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SHORT COMMUNICATION

A rapid, efficient, and cost-effective method for titering third-generation lentiviral vectors

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Abstract

Lentiviral vectors are useful vectors for stable transduction and permanent expression in dividing and non-dividing cells. In particular, third-generation lentiviral vectors have been engineered to be significantly safer than their second-generation counterparts, incorporating several safety features not present in earlier versions. For example, the *tat* gene, which is essential for the replication of wild-type human immunodeficiency virus type 1, has been deleted, and vector packaging functions have been distributed across three separate plasmids, further enhancing safety. In both research and clinical settings, having a reliable and accurate method for titering lentiviral vectors is critical. We have developed a method using the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element as a template for a real-time quantitative polymerase chain reaction, coupled with TRIzol lysis buffer for ribonucleic acid isolation. This method yielded results comparable to those from a commonly used commercial kit, offering advantages of speed, cost-effectiveness, and accuracy. It presents a viable, economical alternative for both research and clinical laboratories.

Keywords: Titer; Third-generation lentiviral vector; Molecular biology; Ribonucleic acid; TRIzol

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1. Introduction

Lentiviral vectors are derived from ribonucleic acid (RNA) viruses belonging to the *Retroviridae* family. Unlike other retroviruses, lentiviruses can transduce both dividing and non-dividing cells, making lentiviral vectors derived from them valuable for a wide range of applications, including gene therapy. Second-generation lentiviral vectors utilize a single packaging plasmid that encodes the *pol*, *gag*, *rev*, and *tat* genes, whereas other virulence factors have been removed.[1-3] Although these vectors are significantly safer than the original lentiviral vectors, the possibility of generating recombinant viruses has not been entirely eliminated. In addition, given that they are almost exclusively derived from human immunodeficiency virus (HIV), safety concerns persist.[4] In contrast, third-generation lentiviral vectors mitigate this risk using three separate packaging plasmids: one for the *gag* and *pol* genes, and two for the *rev* and *env* genes. Further deletions in the 3' long terminal repeat of the vector plasmid make the vectors self-inactivating, providing an additional safety feature.[4,5]

Over the past four decades, the number of clinical trials using viral vectors for gene therapy has grown significantly. Throughout this time, numerous breakthroughs have

been made, alongside some setbacks. Despite these early challenges, intensive research efforts have continued, leading to the approval of several viral vector-based therapies, with many others currently in late-stage clinical trials.[6]

Lentiviral vectors are generated by transfecting human embryonic kidney 293 (HEK293T) cells with the transfer plasmid and packaging plasmids. A critical step in the lentiviral vector production process is calculating an accurate and reproducible vector titer, which can be time-consuming. Several methods for measuring viral vector titer have been described, including enzyme-linked immunosorbent assays to measure the p24 antigen (a protein component of HIV), reverse transcriptase activity, dot blotting, fluorescence-activated cell sorting (FACS), and quantitative polymerase chain reaction (qPCR), which are among the most accurate methods.[7,8] FACS analysis is not suitable for vectors lacking fluorescent reporter genes,[7] making qPCR the most universally reliable method for quantifying lentiviral titer.[6,9] For commercial viability, large-scale production of the vector at high titers is essential. However, many commercially available qPCR-based kits for titering lentiviral vectors are prohibitively expensive. Here, we present the development of a rapid, cost-effective, efficient, and accurate method of lentiviral vector titering. We utilized the third-generation lentiviral vector pRRL.sin.cPPT.LSP.IRES.mVenus.WPRE and compared the results from our TRIzol-based qPCR method with those obtained using the commonly used commercial qPCR Lentivirus Titer Kit (catalog number LV900; Applied Biological Materials [ABM] Inc., Canada). The TRIzol-based method described is not only rapid and cost-effective but also provides titer calculations that are as accurate as those generated by the ABM kit.

2. Materials and methods

2.1. Materials

2.1.1. Plasmids

pRRLSIN.cPPT.PGK-GFP.WPRE, pMDLg/pRRE, pRSV/REV, and pMD. 2/VSV.G were purchased from Addgene, United States.

