SHORT COMMUNICATION

Measurement of lentiviral vector titre and copy number by cross-species duplex quantitative PCR

I Christodoulou[1], P Patsali[2,3], C Stephanou[2,3], M Antoniou[3], M Kleanthous[1,2,4] and CW Lederer[1,2,4]

Lentiviruses are the vectors of choice for many preclinical studies and clinical applications of gene therapy. Accurate measurement of biological vector titre before treatment is a prerequisite for vector dosing, and the calculation of vector integration sites per cell after treatment is as critical to the characterisation of modified cell products as it is to long-term follow-up and the assessment of risk and therapeutic efficiency in patients. These analyses are typically based on quantitative real-time PCR (qPCR), but as yet compromise accuracy and comparability between laboratories and experimental systems, the former by using separate simplex reactions for the detection of endogene and lentiviral sequences and the latter by designing different PCR assays for analyses in human cells and animal disease models. In this study, we validate in human and murine cells a qPCR system for the single-tube assessment of lentiviral vector copy numbers that is suitable for analyses in at least 33 different mammalian species, including human and other primates, mouse, pig, cat and domestic ruminants. The established assay combines the accuracy of single-tube quantitation by duplex qPCR with the convenience of one-off assay optimisation for cross-species analyses and with the direct comparability of lentiviral transduction efficiencies in different species.

INTRODUCTION

Lentiviral vectors (LVs) allow curative gene therapy (GT) through genome integration, accept large payloads at high vector titre, readily transduce non-dividing cells, have a favourable safety profile compared with other integrating viral vectors and, depending on pseudotype, have wide tissue and host tropism, and thus wide-ranging applicability in the field of GT.[1] Efficacy and safety of their application are critically determined by the average vector copy number (VCN) in the corrected cell product, with more integration sites generally giving higher transgene expression levels in gene augmentation approaches, while also increasing the number of viral integration sites and thus the risk of insertional mutagenesis.[2] The desired VCN, as dictated by the required expression levels and percentage of corrected cells on the one hand and by the efficacy, long-term stability and homogeneity of transgene-derived gene expression on the other, is approximated by application of a specific number of biologically active vector particles to a known number of target cells. The actual VCN in the cell product in combination with culture and transplantation regimens, and with a possible growth advantage of corrected cells in turn determine the average VCN in the target tissues of treated patients post GT.

Initial biological vector titre, actual VCN in the cell product and post-GT VCN in the patient are usually determined by the same quantitative real-time PCR (qPCR) procedure, optimised for the respective system under study.[3] The principle of VCN determination for integrating vectors is the quantification of proviral vector genomes in extracted genomic DNA (gDNA) and their normalisation by the number of haploid host genomes against a standard curve of plasmid dilutions or of transgenic material of known copy number. The choice of LV and in particular of endogene target sequences differs considerably between laboratories, but the underlying qPCR assays almost invariably rely on (for example, TaqMan, Applied Biosystems, Waltham, MA, USA) probe technology instead of double-stranded DNA-integrating dyes, in order to minimise the detection of spurious byproducts that is common to the latter detection method. The application of labelled probes in turn opens up the possibility of duplexing LV- and endogene-specific qPCR assays labelled with different detection dyes, in order to allow same-tube detection of both sequences and with the inherent benefits of removing same-sample tube-to-tube variation and reducing reagent cost and processing time. However, to date only the Kohn group has developed a duplex protocol for LV VCN determination,[4] which has as yet not been used independently.[5–8] Strikingly and although any GT clinical trial is as a rule preceded by preclinical validation in non-human disease models, only one assay, incompatible with duplex application, has as yet been published that uses the same endogene qPCR assay for human and animal LV-GT samples.[4] Application of independent qPCR assays, however, precludes a direct comparison of VCN quantitation between species and, on the practical side, duplicates the effort and cost of assay optimisation. As an overriding dilemma, the absence of a community-wide standard quantitation assay and the habitual underdocumentation of technical details pertaining to VCN determination in outcome-focussed publications compromise the comparability of VCN and vector activity per copy between different research groups. This study therefore set out to establish

[1]Cyprus School of Molecular Medicine, Nicosia, Cyprus; [2]Department of Molecular Genetics Thalassaemia, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus and [3]King's College London, Gene Expression and Therapy Group London, UK. Correspondence: Dr CW Lederer, Department of Molecular Genetics Thalassaemia, The Cyprus Institute of Neurology and Genetics, 6 International Airport Avenue, Nicosia 1683, Cyprus.
E-mail: Lederer@cing.ac.cy
[4]These authors contributed equally to this work.
Received 17 October 2014; revised 1 April 2015; accepted 6 May 2015; published online 23 July 2015

a duplex, cross-species assay for LV VCN quantitation towards the establishment of a community standard for VCN reporting.

