Development of plasmids for quantitative detection of integrated lentiviral vectors and evaluation of culture time to perform vector titer by real-time quantitative polymerase chain reaction assay

Elena Baiamonte, Mariella Bagliesi, Valentina Motta, Barbara Spina, Alice Pecoraro

UOC Ematologia per le Malattie Rare del Sangue e degli Organi Ematopoietici, Dipartimento di Oncologia ed Ematologia, AO Ospedali Riuniti Villa Sofia-Cervello, Palermo, Italy

Abstract

The accurate assessment of provirus copy number per cell (VCN/cell) is a fundamental issue in transgenesis as well as in gene therapy studies based on stably integrated vectors. To this end, real-time quantitative polymerase chain reaction (qPCR) is a powerful method but it is sensible to differences in quality or concentration of the two-plasmid preparations used for the construction of the standard curves. In order to minimize technical errors we included genome specific sequences (mouse or human) and vector specific sequences in the same plasmid. We evaluated the specificity and sensitivity of these bivalent plasmids by qPCR analysis on mouse and human genomic DNA containing a known number of a reporter lentiviral vector and we found that the system is reliable to measure up to 0.1 VCN/cell. Here we have applied this assay to measure vector titer of virus stock preparations and to determine the optimal cell passages at which viral titration effectively reflects the number of integrated vectors.

Introduction

Transgenesis studies have represented the milestone to understand the molecular bases of cellular functions. *Ex vivo* genetic correction of autologous cells is an application of transgenesis in clinical setting, and in the last decade has represented an effective challenge to cure diseases otherwise incurable. Stable genetic modification of hematopoietic stem cells (HSC), mostly achieved with retroviral vectors (RV) [either γ -retroviral, γ -RV or

lentiviral vector (LV)] is one example. This class of vectors integrated into the genomic DNA provides a permanent gene modification of the target cells and their abundant progeny, and consequently a long-term therapeutic effect. To date, HSC gene therapy mediated by RV has been successfully employed in nearly 100 patients affected by immune system or metabolic disorders and other monogenic diseases including β -thalassemia.¹ Gene transfer efficiency (percentage of genetically modified cells) and vector copy number integrated per cell (VCN/cell) are factors that contribute to the successful outcome of the therapy in terms of efficacy and safety. Thus, an accurate measure of the transduction efficiency and of the relative number of vectors per cell is mandatory in preclinical and in clinical studies; in the latter, both during the quality control performed before the infusion of modified cells into the patient and after treatment to follow the gene transfer over time. For this reason, several research groups have developed *in vitro* assays to evaluate gene transfer, and real-time quantitative polymerase chain reaction (qPCR) is the most powerful tool especially in presence of very small amount of template.² Moreover this fluorescence-based PCR method to detect nucleic acids is highly sensitive, specific and relatively easy to perform in automated machineries. The accuracy of quantification using this fluorescent based method greatly depends on the construction of standard curves prepared by a number of serial dilutions of two plasmids: one containing vector specific sequences, and the other containing cell host genome specific sequences as control. Minimal differences in the quality of these plasmid preparations or in the quantization of the plasmid concentrations may strongly affect the result. In order to minimize technical errors we constructed recombinant plasmids that include both sequences: vector specific sequences derived from the viral GAG sequence, and host cell specific sequences. Here we report that real time qPCR, using these plasmids, is highly specific and sensible to detect the VCN/cell of a GFP recombinant LV-GFP. We have applied this assay to measure vector titer of virus stock preparations and to determine the optimal cell passages for a correct evaluation of integrated lentiviral vectors.

Materials and Methods

Plasmid constructs

To generate the mouse specific plasmid first we cloned a 1665 bp PCR fragment of mouse albumin in the pGEM-Teasy (Promega) MCS (primers FW: 5'-TGCTCAGCCTCCCAAGTA-3' REV: 5'-GGTCTCCTTATCGTCAGCCT-3') obtaining the intermediate pGEM-Teasy-mALB. Then a 2122 bp PstI-NdeI GAG

Correspondence: Alice Pecoraro, UOC Ematologia per le Malattie Rare del Sangue e degli Organi Ematopoietici, AO Ospedali Riuniti Villa Sofia-Cervello, via Trabucco 180, 90146 Palermo, Italy. Tel.: +39.091.6802744 - Fax: +39.091.6880828. E-mail: a.pecoraro@ospedaliriunitipalermo.it

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restriction fragment obtained of pRLS SIN cPPT PGK eGFP WPRE LTR (LV-GFP) vector was cloned in the MCS of pGEM-Teasy-mALB obtaining the pGEM-Teasy-mALB-GAG. The same procedure was used to generate the human specific plasmid pGEM-Teasy-hALB-GAG; in this case a 1051 bp PCR fragment of human albumin was cloned (primers FW: 5'-CAGAAGGAAGTAATGTGTGT-3' REV: 5'-TGTGATAGCAATAGGGAGTT-3'). The two fragments, GAG and albumin were inserted in a way that the two regions complementary to the fluorescent probes were positioned at a distance of 2 Kb one to each other.