2.1.2. Commercial kit

Applied Biological Materials Inc qPCR Lentivirus Titer Kit (catalog number LV900, Canada) was utilized in this study.

2.1.3. Chemicals

All chemicals and solutions, such as ethanol, were used in their pure form and were purchased from Merck Life Science Pty Ltd, Australia.

TRIzol reagent (catalog number 15596026) was purchased from Thermo Fisher Scientific, Australia.

2.1.4. Cells and cell culture

Human embryonic kidney 293 cells, 0.45 μm Nalgene filters, and OptiMEM 1 Reduced Serum Medium were purchased from Merck Life Science Pty Ltd, Australia.

Fetal calf serum (FCS), Dulbecco's Modification of Eagles Medium (DMEM), and penicillin/streptomycin (pen/strep) were purchased from Thermo Fisher Scientific, Australia.

CF2 CellSTACK 2 chambers (catalog number CLS3310) were purchased from Merck Life Science Pty Ltd, Australia.

2.1.5. Molecular reagents

PowerUp SYBR Green Master Mix was purchased from Thermo Fisher Scientific, Australia.

DNase/RNase free water, Micro Amp Fast Optical 96-well reaction plates, Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) forward primer (AGCTCCTTTCCGGGACTTTC), and WPRE reverse primer (AGCCATGGAAAGGACGTCAG) were purchased from Thermo Fisher, United States.

2.1.6. Equipment

Tangential Flow System (TFF; catalog number OS100T12) was purchased from Pall Corporation, Australia.

Centrifuge (catalog number Eppendorf 5424R) was purchased from Eppendorf South Pacific Pty Ltd, Australia.

Nanodrop One and QuantStudio 6 Flex Real-Time PCR systems were purchased from Thermo Fisher Scientific, Australia.

2.2. Lentiviral vector production

The third-generation lentiviral vector, pRRLSIN.cPPT.PGK-GFP.WPRE, was produced as follows:

The calcium phosphate ($CaPO_4$) precipitation method was used for the transient transfection and packaging of the four lentiviral plasmids in 293T cells.

(i) Day 1 – Preparation for transfection: 293T cells were split in DMEM (180 mL) containing 10% FCS and 1% pen/strep at a density of 6.0×10^7 cells per CF2 CellSTACK two chambers

(ii) Day 2 – Transfection: The medium on the 293T cells was changed to OptiMEM 1 (180 mL) containing 4% FCS. The transfection reagents (tubes 1 and 2) were prepared as shown in Table 1. The contents of tube 2 were slowly mixed into tube 1, and the mixture was left at room temperature for 30 min to

Table 1. Transfection reagents for lentiviral vector production. (A) tube 1, and (B) tube 2

(A) Tube 1		
Transfection reagents	**Amount/volume in CF2**	**Size (kb)**
pRRL.sin.cPPT.LSP. IRES.mVenus.WPRE	600 µg	8.512
pMDLg/pRRE	600 µg	8.89
pRSV/REV	320 µg	4.174
pMD2/VSV.G	400 µg	5.924
2M $CaCl_2$	1.2 mL	-
dH_2O	To – 8 mL	-
Total	8 mL	-
(B) Tube 2		
Reagents	**Volume in CF2**	
1M HEPES	0.4 mL	
2M NaCl	1 mL	
150mM Na_2HPO_4	120 µL	
dH_2O	6.508 mL	
Total	8 mL	

Abbreviations: $CaCl_2$: Calcium chloride; CF2: CF2 CellSTACK 2 chambers; dH_2O: Distilled water; CF2: CF2 CellSTACK 2 chambers; dH_2O: Distilled water; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaCl: Sodium chloride; Na_2HPO_4: Disodium phosphate.

allow complete precipitation. The mixture was then added to OptiMEM 1 medium without FCS; then it was transferred onto the 293T cells. The cells were incubated in a humidified chamber at 37°C with 5% carbon dioxide for 4 h. After incubation, the OptiMEM 1 medium (without FCS) was replaced with fresh OptiMEM 1 medium, and the cells were then incubated overnight at 37°C with 5% carbon dioxide

(iii) Day 3 – 5 – Virus harvesting: The virus-containing medium was harvested on days 3 – 5, replenished with fresh OptiMEM 1 medium (without FCS) after each harvest. The harvested medium may be stored at −80°C until ready for filtration through 0.45 µm Nalgene filters to remove cell debris.

