Quantitative Determination of Lentiviral Vector Particle Numbers by Real-Time PCR

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ABSTRACT

Here, we describe a quantitative, DNA-based, real-time PCR approach to determine the number of lentivirus particles that are present in vector preparations. In this approach, the minus strong-stop cDNA fragment that is present in viral capsids serves as template for PCR. Using this technology, we found that only 0.1%–1% of the virus particles that are present in vector preparations are infectious. The approach described here is rapid, reliable, and simple in concept and can be used to estimate both vector particles in supernatants and the number of infectious particles. Also, this approach can easily be adapted to a high-throughput system by using 96-well plates and a 2-h running time.

INTRODUCTION

In the past few years, lentivirus-based vectors have gained increasing attention because their karyophilic properties allow their use for the transduction of quiescent cells (2,4,6,8,13). Lentiviral vectors are usually prepared by transient transfection of plasmid DNAs into 293T cells. The titer of the viral preparations is estimated from the number of infected cells expressing a transduced marker gene. This procedure generally takes 4–5 days to complete.

A faster procedure is the direct estimation of the number of virus particles in the lentivirus supernatants. This can be done by dot blot analysis of RNA extracted from viral particles (7). The comparison of signal intensity to a standard RNA/DNA allows the estimation of viral particles in the test samples. However, this procedure is time consuming and only semi-quantitative. Recently, a quantitative, real-time, PCR-based strategy was used to evaluate vector particles in retroviral vector supernatants by a single-tube RT/DNA PCR with RNA isolated from vector supernatants (5,9). The critical step in this procedure is the isolation of RNA from the viral particles, which makes it susceptible to all the bias associated with RNA work. Moreover, the use of this method for the analysis of multiple probes in parallel is cumbersome and time consuming.

In this study, we established a real-time PCR for the quantification of lentiviral particles. The procedure makes use of the minus strong-stop cDNA fragment that is present in retroviral capsids as template for the real-time PCR. The protocol described here does not require an RT step and, thus, is faster than other published methods.

MATERIALS AND METHODS

Virus Production and Transduction of Target Cells

293T cells were co-transfected with 20 µg lentivirus construct pRRL-CGW-SIN, 13 µg packaging plasmid pCMV Δ8.9.3, and 7 µg VSV.G-expressing plasmid pMD.G (12) using the calcium phosphate DNA precipitation method (3). The cell culture supernatant was replaced 8 h after transfection with medium containing 10 mM sodium butyrate. The lentiviral particles were harvested in XVIVO-10 containing 1% human serum albumin (HSA) every 24 h for three consecutive days, pooled, cleared by low-speed centrifugation (1000× *g*), and filtered through a 0.45-µm, pore-size filter. The biological titer was assessed as follows: 293T cells were plated at a density of 1×10^5 cells into gelatin-coated, 24-well plates 24 h before transduction. Transduction was performed with serial dilutions of virus supernatant (10^6 to 10^1 dilutions) in the presence of 8 µg/mL polybrene for 6 h. Cells expressing enhanced green fluorescent protein (EGFP) were counted 72 h later under fluorescence microscopy. The number of integrated proviruses was determined by real-time PCR.

Real-Time PCR

The TaqMan® probes and PCR primers were developed using the Primer Express software version 1.0 (Applied Biosystems, Foster City, CA, USA). We used forward primer 5′-AGCTTGCCTTGAGTGCTTCA-3′ and reverse primer 5′-TGACTAAAAGGGTCTGAGGGA-3′ together with the probe 5′-FAM-TGCCCGTCTGTTGTGTGACTCTG-TAMRA-3′ (Applied Biosystems). Real-time PCR was performed using an ABI PRISM® 7700 Sequence Detector and software version 1.6.4 (Applied Biosystems). Amplification mixtures (50 µL) contained 1 µL virus supernatant at various dilutions, 1× TaqMan buffer, 200 µM dNTPs, 5.5 mM $MgCl_2$, 0.9 µM each primer, 200 nM TaqMan probe, and AmpliTaq Gold® (0.025 U/µL). Reaction conditions were 2 min at 50°C, an initial denaturation step at 95°C for 10 min, followed by 40 cycles of a two-step PCR (95°C for 15 s and 60°C for 60 s).

