Lentiviral vectors titration using real-time PCR

Amir Allahverdi[1], Fatemeh Eskandari[1], Mohammad Hossein Moghadasi[1], Mehdi Azad[2], Mehdi Goudarzi[3], Saied Abroun*[,1], Masoud Soleimani[1]

[1]Department of Hematology, Faculty of Medicine Sciences, TarbiatModares University, Tehran, Iran.
[2]Department of Medical laboratory sciences, Faculty of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, Iran.
[3]Department 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 genetransfer 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 titeration. 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 studyshowed that theusing ofantibioticresistance genesviral particles titration maybeefficient with highly accuracy.

Keywords Lentivirus; qPCR; Titration methods; Puromycin.

INTRODUCTION

Lentiviral vectors (LVs) are useful vehicle for genetransfer agenet to dividing and non-dividing cells. Lentiviruses were produced by transfected 293T packaging cells with plasmids containaning lentiviral vectors companents. 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, fluorescenceactivated 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 techniqueto 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 oftransgenes. 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 fordetection copy number of virus particles.

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

MATERIALS AND METODS

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^{10}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 4×10^{6}/ml were seeded at 100 mm dishes**(**JET BIOFIL,china) containing L-DMEM supplemented with 10% FBS. When the cellsreached 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 24hr 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 poly ethylene 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 dissolvedat1ml ofDMEM-F12 medium and aliquted in sterile 1.5µl microtubes that stabled for one year at -70°C or at 4°C for a week. HEK 293Tcells were seeded at the density of 6×10^{4} /mL per well in 12-well tissue culture plates **(**JETBIOFIL, 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, Forwardpuromycin5'-TAAATATAGTCAATGTCCCTCAGC -3' and reverse 5'- TGTGGTTCTGTGTTGGTAGC-3',174bp.Standard curve was generated by using 10 log including serial dilution (7.23×10^{7}-7.23×10^{9} particle/µl) ofplasmidEX-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)

