Lentiviral vectors titration using real-time PCR

Amir Allahverdi¹, Fatemeh Eskandari¹, Mohammad Hossein Moghadasi¹, Mehdi Azad², Mehdi Goudarzi³, Saied Abroun*¹, Masoud Soleimani¹

¹Department of Hematology, Faculty of Medicine Sciences, Tarbiat Modares University, Tehran, Iran.
²Department of Medical laboratory sciences, Faculty of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, Iran.
³Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Science, Tehran, Iran.

*Corresponding Author: email address: abroun@modares.ac.ir (S. Abroun)

ABSTRACT

Lentiviral vectors (LVs) are useful vehicle for gene transfer to dividing and non-dividing cells and genetic manipulations. However, the use of lentiviruses in studies requires an accurate titration technique. Quantitative real-time PCR (qPCR) is a sensitive technique for the indication and quantitation of retrovirals particles. In this study, we used the qPCR for lentiviral vector titration. The puromycin resistance gene as templates for an SYBR green-based real-time qPCR method and detect lentiviral copy number integrated lentiviral DNA. Consequently, this study showed that the using of antibiotic resistance genes viral particles titration maybe efficient with highly accuracy.

Keywords Lentivirus; qPCR; Titration methods; Puromycin.

INTRODUCTION

Lentiviral vectors (LVs) are useful vehicle for gene transfer agent to dividing and non-dividing cells. Lentiviruses were produced by transfected 293T packaging cells with plasmids containing lentiviral vectors component. Lentiviral transduction efficiencies of up to 95%, with low levels of cell toxicity in transduced cells[1]. However, in order to perform transgenic manipulating, we need methods to analyze the titration of lentiviral vectors.

Various methods for titration have been reported[2, 3], including p24 antigen ELISA (enzyme-linked immune sorbent assay), RNA titers, reverse transcriptase (RT) activity, fluorescence-activated cell sorting (FACS), and quantitative polymerase chain reaction modifications (qPCR). However, some of these techniques aren’t appropriate for estimating the numbers of viral particles. For example, nonfunctional and functional particles measure by p24 antigen ELISA, RNA titration and RT-assay[4, 5]. The common and simple technique to quantify functional vector titers utilizes eGFP fluorescence and fluorescence-activated cell sorting (FACS)[6]. However, FACS analysis of transgene expression is limited to fluorescent proteins and may not recognize cells that containing multiple copies of transgenes. The most precise and difficult tittering method is detection of vector DNA integration in transduced cells[7]. Usually, the copy number of a functional vector is the number of vector particles required to transduce a single cell in a defined volume, and the number of integrated DNA lentiviral copies per cell by estimate by real-time PCR assay[8]. Antibiotic resistance gene as selection marker presents in all generation of LV and can be used for detection copy number of virus particles.

In this study, we described a quantitative quantitative PCR (qPCR) by using primer sequences that are specific for the puromycin resistance gene to measure the copy numbers of lentivirus that integrated into the genome after production and transduction.

MATERIALS AND METHODS

Lentiviral vector

EX-M0942-Lv105 plasmid was purchased from (Genecopoeia, USA). PsPAX2 and pMD2.G vectors for viral packaging were purchased from (Invitrogen, USA). These vectors were transformed in DH5α. Plasmids
were purified by using a kit (real-biotech, plasmid mini kit, Taiwan). One single copy of EX-M0942-Lv105 containing 7730 bp. So, 7.23×10^9 copies were per micro liter were obtained. This calculation was needed for viral particles titration. Serial dilutions of the plasmid (7.23×10^9 -7.23×10^7) were prepared and then lentiviral standard curve was created for determining the copy number of integrated lentiviruses.

**Cell culture**

HEK-293T cells with density of 4 x 10^6/ml were seeded at 100 mm dishes (JET BIOFIL, China) containing L-DMEM supplemented with 10% FBS. When the cells reached 80% confluency, transfection with plasmid EX-M0942-Lv105 and the two packaging viral vector (pMD2.G and psPAX2) was performed through the calcium-phosphate protocol [9]. The cell supernatant were collected every 24 hr and fresh medium containing serum was added to the cells. After 72 hours, the total viral medium was centrifuged (10min2100g) and filtrated with 0.45 micron syringe filter. The concentration of virus was performed using polyethylene glycol (PEG) 50% (Sigma, USA) and NaCl 5M (Merck, Germany) were added to the supernatant and incubated for 16-20 hours on Shaker at 4°C. Then samples were centrifuged (10 min 4100g at 4°C). Sediment dissolved at 1ml of DMEM-F12 medium and aliquoted in sterile 1.5μl microtubes that stabled for one year at -70°C or at 4°C for a week. HEK 293T cells were seeded at the density of 6x10^4/ml per well in 12-well tissue culture plates (JET BIOFIL, China). 50μl of concentrated virus were added to the cells. After 16 hr, the culture medium replaced with fresh DMEM media supplemented with 10% FBS. After 96 hr incubation (reducing the contamination from plasmid DNA) at 37°C and 5% CO2, cells were harvested and DNA was isolated.

