGMP QC laboratories testing the lentiviral vectors themselves and in post-transduced cellular products.

MATERIALS AND METHODS

ddPCR run and data analysis

Reactions were prepared with appropriate supermix for singleplex reactions (ddPCR Supermix for Probes, no dUTP, Bio-Rad Catalog 1863010) or duplex reactions (Multiplex Supermix, Bio-Rad Catalog 12005939). PCR primers and probes (Thermo Fisher TaqMan Gene Expression Assay) were custom ordered in which the RRE target contained the probe with the FAM reporter, and GAPDH contained the probe with the VIC reporter. Both primer and probe assays contained the MBG-NFQ quencher. The addition of dithiothreitol (DTT) to the mastermix was used for duplex reactions.

Thermal cycling for all ddPCR experiments was performed using a Bio-Rad QX ONE workstation and the following amplification conditions: 10 min at 95°C followed by 40 cycles for 15 s at 95°C and 1 min at 52°C. The raw QX ONE data file was exported and analyzed using

Table 2. IU titer results for ddPCR and flow cytometry represented as averages with associated percent difference for each lentivirus ID production

Lentivirus ID	Titer by ddPCR (IU/mL)	Titer by flow cytometry (IU/mL)	% difference
LV CAR-1a	1.43E7	5.09E6	181%
LV CAR-1b	8.18E6	7.35E6	11%
LV CAR-2	4.06E7	3.52E7	15%
LV CAR-3	2.76E8	2.14E8	29%
LV GFP	4.05E8	3.11E8	30%

regulatory edition software. Samples were identified using the Plate Layout tab, and thresholds for each target were determined using the negative control to separate the positive droplet population containing the gene of interest to the negative droplet population.

Positive control establishment

Serial dilutions of the purified LPS were generated in nuclease-free water, TE buffer (Thermo Fisher Scientific, Catalog AM9858), or SDB diluents. The SDB contains GeneAmp 10X PBC Buffer (Thermo Fisher Scientific, Catalog N8080129), Pluronic F-68 Non-ionic Surfactant (Thermo Fisher Scientific, Catalog 24040032), and Salmon Sperm DNA, sheared (Thermo Fisher Scientific, Catalog AM9680) for stabilization. Six, 10-fold plasmid dilutions spanning 0.7-7E4 theoretical target copies/µL were generated as determined using Equation 1 and the measured concentration of the stock solution (Nanodrop, Thermo Fisher Scientific). Molecular weight was set to 660 g/mol nucleotides for double-stranded plasmid DNA.

$$\text{Copy Number of Target} = \frac{\left(\text{Amount of plasmid (ng)} * \left(6.0221 * 10^{23}\frac{\text{molecules}}{\text{mol}}\right)\right)}{\text{Molecular Weight}\left(\frac{\text{g}}{\text{mol}}\right) * 1 * 10^{9}\left(\frac{\text{ng}}{\text{g}}\right)} \quad \text{(Equation 1)}$$

Singleplex and duplex ddPCR reactions containing the lenti-specific target (RRE or transgene) and/or housekeeping gene were set up for the serial dilution series and run using the Bio-Rad QX ONE ddPCR instrument.

Suitable LPS concentration within the center of the instrument range was determined based on Equation 2. Lambda was identified for each plasmid serial dilution and plotted against measured target concentration. A Lambda value of 0.7 corresponds to the point in which there is a half positive and half negative population. Concentration was calculated using the best-fit equation.

$$\text{Lambda} = -\ln\left(\frac{\text{Number of Negative Droplets}}{\text{Number of Total Droplets}}\right) \quad \text{(Equation 2)}$$

To determine assay LLOQ of the RRE ddPCR assay, six 10-fold dilutions of the LPS were generated and spiked into 250 ng of HT1080 gDNA. Duplex reactions containing both targets were run, and recovery (%) was calculated based on the theoretical concentration of the RRE target, Equation 3.

$$\%\ \text{Recovery} = \frac{\text{Measured Target Copies}}{\text{Theoretical Target Copies}} * 100 \quad \text{(Equation 3)}$$

As a positive control, subsequent infectious titer and VCN assays include the LPS dilution containing 7.2E3 copies/µL. Acceptance criteria is 70%–130% recovery with replicates containing less than 30% CV.