RESULTS AND DISCUSSION

Aiming to establish a qPCR assay with cross-species compatibility for human and murine sequences, we chose a published LV qPCR assay,[9] qLV (see Table 1), and selected the highly conserved poly (rC)-binding protein 2 (*PCBP2*) gene from the literature,[10] and a *PCBP2* region of high sequence identity and sufficient length for optimised endogene primer and probe design. The corresponding qPCR assay, qPCBP2, has perfect sequence identity of primers and probes for 35 different mammalian species (see Figure 1a), including human, apes, new- and old-world monkeys, Chinese and golden hamster, mouse, pig, domestic and wild ruminants, delphinidae, three bat species, hedgehog, shrew and armadillo, and sequence identity of the entire amplicon for 33 of these. In the following, we validate the VCN assay in human and murine cells and demonstrate the functionality of the qPCBP2 assay in three additional species, but all of the above species are either naturally or experimentally infected with lentiviruses (see, for example,[11]) and thus possible assay targets.

Duplex PCR is subject to competition of all same-tube amplicons, which for quantitative assays entails the confirmation of linear amplification for all products of interest within the prospective range and ratios of target concentrations. Following a simple workflow (see Supplementary Materials and Methods 1), we used gDNA from human and murine erythroleukaemia cell lines (HEL and MEL, respectively) to work towards a standard assay for 100 ng gDNA of lentivirally transduced sample. To this end, we tested the amplification of simplex versus duplex reactions for combinations of 50–200 ng gDNA, in order to allow for limiting sample amounts or considerable imprecision in spectrophotometrically determined DNA quantities, with lentiviral plasmid DNA (see Supplementary Materials and Methods 2). We used LV plasmid amounts equivalent to VCN 0.16 up to 20 in 100 ng of gDNA, adjusting plasmid amounts for the respective genome size and arguing that a VCN outside this range would not be used for titre calculations, and that for therapeutic applications a lower VCN would be insufficient and a higher VCN would carry an unacceptably high risk of insertional mutagenesis.

Initial attempts to establish a duplex protocol based on the kit routinely used in our laboratory for simplex detections (TaqMan 2× PCR Master Mix, Applied Biosystems) led to a depression of amplicon detection in duplex compared with equivalent simplex reactions when the alternative template became more abundant within physiological and routine experimental parameters (VCN ⩾ 1.6 or gDNA ⩾ 100 ng; data not shown). This nonlinear behaviour of duplex reactions persisted despite modification of a multitude of parameters relevant to qPCR efficiency and amplicon competition (see Materials and Methods), but

disappeared after a switch to the 2× Multiplex PCR Master Mix (Qiagen, Hilden, Germany).

The dedicated multiplex kit in combination with qLV and qPCBP2 allowed us to established a standardised duplex protocol (see Supplementary Materials and Methods 3 and 4) with extreme correspondence between expected and observed values for LV detection (Supplementary Figure S1) and gDNA detection (Supplementary Figure S2), and between simplex and duplex detection of LV (Figure 2a and Supplementary Figure S3) and gDNA (Figure 2b) within the above parameters. The qPCR duplex assay displayed average amplification efficiencies of $(96.8 \pm 3.2)\%$ for qLV and of $(93.3 \pm 4.8)\%$ for qPCBP2 across all combinations of VCNs and gDNA template types and concentrations tested, as shown in Supplementary Table S1, which also gives detailed results for efficiencies and quality of fit for individual combinations.