2.2.1. Vector concentration and purification

The TFF system was used for filtration and concentration of the vector.

(i) Before starting the vector concentration, the flow rate of the TFF apparatus was ensured to be between 700 and 900 mL/min

(ii) The TFF was washed with 1,000 mL phosphate-buffered saline (PBS), followed by 500 mL OptiMEM 1 medium

(iii) The tank was filled with 7 L of medium collected from the transfected 293T cells. Once the medium was filtered, additional medium was added until 100 mL remained in the tank, after which the remaining medium was collected

(iv) 100 mL of OptiMEM 1 was added, and filtration was continued for another 15 min. This step was repeated twice

(v) The filtered medium was centrifuged at 50,000× g for 2 h at 4°C

(vi) The virus pellet was resuspended in 4 – 6 mL of OptiMEM1 medium, and the filtered viral supernatant was stored at −80°C

(vii) The TFF apparatus was cleaned with 1 L of 0.5 M sodium hydroxide for 30 min, and then the process was repeated once. Next, the apparatus was washed with 2 L of 0.1 M sodium hydroxide for 1 h, followed by two washes (1 h each) with 2 L of PBS each time. Finally, the TFF was run.

2.3. Methods for titering the lentiviral vector

2.3.1. qPCR lentivirus titer kit method

This methodology took approximately 2.5 h to complete, at a cost of Australian dollars (AUD) $1,958.00 per 96-well plate. The cost included the kit, quantitative standards, and associated freight charges (both international and local).

Following the manufacturer's instructions for titering lentiviral vectors (ABM kit; LV900; Canada), the supplied standards were diluted at 10^9, 10^8, 10^7, and 10^6 copies/mL for qPCR analysis and the production of a quantitative standard curve. Lentiviral vectors (1 µL) were diluted 1:1,000. Viral vector samples that produced large pellets after centrifugation were diluted 10,000 times. Quantitative analysis was performed when the R^2 value of the standard curve was >0.99, with an R^2 of 0.998 being typical for the standard curve.

2.3.2. TRIzol method

The following methodology required 1 h for RNA isolation, 2 h 30 min for qPCR, at a cost of AUD$115.20 per 96-well plate. The TRIzol reagent was priced at $AUD296.00, and the primers cost approximately AUD$30.00. The total cost was approximately AUD$300.00 for 500 samples or $AUD58.00 for 96 samples. This was approximately 34 times less costly than using the ABM kit, which also measures 96 samples. The method is illustrated graphically in Figure 1.

i. The vector (1 µL) was diluted 1:1000 with PBS and mixed. Depending on the size of the pellet, the vector may have needed to be diluted between 1:100 and 1:10,000

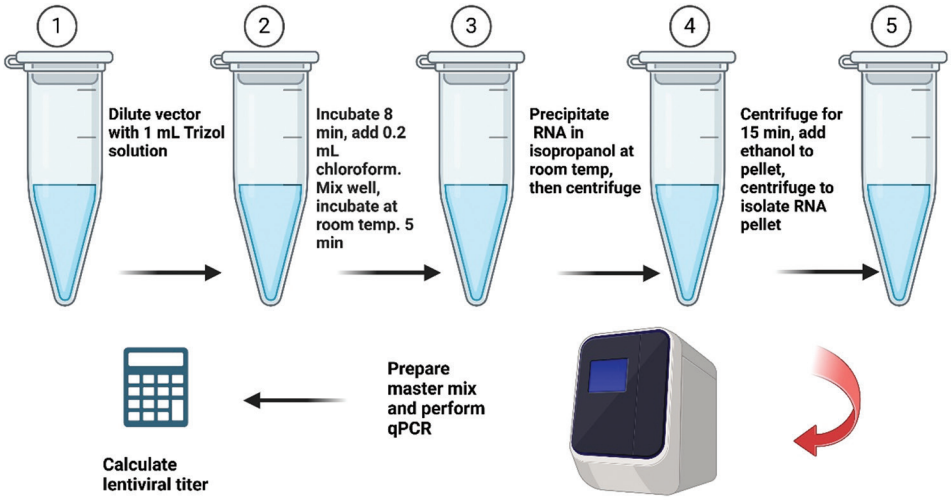

Figure 1. Graphical representation of the TRIzol method of lentiviral vector titration. Created in BioRender by Ann M. Simpson (2024). https://BioRender.com/t86k558.