Lentiviral vector preparations

Five lentiviral preparations were generated and further characterized by infectious titer. Upstream methods involved transfection of viral producing cells (VPCs) (HEK293) with the gene transfer plasmid, helper plasmids, and envelope plasmid following the LV-Max Transfection Kit (Thermo Fisher Scientific, Catalog A35348). Table S1 indicates the lentivirus vector name, associated transgene, and transgene size. A prescribed plasmid dilution ratio, in addition to the transfection reagent/booster, was added to the VPCs and incubated for 72 h before harvest. Downstream processing involved VPC harvest, endonuclease treatment, clarification, chromatography purification, concentration, and final filtration. Preparations LV CAR-1a and LV CAR-1b differ based on downstream purification method parameters for the same lentivirus transgene.

Infectious titer assay

A fibrosarcoma cell line, HT1080 (ATCC, Catalog CCL-121), that is permissive to lentiviral infection was cultured in complete media including DMEM (Thermo Fisher Scientific, Catalog 10569010) with 10% fetal bovine serum (Thermo Fisher Scientific, Catalog A5209402) at 37°C, 5% CO_2. Cells were expanded to a qualified working cell bank at passage six, which was authenticated by karyotype, short tandem repeat testing (Wicell Characterization Services) and mycoplasma. HT1080 cells were passaged at approximately 80%–90% confluency using TrypLE (Thermo Fisher Scientific, Catalog 12563011). Four hours before lentiviral infection, 7E4 HT1080 cells seeded per well to allow for attachment. Ten-fold serial dilutions of the thawed virus and complete media were performed to span 1E2 to 1E7 viral dilutions. Lentiviral transduction was performed by aspirating the cell media and replacing it with 400 µL of the corresponding viral dilution. The 24-well plates were centrifuged at 900 × *g* for 30 min at 20°C and incubated at 37°C, 5% CO_2 prior to cell harvest.

For ddPCR analysis, HT1080s were harvested 4 days post-transduction, and genomic DNA was extracted in a multi-step process. Cell pellets were resuspended in 200 µL 1X dPBS (Thermo Fisher Scientific, Catalog 14190-144), and the Thermo Fisher PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Catalog K182002) was used. Kit components including Proteinase K (Thermo Fisher Scientific, Catalog K182002) and RNase A (Vendor, Catalog K182002)

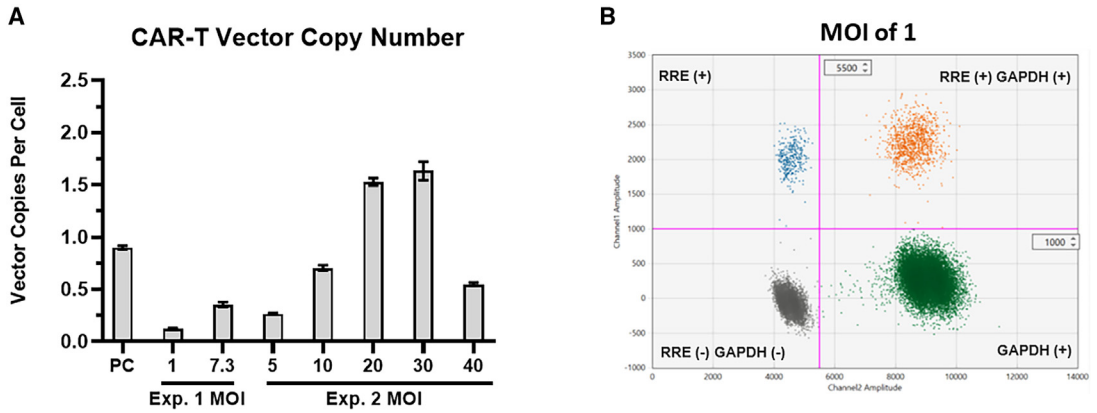

Figure 6. Vector copy number of transduced CAR-T cells

(A) VCN of CAR-T cells generated with LV CAR-2 at various MOIs using flow cytometry titer result. Experiment (Exp.) 1 includes the VCN of two separate batches of CAR-T cells generated and cultured for 10 days, at an MOI of 1 or 7.3. Experiment (Exp.) 2 MOI study includes the VCN of CAR-T cells in which saturation is achieved at MOI 30. Data represent the mean ± standard error (n = 3 per MOI). VCN of a GFP-LV transduced Jurkat cell line labeled PC contains a copy number of 0.89 copies/cell and 3.6% CV among three experiments. (B) Representative 2D amplitude plot of CAR-T gDNA generated at an MOI of 1 corresponding to an average VCN of 0.1 vector copies per cell.