RESULTS AND DISCUSSION

We designed a real-time PCR approach to estimate the number of lentiviral particles that are present in supernatants of producer cells. As template for the reaction, we used the minus strong-stop cDNA (U5/R region) that is present within lentiviral capsids (10,11). To validate our assay and estimate the detection limit and linear range of the methodology, serial dilutions of the lentiviral transgene plasmid, pRRL-CGW-SIN (12), were made and subjected to real-time PCR in triplicate as described in Materials and Methods. A linear relationship between the copy number and fluorochrome signal intensity was observed over 5 logs (Figure 1A; 2×10^6 to 2×10^1 molecules/PCR). Less than 20 plasmid copies could be detected, whereas samples containing no plasmid DNA did not show any signal above background. Figure 1B shows the standard curve that was constructed from these data.

The next cell culture supernatants containing lentiviral vector particles obtained at different time points after transfection of 293T cells were diluted 1/10 and subjected to real-time PCR as indicated in Materials and Methods. As expected, a fluorescence signal (ΔRn) was observed in all samples (Figure 2A). Virus samples collected 24–48 h after transfection showed lower thresh-

Table 1. Comparison between Total Particle Numbers and Infectious Viruses as Determined by Real-Time PCR or by a Biological Assay

	Real-Time PCR[a] (Virions/mL)	PCR Titer[b] (Infectious Virions/mL)	Biological Titer[c] (Infectious Virions/mL)
1st Harvest	$0.1–1.2 \times 10^9$	$1.6–8.0 \times 10^6$	$0.1–1.0 \times 10^7$
2nd Harvest	$1.0–9.0 \times 10^8$	$0.9–3.0 \times 10^6$	$0.6–6.0 \times 10^6$
3rd Harvest	$3.0–8.0 \times 10^7$	$3.0–9.0 \times 10^5$	$1.0–8.0 \times 10^5$

Lentiviral particle numbers and titer as determined by real-time PCR in comparison to the biological titer on 293T cells. Particle numbers were determined as described in Materials and Methods from viral supernatants (n = 5) collected at different time points after transfection of 293T cells. For the determination of the number of infectious particles by real-time PCR, genomic DNA was extracted from transduced 293T cells and subjected to real-time PCR as described for the viral particles. To correct for the genomic DNA input, the albumin gene was amplified in parallel (1). Biological titers were determined in triplicate by endpoint dilution.

[a]From vector supernatant.
[b]From genomic DNA of transduced cells.
[c]From EGFP-positive cells.