Finally, and using gDNA from clones of MEL and HEL cells transduced with LVs typical for GT applications, we ascertained the consistency of the measured number of integrated proviral copies with those obtained through an independent simplex assay (see Figures 1b–e and Supplementary Figure S4). Subsequent tests of the assay with gDNA from additional species (see Supplementary Figure S5, demonstrating use with bovine, caprine and ovine gDNA) confirm its potential applicability to a wide range of model systems. The duplex VCN assay has since been adopted in our laboratory for the determination of LV titres after vector production and of VCN after transduction of human peripheral blood-derived CD34^{+} cells and murine bone marrow-derived lineage-negative cells, and has proven robust and economical. Besides these benefits in its local application, the wide species compatibility of this assay allows its wider adoption and thus its establishment as a standard for comparing derived experimental and vector parameters, such as transduction efficiency and activity per vector copy, between independent lentiviral GT studies.
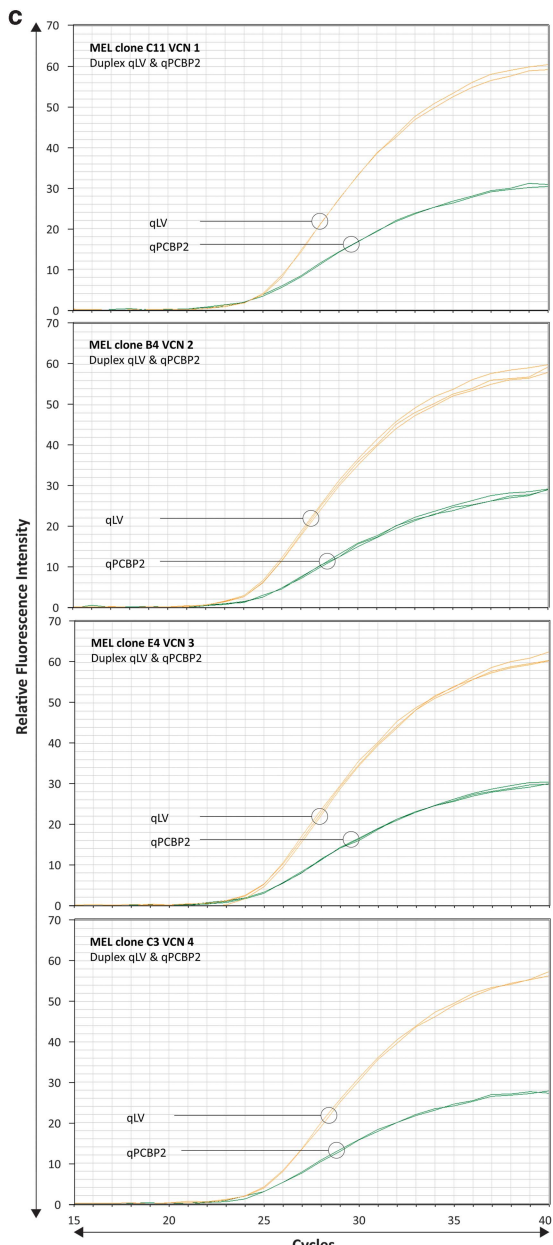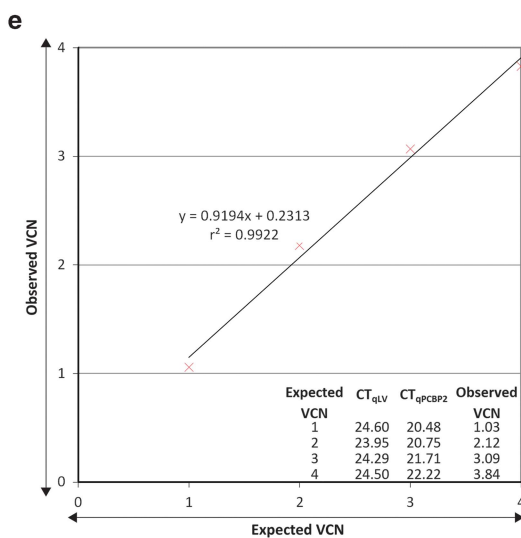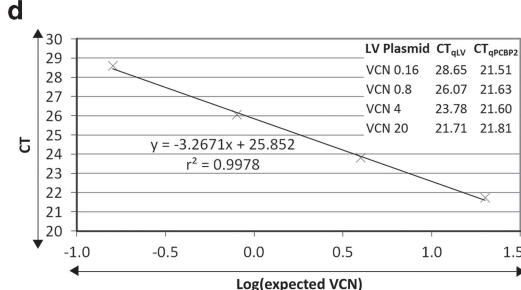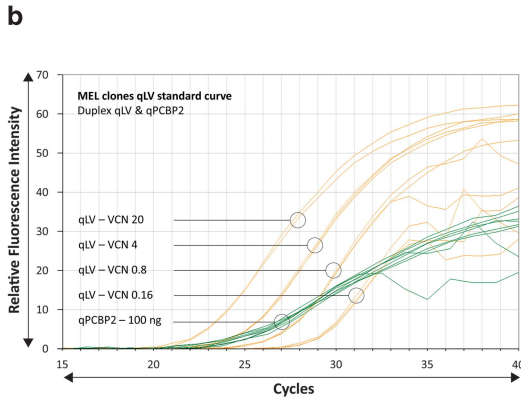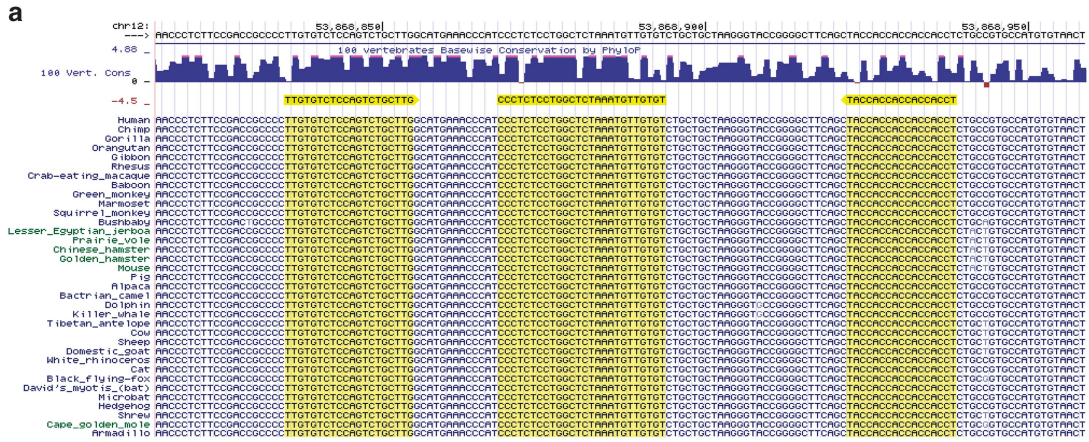
MATERIALS AND METHODS