were added to the samples and incubated at room temperature prior to lysis with PureLink Genomic Lysis buffer (Vendor, Catalog K182002). Ethanol was added to the lysate, and the solution was transferred to a PureLink Spin Column (Vendor, Catalog K182002) for purification. Samples were centrifuged at 10,000 × *g* for 1 min at 20°C, two wash buffers were separately added with centrifugation steps at 16,000 × *g*. Genomic DNA was eluted using distilled water, and DNA concentration was measured using UV spectroscopy (Nanodrop, Thermo Fisher).

ddPCR reactions for the transgene/RRE and GAPDH targets were carried out as described above. Equation 4 includes the calculation to obtain infectious titer, and average values among viral dilutions within range were included.

$$\text{IU} \Big/ \text{mL} = \frac{\text{Viral Gene Copies}}{\text{Housekeeping copies}} * 2 * \frac{\#\text{ cells seeded at transduction}}{\text{mL virus}} \quad \text{(Equation 4)}$$

For flow cytometry analysis, HT1080 cells were harvested 4 days post-transduction and centrifuged at 200 × *g* for 4 min at 20°C to pellet. Cell counts and viability were obtained prior to aliquoting approximately 1E6 cells per tube. Antibody staining that targets CAR+ surface antigen or other markers on transduced cells include EGFRt, V5 tag, FMC63, and anti-CD34, for lentivirus preparations 1 through 5, respectively. The Attune flow cytometer was used for data collection, and gates were established in FlowJo software to determine the percentage of positive cells containing the target. Post-transduction titer was determined using Equation 5.

$$\frac{\text{IU}}{\text{mL}} = \left(\%\text{ positive cells} * \frac{\#\text{ cells seeded at transduction}}{\text{Volume of Inoculum (mL)}}\right) * \text{Lentivirus Dilution Factor} \quad \text{(Equation 5)}$$

ddPCR VCN assay

T cells, isolated using positive selection from PBMCs, were transduced with CD19 CAR lentivirus at various MOIs. Positive selection involved Dynabead magnetic beads (Thermo Fisher Scientific, Catalog 11141D) that bind to the T cells to be isolated and used to separate these cells from the total cell population. Cells were harvested 4 to 10 days post-transduction for further analysis.

Following gDNA extraction as described in the infectious titer assay section, ddPCR was performed to determine the number of integrated viral gene (RRE) and housekeeping gene (GAPDH) copies. VCN per cell was determined using Equation 6, which accounts for a multiplication factor of 2 due to the presence of two GAPDH copies per cell.

$$\text{VCN Copies per Cell} = \frac{\text{Viral Gene Copies}}{\text{Housekeeping Copies}} * 2 \quad \text{(Equation 6)}$$

Statistical analysis

Graphs were generated using GraphPad Prism in which data represent average values with standard error bars above and below the mean. GraphPad Prism was also used to perform statistical analysis. For the assessment of LPS by target, one-way ANOVA with a confidence level of 0.95 was completed among diluents (Figure 1). Paired two-tailed Student's t tests were used to determine differences between singleplex and duplex reactions (Figure 1). Differences among target % recovery based on diluents were assessed using a one-way ANOVA with a confidence level of 0.95 followed by post hoc comparison (Figure 2). For infectious titer comparison between ddPCR and flow cytometry, paired two-tailed Student's t tests were completed as indicated (Figure 5).

DATA AND CODE AVAILABILITY

Further inquiries can be directed to the corresponding author.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2023.101120.

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AUTHOR CONTRIBUTIONS

J.K. and U.L. contributed to the assay design and analysis of the study results. U.L. and R.S. were involved with the linearized plasmid standard synthesis and target selection. J.K. performed the experiments including the positive control establishment, ddPCR assay parameter determination, and IU titer/VCN assessment by ddPCR. All authors contributed to the article and approved the submitted version.

DECLARATION OF INTERESTS

J.K., S.M., U.L., and R.S. were employed by Thermo Fisher Scientific.

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