Cell lines

The murine erythroleukemia cell line MEL-58546, the human HeLa cell line and the human 293T cell line were grown at 37°C in the presence of 5% CO₂, in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 2 mmol/L l-glutamine, 0.1 mmol/L non-essential amino acids and the antibiotics (penicillin/streptomycin 10 g/mL) (all materials supplied by Gibco-Invitrogen, Life Science Technology, Grand Island, NY, USA).

Generation of MEL and HeLa cells containing a known vector copy number

A total of 5×10⁵ MEL and HeLa cells were

transduced with LV-GFP vector at a modest MOI to limit the VCN/cell as reported elsewhere.³ Sixteen hours after transduction the cells were washed and plated at one cell per well in 96-well flat-bottomed dishes. Individual clones were expanded and screened for the presence (GFP fluorescent cells) and for the integrated vectors by Southern blot analysis using specific radiolabeled probes.

Vector production and titration via GFP expression and via quantitative polymerase chain reaction

Viral stocks were generated by co-transfection into 293T cells of the gene transfer plasmid (LV-GFP) with the envelope plasmid (VSV-G) and the packaging plasmids (pMDLg/pRRE and pRSV-REV). Vector production was performed as reported elsewhere.⁴ Viral titers were determined by transduction of HeLa cells with serial dilution of the vector stock, and GFP expression was measured by flow cytometer (FC-500; Beckman Coulter, Inc., Brea, CA, USA) at day 5 of culture. The titer was calculated using the following formula: number of cells $\times$ dilution factor $\times$ percentage of GFP positive cells. To measure vector titers by real time qPCR an aliquot of cells after transduction was expanded and splitted every two days and genomic DNA were prepared at 8, 12, 14 and 16 days of culture that correspond to 2, 3, 4 and 5 cellular passages respectively. Virus titers were measured using the following formula: number of cells $\times$ dilution factor $\times$ VCN, using the primers described in the section below.

Quantitative real-time polymerase chain reaction analysis

Real-time quantitative PCR was performed in ABI PRISM 7900 sequence detection system (Applied Biosystems, Inc., Foster City, CA, USA) using the following primers and probes: GAG-Fwr: 5'-GGAGCTAGAACGATTTCGACAGTTA-3', GAG-Rev: 5'-GGTTGTAGCTGTCCCACTATTGTGTC-3', GAG probe: 5'-FAM-ACAGCCTTCTGATGTTTCTAACAGGCCAGG-TAMRA-3'; mALB-Fwr: 5'-GGAAAAGTGCTGCGCT-GAAG-3', mALB-Rev: 5'-TTGGCTCATGGAACCTAC-CA-3', mALB probe: 5'-VIC-AATCCTCCCGCATGC-TACGGCA-TAMRA-3'; hALB-Fwr: 5'-TGAAACAT-ACGTTCCCAAGAGTTT-3', hALB-Rev: 5'-CTC-CTTCTCAGAAAGTGTGCATAT-3', hALB probe: 5'-VIC-TGCTGAAACATTCACCTTCCATGCAGA-TAMRA-3'. Each DNA sample was run in triplicate in 25 μ L reaction volume using Taqman Universal PCR Master Mix (Applied Biosystems). Thermal cycling was started for 10 min at 95°C, followed by 40 thermal cycles of 15 s at 95°C and 1 min at 60°C. Vector copy number/cell was calculated using the following formula: (quantity mean of GAG sequence/quantity mean of ALB sequence) $\times$ 2 (or $\times$ 3). 2-fold factor was used in the case of DNA from the diploid cells (MEL); 3-fold factor was used in the case of DNA from the triploid cells (HeLa).

Results and Discussion

Plasmid constructions

We constructed a mouse specific plasmid (pGEM-Teasy-mALB-GAG) and a human specific plasmid (pGEM-Teasy-hALB-GAG) that carry the sequences from mouse or human albumin respectively, and the viral specific fragment derived from GAG sequences into pGEM-Teasy. We cloned the two fragments in a way that probe specific regions were positioned at a distance of 2 Kb one to each other.