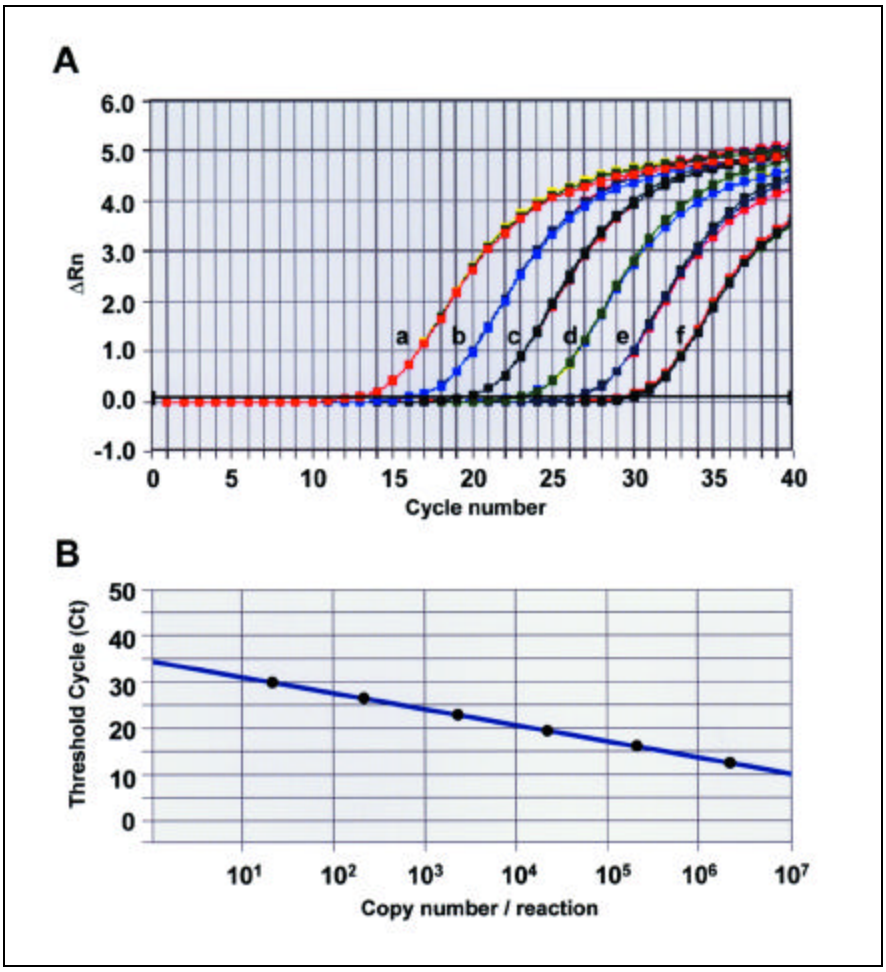

Figure 1. Standard amplification plot for titer determination of lentiviral vector using real-time PCR. (A) Serial dilutions (2×10^6 to 2×10^1 copies; curves a–f) of the lentiviral transgene plasmid pRRL-CGW-SIN in herring sperm DNA (100 µg/mL) were prepared in triplicate and subjected to real-time PCR with the probe and primer combinations indicated in Material and Methods. ΔRn denotes the normalized reporter signal (Rn), minus the baseline signal detected during the first 15 PCR cycles (5). The threshold of ΔRn is shown as a horizontal lane. (B) Standard curve constructed from the amplification plot depicted in A. The correlation coefficient is 1.000. C_t shows the fractional cycle number where a significant increase in Rn above a baseline signal (horizontal lane) can first be detected.

old cycle (C_t) values than those collected at later time points (Table 1). The number of virus particles in the supernatants was then estimated from the number of cycles required by the samples to reach the threshold value. According to this, supernatants collected 24–48 h after transfection contained 1.3×10^8 virus particles/mL, while a reduction in the amount of viral particles was observed in supernatants collected at later time points (Table 1). The same results were obtained in a series of experiments (n = 6) using two different plasmid preparations.

A particular concern of this methodology is contamination with plasmid DNA because supernatants containing virus particles were collected from transiently transfected cells. To check for the presence of contaminating plasmid DNA in virus preparations, supernatants collected from 293T cells 48 h after transfection were treated with DNase I (37°C for 15 min). The threshold values reached by these samples differ only by one PCR cycle from those obtained from untreated samples (data not shown). Similarly, no fluorescence signals were obtained from samples collected from 293T cells that were transfected with vector plasmid alone. These results suggest that the ΔRn signals obtained in the real-time PCR are derived from DNA fragments that are present within the virions and not from contaminating plasmid DNA.