ii. 1 µL of the diluted vector was added to 0.5 mL of TRIzol reagent. The mixture was shaken vigorously for 30 s to ensure complete lysis of the cells. The TRIzol solution, used in place of the ABM lysis solution, was superior for the lysis and extraction of viral RNA
iii. The sample was incubated at room temperature for 8 min, with intermittent mixing, to allow complete dissociation of the nucleoprotein complex
iv. 0.2 mL chloroform was added to the tube, mixed thoroughly by inverting several times, and incubated at room temperature for 5 min
v. The sample was centrifuged at 21,100 × g for 15 min at 4°C
vi. The colorless upper aqueous phase was transferred into a new 2 mL microcentrifuge tube
vii. The RNA was precipitated by adding 0.5 mL of isopropanol, mixing well by inversion, and the tube was incubated at room temperature for 10 min
viii. The sample was centrifuged at 21,100 × g for 15 min at 4°C
ix. If the pellet was small and not visible, the aqueous phase was carefully removed, leaving approximately 200 µL of liquid in the tube without disturbing the pellet
x. 1 mL of 75% (volume/volume) ethanol was added to the tube, and the mixture was inverted 3 – 5 times
xi. The sample was centrifuged at 211,309 × g for 10 min at 4°C
xii. The liquid was carefully aspirated and discarded, and the sample was allowed to dry for 5 min at room temperature
xiii. The RNA pellet was resuspended in 20 µL of ultrapure DNase/RNase-free water
xiv. 1 µL was used to test the RNA quantity and quality through Nanodrop spectroscopy or another appropriate method
xv. The RNA was stored at −20°C or −80°C.

The vectors titered in this study included clinical-grade samples that had been produced commercially for use in animal models at the Viral Vector Manufacturing Facility, Westmead Health, New South Wales, Australia.

2.3.3. qPCR analysis

i. Using the WPRE-containing plasmid pRRLSIN.cPPT.PGKGFP.WPRE of known concentration and serial dilutions of the plasmid were prepared in the range of 10^5 – 10^9 copies/mL
ii. Standards were amplified using the WPRE forward and WPRE reverses primers to generate a standard curve
iii. qPCR reactions were carried out using the Power Up SYBR Green Master Mix (2×) after reverse transcription, following the manufacturer's instructions
iv. Reactions were prepared by pipetting from a master mix, which was made for the appropriate number of reactions to be analyzed (standards and viral vector samples). Table 2 lists the components of one well.

2.3.4. Calculating lentiviral titer

All standards and samples were analyzed by qPCR in either duplicate or triplicate reactions. After qPCR, the mean cycle threshold (Ct) values for the standards were plotted against the deoxyribonucleic acid (DNA) copies

(copies/mL) to generate a standard curve. The mean Ct value for each sample was then interpolated on the standard curve to determine the copy number and titer (copies/mL). If the samples had been diluted before PCR, the final concentration and/or titer were adjusted according to the appropriate dilution factor.

2.4. Statistical analysis

Data (n = 9 for each method) were subjected to statistical analysis to determine whether there was a significant difference in the viral titers obtained by the two methods. A Mann–Whitney U test was applied to the data to identify significant differences. The standard curves were constructed using Graph Pad Prism7 software (United States) and were used for interpolation and titer calculations only if the R^2 value was >0.99.