Standard curves setting

Using pGEM-Teasy-mALB-GAG and pGEM-Teasy-hALB-GAG we setted standard curves using a serial of log dilutions starting from a concentration of 10^7 copies of plasmid/ μ L. The analysis of the amplification reactions showed that there was no quenching between the two fluorescent signals generated by the adjacent probes at the distance of 2 Kb. In both mouse and human curves, the Ct (threshold Cycle) values interpolated a straight line with slope values of 3.33 and $r^2=0.99$. These values revealed an optimal standard curve construction.

Quantization of the absolute number of integrated vector per cell

We transduced MEL and HeLa with a LV-GFP vector and we identified cells bearing the vector by flow cytometry. We screened individual clones to determine the VCN cell by Southern blot analysis (data not shown). We used genomic DNA extracted from clones that resulted in 1, 2, 3, and 4 copies/cell to perform real time qPCR assays using our bivalent plasmids. The results from several experiments using different plasmid preparations and dilutions always confirm the number

of vectors determined by Southern. In different replicates of several experiments we obtained the following mean values: 1 ± 0.2 ; 2 ± 0.1 ; 3 ± 0.3 ; 4 ± 0.5 . We then tested the sensitivity of the method by making a serial dilution (from 1:1 to 1:16) of DNA from cells containing 1 vector/cell and we were able to measure up to 0.1 VCN/cell.

Virus titration: evaluation of the optimal culture day to measure only integrated vectors in the host genome

We titrated LV-GFP vector preparations in HeLa cells by two methods: by measuring the expression of GFP transgene as percentage of fluorescent cells and by real-time qPCR using the standard curves prepared with pGEM-Teasy-hALB-GAG. Comparing the values obtained we observed that titers by real-time qPCR resulted 3 times higher than titers obtained measuring the percentage of GFP positive cells. Moreover we aimed to determine the optimal time of culture at which is convenient to measure the number of integrated vector per genome. In fact, it is known that early after transduction the viral cDNA generated by reverse transcription, circularizes and forms the so-called *DNA circles* that do not necessarily integrate in the genome and are destroyed later during the various cell divisions.⁵ For this reason we measured the VCN/cell by real-time qPCR at different days after HeLa cells transduction and we found that before day 12 the VCN/cell is over-estimated and that after this time the number of vector per cell remains constant over time. This finding was reproducible in a set of 5 different experiments. Probably after 12 days of culture (3 cell passages) the *DNA circles* are lost during cell divisions (Figure 1). Thus we conclude that, this is the correct time point after transduction at which the evaluation of VCN/cell by qPCR must be performed in order to be sure that only integrated copies are measured.

Figure 1. Vector copy number per cell (VCN/cell) analysis in HeLa cells at different time point after transduction with different viral stock preparations. VCN/cell are measured by quantitative polymerase chain reaction in DNA from HeLa cells at day 8, 12, 14 and 16 after transduction with the lentiviral vector-GFP vector. On the ordinate axis is reported the VCN/cell, on the abscissa are reported 5 different experiments (1 to 5). Day 8, 12, 14, and 16 corresponds to 2, 3, 4, and 5 cell passages respectively.

Conclusions

One of the crucial aspects in transgenesis studies is to determine the exact amount of genetically modified cells and the number of transgenes per cell. Real-time qPCR is a usefulness assay to evaluate the quantity of vector integrated in the host genome also in the presence of a minimal amount of nucleic acids.

The correct quantification by this method depends on the construction of standard curves prepared by serial dilutions of two plasmids, one carrying vector specific sequences and the other carrying host cell genome specific sequences as control.

The correct setting of an absolute standard curve procedure requires several parameters including the DNA purity since RNA contamination may inflate DNA concentration and ultimately the dilutions necessary to prepare a correct standard curve. Difference in the concentrations of the two plasmids necessary to prepare the standard curves is directly reflected in quantification errors by qPCR assay.

In order to limit technical errors we inserted the aforementioned sequences, vector specific

sequences (GAG region) and host cell genome specific sequences, in the same plasmid. In particular we generated a mouse specific plasmid and a human specific plasmid. Here we showed that there was no quenching between the two probes (GAG and ALB) when the two sequences are positioned at 2 Kb of distance one to each other. The VCN performed by real time qPCR using these plasmids was validated by Southern blot analysis and was highly specific and sensitive (up to 0.1 VCN/cell). Here we used this method for the titration of LV-GFP vector preparations and observed that titer values obtained by qPCR were 3 times higher than those obtained by measuring the frequency of GFP expressing cells.

We also determined that 12 days of culture (or 3 cell passages) after transduction are necessary to determine the correct number of integrated lentiviral vectors per cell. Before this time the values of VCN/cell result overestimated due to the presence of not integrated *DNA circles* that are subsequently lost during the cell divisions. In conclusion the bivalent plasmids we constructed are a useful tool to minimize technical errors that can occur in qPCR.

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