The same probe and primers were used to determine the number of integrated proviral genomes, which reflects the number of infectious particles that are present in the viral preparations. Genomic DNA was extracted from trans-

duced 293T cells 72 h after transduction. Serial dilutions of genomic DNA in herring sperm DNA were subjected to real-time PCR as previously described for the viral supernatants. Values were corrected for the input DNA by amplifying albumin DNA in parallel (1). Table 1 shows the number of virus particles obtained from three independent virus harvests within the first 24–72 h after the transfection of 293T cells. From these, only 1% was found to be infectious as estimated by real-time PCR from genomic DNA of transduced cells or from the number of EGFP-positive cells. Similar results have been described for Moloney murine leukemia virus-based vectors using the direct DNA-PCR methodology to estimate the total number of virus particles versus the number of infectious particles (10).

One of the most interesting applications of this methodology is the quantification of particle numbers during the downstream processing of viral supernatants. For example, we have used this technology to estimate the recovery of virus particles after the concentration of viral samples. A virus supernatant was concentrated 8.6-fold by anion exchange chromatography (unpublished data) and, subsequently, 16.6-fold by ultrafiltration. A clear decrease in the number of cycles required to reach the C_t values was evident from the concentrated samples (Figure 2B, curves II and III), reflecting the net increase in particle numbers. The number of particles increased upon concentration from an initial 5.1×10^7 virions/mL (biological titer: 5.0×10^5 TU/mL) to 1.7×10^8 and 2.9×10^9 virions/mL (biological titer: 4.5×10^7 TU/mL), respectively. The recovery of virus particles after concentration over the anion exchange column was 38.9%, whereas no particles were lost during the ultrafiltration step.

Next, we compared the quantification of lentiviral particles by the RT/DNA-PCR method described by Sanburn et al. (9) and the direct DNA-PCR method described in this work. Viral RNA was extracted from supernatants of producer cells and reverse transcribed as described by Sanburn et al. (9), with the exception that the primer pairs described here were used for the RT/DNA-PCR. In parallel, equivalent amounts of virus supernatant

Table 2. Comparison of Lentiviral Vector Particle Numbers Using the Minus Strong-Stop cDNA or Viral RNA as Template for the Real-Time PCR

Name of Sample	PCR Titer from Vector Supernatants (Virions/mL)	PCR Titer from RNA Isolated from Vector Supernatants (Virions/mL)
Supernatant no. 1	9.0×10^8	2.1×10^9
Supernatant no. 2	6.1×10^8	1.1×10^9
Supernatant no. 3	4.5×10^8	9.0×10^8