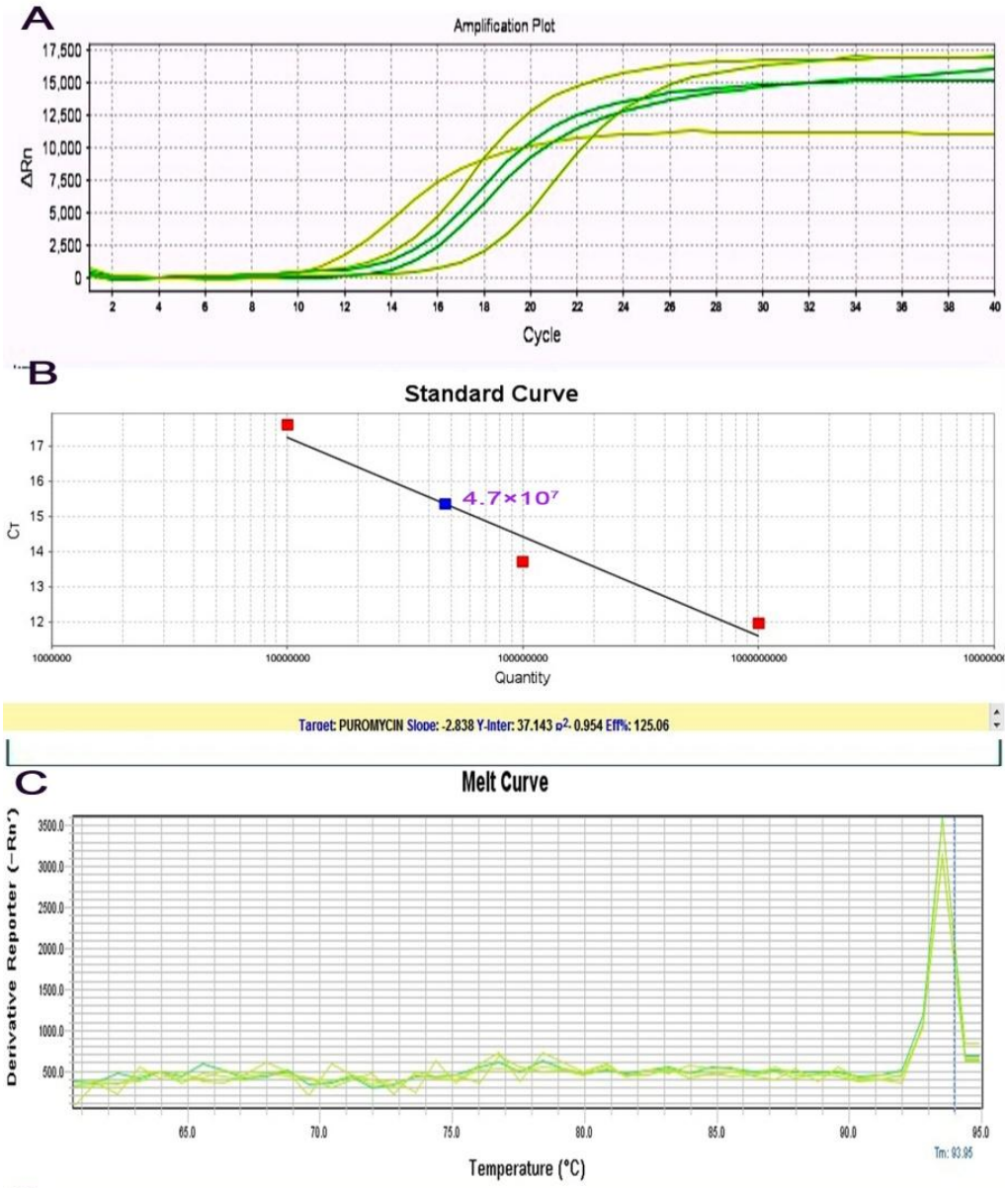

Figure1. Quantitation of lentivrus vector samples by real-time PCR. (A)Amplification plot of samples with each dilution represented in order from left to right on the graph. The x-axis represents the cycle of PCRamplification curve. (B) A reprehensive ofstandard curve for quantification of viral samples. 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 lenitiviral vector DNAs, and Any cell which transduced will grow in the presence of that particular antibiotic[11]. Puromycinresistance gene can be usedtocalculate the total functional leniviral 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 transcriptionand 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 presenceof 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 allowsfor 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

1. McMahon J, Conroy S, Lyons M, Greiser U, O'shea C, Strappe P, et al. Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral vectors. Stem Cells Dev. 2006;15(1):87-96.

2. Geraerts M WS, Baekelandt V, Debyser Z, Gijsbers R. . Comparison of lentiviral vector titration methods. BMC biotechnology. 2006;6(1):34.

3. Delenda C, Gaillard C. Real-time quantitative PCR for the design of lentiviral vector analytical assays. Gene Ther. 2005;12:S36-S50.

4. Nair A, Xie J, Joshi S, Harden P, Davies J, Hermiston T. A rapid and efficient branched DNA hybridization assay to titer lentiviral vectors. J Virol Methods. 2008;153(2):269-72.

5. Goff S, Traktman P, Baltimore D. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. J Virol. 1981;38(1):239-48.

6. Kutner RH, Zhang X-Y, Reiser J. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. Nat Protoc. 2009;4(4):495-505.

7. Riviere I, Brose K, Mulligan RC. Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. Proc of the Natl Acad of Sci. 1995;92(15):6733-7.

8. Sastry L, Johnson T, Hobson M, Smucker B, Cornetta K. Titering lentiviral vectors: comparison of DNA, RNA and marker expression methods. Gene Ther. 2002;9(17):1155-62.

9. Jordan M, Schallhorn A, Wurm FM. Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic Acids Res. 1996;24(4):596-601.

10. Sacchi P, Soglia D, Maione S, Meneguz G, Campora M, Rasero R. A non-invasive test for sex identification in Short-toed Eagle (< i> Circaetus gallicus</i>). Mol cellul probes. 2004;18(3):193-6.

11. Stewart SA, Dykxhoorn DM, PALLISER D, MIZUNO H, YU EY, AN DS, et al. Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA. 2003;9(4):493-501.
12. White SM, Renda M, Nam N-Y, Klimatcheva E, Zhu Y, Fisk J, et al. Lentivirus vectors using human and simian immunodeficiency virus elements. J Virol. 1999;73(4):2832-40.
13. Lizée G, Aerts JL, Gonzales MI, Chinnasamy N, Morgan RA, Topalian SL. Real-time quantitative reverse transcriptase-polymerase chain reaction as a method for determining lentiviral vector titers and measuring transgene expression. Hum Gene Ther. 2003;14(6):497-507.
14. MacNeil A, Sankalé J-L, Meloni ST, Sarr AD, Mboup S, Kanki P. Genomic sites of human immunodeficiency virus type 2 (HIV-2) integration: similarities to HIV-1 in vitro and possible differences in vivo. J virol. 2006;80(15):7316-21.