**DNA extraction**

DNA extracted from HEK-293T cells by Spin Blood Mini Kit (Invisorb®, Germany) and stored at -20°C until use according to manufacturer’s protocol as previously described[10]. Standard samples of plasmid and oligonucleotide DNA were extracted in decimal concentrations to cover all possible measurement ranges.

**Real-time PCR**

The concentration of lentiviral vector was assessed with puromycin primer. The primer sequence was as follow, Forward puromycin 5’- TAAATATAGCTCAAGTCCCTACG -3’ and reverse 5’- TGTGGTTTCGTGTGGTAGC-3’ , 174bp.Standard curve was generated by using 10 log including serial dilution (7.23×10^7 - 7.23×10^9 particle/μl) of plasmidEX-M0942-Lv105 containing puromycin resistance gene sequence. The standard curve was calculated by Applied Biosystems Step One software v2.2. Recombinant DNA (unknown sample) that extracted from HEK-293 and standard samples run on Applied Biosystems Step One (Applied Biosystems, USA) with a SYBR-Green kit (Takara, Japan) using the in a total reaction volume of 10μl. All reactions were performed with annealing at 60°C for 40 cycles and the melting curve analysis was achieved at the end of each reaction.

For quantifying of copy numbers of viral particles Step One software v2.2. Primers specific for puromycin resistance gene were shown in Table 1. The qPCR values are mean ± SEM.

**RESULTS**

**Lentivector titration using qPCR**

The number of lentiviral vector copies was measured by the standard curve was created automatically with the Applied Biosystems (Step One v2.0) software in each run by plotting the Ct number against the copy numbers of each standard and quantification of viral DNA for unknown samples was inferred from the regression line (Figure.1A, B). Melting Curve for standard and unknown samples occurs at 93.85°C. Also we can observe that no contaminating products were present in this reaction. (Figure.1C)
Figure 1. Quantitation of lentivirus vector samples by real-time PCR. (A) Amplification plot of samples with each dilution represented in order from left to right on the graph. The x-axis represents the cycle of PCR amplification curve. (B) A comprehensive of standard curve for quantification of viral samples. Three 10-fold-dilutions (7.23×10^7-7.23×10^9 copies of the plasmid /μl) were used as standards sample and viral titer was 4.7×10^7 particle/μl (red dots showed with red dots and unknown sample showed by blue dot on diagram). (C) Melting curve analysis of the samples. The products from vector and viral samples have the same melting point peak at 93.85°C.

**DISCUSSION**

In order to define an optimal functional titering method for lentiviral vectors, we have developed another a quantitative real-time PCR method for DNA tittering by using antibiotic resistance gene primers. Antibiotic resistance genes are often encoded on lentiviral vector DNAs, and Any cell which transduced will grow in the presence of that particular antibiotic[11]. Puromycin resistance gene can be used to calculate the total functional lentiviral particles. The SYBR green-based real-time qPCR method reported here, based on puromycin as template provides, accurate, and repeatable method of lentiviral titration.
The most prevalent evaluation of functional viral titration based on protein expression like GFP marker which is not available in all lentiviral vectors [12]. A few reports have described the application of the qPCR method for lentiviral titration [3, 13]. Those studies describe a quantitative RT-PCR method using primer sequences specific for the WPRE and Gag which gives functional transgene expression of lentiviral particles. However, titration on the mRNA level may not suitable for estimating the copy number of lentivirus on the DNA level. All integration events do not lead viral gene expression, because a significant proportion of provirus integrates into regions of the genome that are not related to gene transcription and could be related to a lack of GFP expression[14]. Furthermore RNA titration method needs two pairs of fluorescent probes, which increases cost of analysis. Kutner et al. provide real-time PCR protocols to titrate lentiviral vectors based on proviral DNA copies present in genomic DNA extracted from transduced cells with fluorescent probes[6].

In this study we described a simple application of SYBR green-based method real-time PCR systems by using primer specific for puromycin resistance gene without fluorescent probe. We used high pure and concentrated plasmid (610μgr/ml) and a dilution series standards samples under high efficiency conditions. Specificity of reaction was checked with melt curve and no significant presence of primer-dimer structures. This novel protocol provides a fast method of quantifying the lentiviral concentration by means of puromycin resistance gene. Other important advantages of this approach are its simplicity, the use of common reagents available in most molecular biology laboratories.

CONCLUSION

In conclusion, the use of puromycin resistance gene as templates for real-time qPCR based on SYBR green allows for a sensitive and accurate assessment of lentiviral copy numbers at integrated lentiviral DNA level. However additional studies are needed to compare this method with other conventional titration methods.

REFERENCES