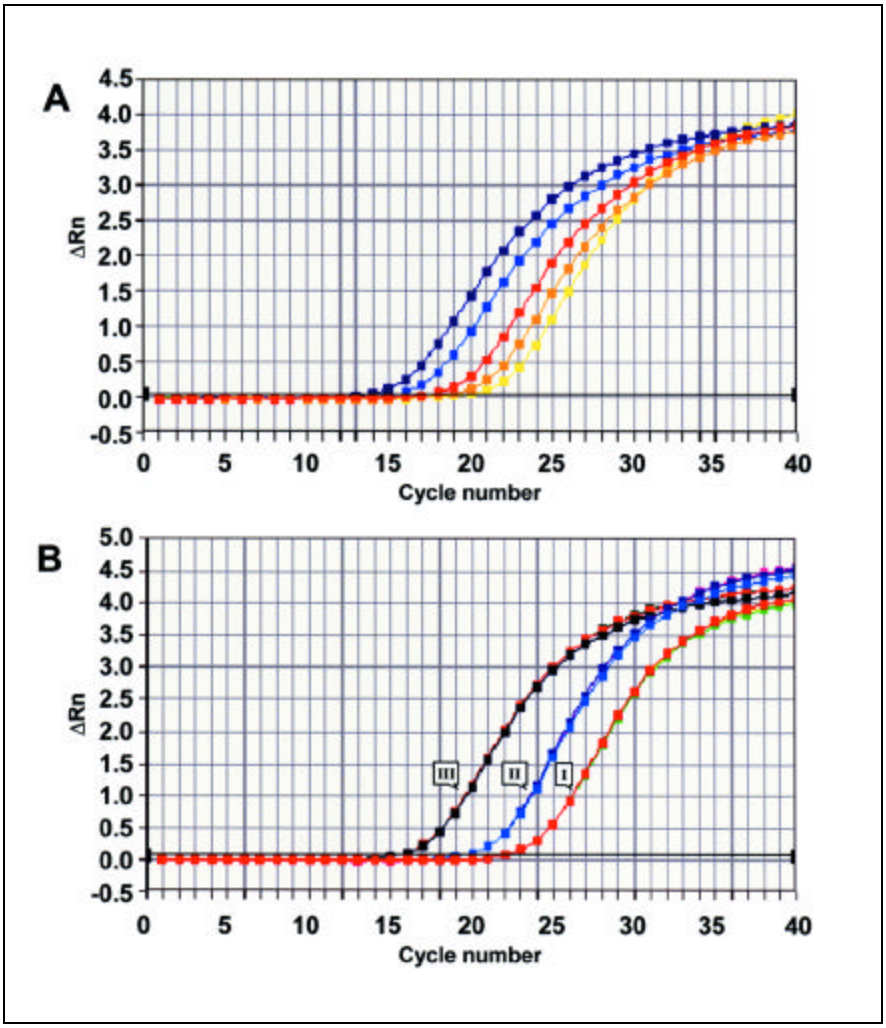

Figure 2. Determination of lentiviral vector particle numbers in supernatants of producer cells by real-time PCR. (A) 293T cells were transiently transfected with two different plasmid preparations. Viral supernatants were collected after 24 (violet and red curves), 48 (blue and orange curves), and 72 h (yellow curve), diluted 1/10, and subjected to real-time PCR. (B) Quantification of virus particles after the downstream processing of a lentiviral vector supernatant. Viral particles were concentrated by anion exchange chromatography, followed by ultrafiltration. A 1:100 dilution of the virus supernatant before and after concentration was subjected to real-time PCR as described in Materials and Methods. The figure shows the change in fluorescence (ΔRn) versus the number of PCR cyles. Curve I, unconcentrated virus; curve II, virus concentrated 8.6-fold by anion exchange chromatography; and curve III, virus concentrated 16.6-fold by ultrafiltration.

were used for the direct DNA-PCR as previously described. The number of virus particles detected by each of the methods was similar, with only twice as many particles detected when the RT/DNA-PCR was used (Table 2). The difference in numbers may be due to the fact that not all capsids contain the minus strong-stop cDNA (11). In spite of this, the direct DNA-PCR method may well be the procedure of choice for the determination of viral particle numbers because of its simplicity, reproducibility, and short processing time.

In conclusion, real-time PCR is a rapid and reliable method to determine the number of virus particles in any vector preparation and can also be used to follow the efficiency of any purification/concentration method used to increase viral titers. This information can be used to estimate not only the quality of the virus preparations but also titers, assuming that only 1% of the total number of particles is infectious. For example, we have found that lentiviral vector preparations containing less than 10^7 particles (infectious particles are approximately 10^5) are not useful for the transduction of a factor-dependent human cell line (TF1) (data not shown). This early information saves time because the biological determination of vector titer would take at least 72 h, while the real-time PCR assay described here takes only 2–3 h to complete.

The method described here can be used for any HXB2-based lentiviral vector because it relies on the presence of regions within the viral genome that are essential for virus replication and, thus, is independent of any insert within the vector. Furthermore, this technology can be adapted for the quantification of HIV-1 particles in HIV-1-infected individuals, provided the nucleotide sequence of the strong-stop cDNA is known.

Because of its simplicity and short processing time, the direct determination of particle numbers by real-time PCR can be used to follow any changes in cell culture conditions, which may improve the ratio between total and infectious particle numbers, and is also suitable for the assessment of any downstream processing of virus particles. Similarly, a large number of samples (up to 96) can be screened within 2 h, thus making this method ideal for the high-throughput analysis of stable producer cell lines.

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