Table 2. Real-time PCR components for one well and qPCR amplification parameters. (A) PCR components for one well, and (B) standard cycling conditions (primer temperature ≥60°C)

(A) PCR components for one well		
PCR component	**Volume**	
	Standards	**Samples**
PowerUp SYBR Green Master Mix (2×)	10 µL	10 µL
Forward primer	0.25 µL	0.25 µL
Reverse primer	0.25 µL	0.25 µL
Template	5 µL	1 µL
Nuclease free water	9.5 µL	13.5 µL
Total	25 µL	25 µL

(B) Standard cycling conditions (primer temperature ≥60°C)			
Step	**Temperature**	**Time**	**Number of cycles**
Reverse transcription	42°C	20 min	1
Enzyme activation	95°C	10 min	1
Denaturation	95°C	15 s	40
Annealing/extension	60°C	1 min	

Abbreviation: PCR: Polymerase chain reaction.

3. Results

3.1. Performance comparison of two lysis buffer formulations for RNA preparations

The titers of vector stocks produced using either method were not significantly different. The titer obtained from the ABM kit was 32.6 ± 8.9 × 10^8 transduction units (TU)/mL, whereas the titer from the TRIzol method was 32.1 ± 4.6 × 10^8 TU/mL (n = 9). Table 3 shows the concentration and purity of RNA isolated using the ABM kit, following the manufacturer's instructions, while Table 4 presents the same data for the five samples processed using the TRIzol method. Statistically, the A260/A280 and A260/A230 ratios were significantly lower for the samples processed with the ABM kit compared to those extracted using the TRIzol method (P < 0.0001). The expected A260/230 ratios for "pure" nucleic acid are typically in the range of 2.0 – 2.2. A ratio lower than this may indicate the presence of contaminants. The higher A260/A230 ratios obtained with the TRIzol method were not significantly different from those of the ABM kit method but were closer to the expected values for "pure" nucleic acid. These ratios did not impact the PCR process in the titering assay. Moreover, the commercial lentiviral preparations used in this study have been successfully used in *in vitro* (cell culture) and *in vivo* transduction experiments without issue.

3.2. Comparison of lentiviral vector titration using the TRIzol method and the commercial kit

Figures 2 and 3 show the qPCR results for the ABM kit and the TRIzol method, respectively, applied to nine samples for lentiviral titer determination. Following the manufacturer's instructions for the Lentivirus Titer Kit (ABM; LV900; Canada), standards (supplied with the kit) were diluted in the range of 10^9, 10^8, 10^7, and 10^6 copies/mL, followed by qPCR. Samples producing small pellets were diluted 1:1,000 times, whereas those producing large pellets were diluted 1:10,000. An R^2 of 0.998 was obtained for the standard curve. For the TRIzol

Table 3. Summary of the concentration and purity of isolated viral ribonucleic acid using the quantitative polymerase chain reaction lentivirus titer kit

Sample	Lysis buffer (µL)	Vector volume (µL)	Vector RNA concentration (ng/µL)	A260/280 ratio	A260/230 ratio	Total volume (µL)
1	18	2	30.8	0.39	0.08	20
2	18	2	48.2	0.53	0.11	20
3	18	2	30.1	0.38	0.08	20
4	18	2	43.3	0.51	0.11	20
5	18	2	29.5	0.36	0.08	20

Abbreviation: RNA: Ribonucleic acid.

Table 4. Summary of the concentration and purity of isolated viral ribonucleic acid using the TRIzol method

Sample	Vector volume (μL)	Vector RNA concentration (ng/μL)	A260/280 ratio	A260/230 ratio	Total volume (μL)
1	1	478.5	2.03	0.45	20
2	1	382.8	1.95	0.35	20
3	1	749.1	1.67	0.27	20
4	1	548.4	1.61	0.24	20
5	1	655.8	1.61	0.24	20

Abbreviation: RNA: Ribonucleic acid.

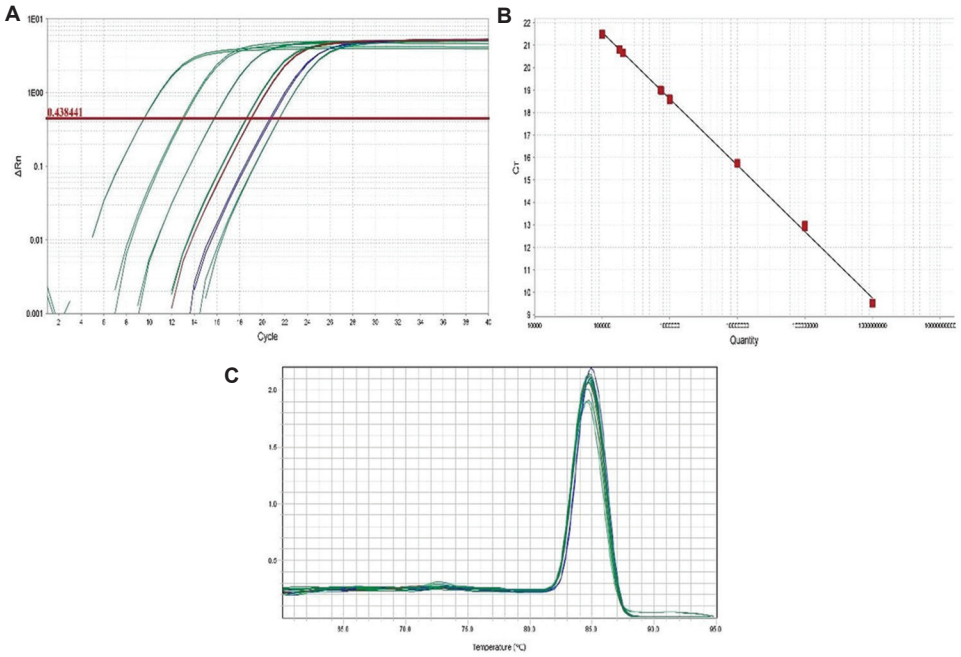

Figure 2. Quantitative polymerase chain reaction results from the Applied Biological Materials kit. (A) Amplification plots for standards and lentiviral vector samples. The amplification plots in green correspond to the standards provided in the kit (from left to right: 10^9, 10^8, 10^7, 10^6, and 10^5 copies/mL). The curves in other colors correspond to the viral samples. (B) Standard curve generated from the amplification plots of the provided deoxyribonucleic acid standards. (C) Melt curves of amplified products. R^2=0.998.

method (Figure 3), the qPCR standards were prepared using plasmid DNA containing the WPRE element, along with the corresponding WPRE-specific primers (forward and reverse). qPCR was carried out using the Power Up SYBR Green Master Mix, and an R^2 value of 0.998 was also obtained for the standard curve.

Both methods worked and produced similar results. The ABM kit calculated a titer of $6.2 \times 10^7 \pm 2.3 \times 10^7$ copies/mL, whereas the TRIzol method calculated $6.4 \times 10^7 \pm 2.3 \times 10^7$ copies/mL ($n = 5$). Analysis of the Ct values from the amplification curves indicated that the results from the two methods did not differ significantly.

4. Discussion

While a large number of methods for lentiviral titration has been described in the literature, including quantification of integrated proviral DNA, titration based on the expression of proteins linked to fluorescent marker genes, and other methods using SYBR Green-based real-time qPCR with the WPRE template[9] – similar to the approach used in this study – this paper highlights several unique aspects.

In this work, we describe the development of a rapid, efficient, and economical method for titering third-generation lentiviral vectors. Although the method itself is not entirely novel, the use of TRIzol reagent in this context is. Some traditional methods of lentiviral titration can take several days to complete,[6,7] whereas commercial kits, such as the one tested here, are quick but very expensive (AUD$1,958 for 96 samples). In addition, these kits provide reagents in specific, limited amounts and volumes. For example, the ABM

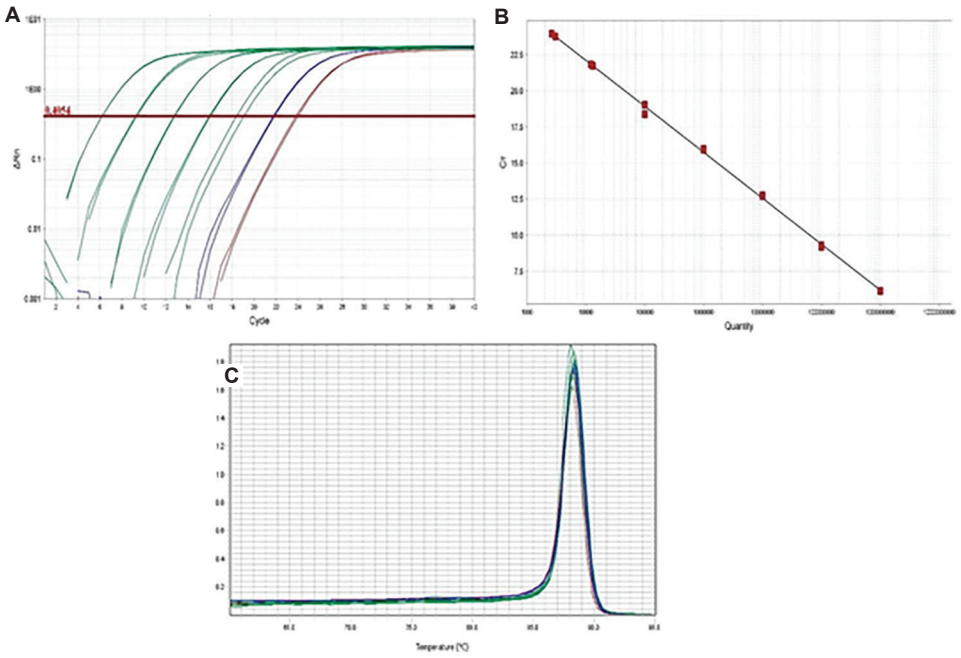

Figure 3. Quantitative polymerase chain reaction results for standards and lentiviral vector samples using the TRIzol method. (A) Amplification plots for in-house standards (green), with concentrations from left to right: 10^9, 10^8, 10^7, 10^6, and 10^5 copies/mL. The curves in other colors represent viral samples. (B) Standard curve generated from the amplification plots of the prepared deoxyribonucleic acid standards. (C) Melt curves of amplified products. R^2=0.998.

kit used in this study provides a limited volume of master mix (1.25 mL), primer mix (200 µL), standard DNA (50 uL), and virus lysis buffer (800 uL). To achieve accurate results, a viral range of 10^7 TU/mL is required, which often requires multiple dilutions of the samples (e.g., ×100, ×1,000, or ×10,000), consuming a significant amount of the sample. In contrast, although our technique also required sample dilutions to obtain the appropriate range, the use of TRIzol reagent is both more affordable and widely available (the procedure costs approximately AUD$115.00 for 96 samples). The lysis buffer used to extract RNA was also significantly better than that provided by the ABM kit. qPCR analysis using TRIzol yielded results that were accurate and not significantly different from those obtained with the ABM kit. The significantly lower A260/A280 ratios observed with the ABM kit may suggest the presence of contaminating proteins, phenol, or other impurities in those samples. Similarly, the lower A260/A230 ratios in the ABM samples may indicate contamination with guanidine hydrochloride or guanidine thiocyanate. While it was beyond the scope of this study to investigate these contaminants further, it would be of interest to test this methodology on other retroviral and lentiviral vector systems in future studies. However, we anticipate that this would not present any major issues, as the technique is simple and efficient for titering these vector systems.

5. Conclusion

We have developed a sensitive and accurate method for measuring viral titers based on the use of WPRE as the template for SYBR Green-based real-time qPCR. This method provides a reliable assessment of lentiviral copy number that is not significantly different from that obtained using a commonly used commercial kit. The use of TRIzol to isolate lentiviral vector RNA for qPCR-based titration is a novel approach. Our method is comparable in time to the commercial kit but is significantly more cost-effective, utilizing commonly available reagents found in most molecular biology laboratories. This benefit makes it an attractive alternative to more expensive commercial kits for many research laboratories. Although the technique was initially designed for small research and clinical laboratories, future studies could explore its scalability for larger-scale processing, potentially incorporating robotics or other forms of automation.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors
Formal analysis: Binhai Ren
Investigation: Binhai Ren
Methodology, Binhai Ren, Najah T Nassif
Visualization: Ann M. Simpson
Writing – original draft: Binhai Ren
Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data can be obtained from the corresponding author.

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