Cell material for DNA extraction was wild-type and LV-transduced HEL (92.1.7 (ATCC TIB-180)) and MEL (48 (ATCC, Manassas, VA, USA, CRL-1913)) cell populations and clones, maintained at 37 °C, 5% CO_2 and 100% relative humidity in RPMI medium, supplemented with 1% penicillin/streptomycin, 10% fetal bovine serum and 1% L-glutamate (all from Life Technologies, Carlsbad, CA, USA). The LVs used for transduction were MA821[T87Q], a derivative of the GLOBE vector[12] encoding a T87Q mutation in the β-globin open-reading frame, and pCCL.sin.cPPT.hPGK.GFP.WPRE[13] for MEL and human erythroleukaemia cells, respectively. Cell clones were isolated by twofold limiting dilution and visual inspection of single-cell status, followed by simplex qPCR quantification using LV plasmid and gDNA standard curves. The clones used here

Table 1. Primer and probe sequences for the duplex cross-species VCN qPCR assay

qPCR assay	Target	Function	Sequence (5′ to 3′)
qLV	HIV gag	FW	TCTCGACGCAGGACTCG
		RV	TACTGACGCTCTCGCACC
		Probe	Yakima-Yellow-ATCTCTCTCCTTCTAGCCTC-*ZNA₄*-BHQ1
qPCBP2	*PCBP2* IVS 13	FW	TTGTGTCTCCAGTCTGCTTG
		RV	AGGTGGTGGTGGTGGTA
		Probe	FAM-CCCTCTCCTGGCTCTAAATGTTGTGT-BHQ1

Abbreviations: BHQ1, black hole quencher-1; FAM, 6-carboxyfluorescein; FW, forward primer; IVS, intervening sequence; probe, 5′ fluorescently labelled and 3′ quencher-linked probe; RV, reverse primer; ZNA, zip nucleic acid cationic spermine residues. Assay qLV is based on sequences published by Amendola et al.,[9] with modifications shown in italics, and recognises the gag sequence of the MA821[T87Q] transfer vector plasmid and of proviral integration sites. Assay qPCBP2 recognises an intronic *PCBP2* region of perfect cross-species identity between mouse, human and 31 additional species for the entire amplicon.

a

b

c

d

LV Plasmid	CT_{qLV}	CT_{qPCBP2}
VCN 0.16	28.65	21.51
VCN 0.8	26.07	21.63
VCN 4	23.78	21.60
VCN 20	21.71	21.81

$$y = -3.2671x + 25.852$$
$$r^2 = 0.9978$$

e

$$y = 0.9194x + 0.2313$$
$$r^2 = 0.9922$$

Expected VCN	CT_{qLV}	CT_{qPCBP2}	Observed VCN
1	24.60	20.48	1.03
2	23.95	20.75	2.12
3	24.29	21.71	3.09
4	24.50	22.22	3.84

showed a consistent quantity ratio for the LV transgene in three independent experiments. gDNA was extracted using either a standard phenol:chloroform:isopropanol–chloroform extraction method (for bovine, caprine and ovine tissues) and the FlexiGene DNA kit (Qiagen, for cell lines). The LV plasmid standard used was the corresponding 9771-bp plasmid encoding the MA821^{T87Q} transfer vector segment, with 3.26 and 3.74 pg plasmid DNA representing a VCN of 1 in 100 ng of human (3.1 Gb per haploid

genome, GRCh37) and murine (2.7 Gb per haploid genome, GRCm38) gDNA, respectively. Experimental procedures followed for vector production in HEK 293T cells, vector titration and lentiviral transduction of cells have been described elsewhere.[12,14] For LV standard curves, fivefold serial dilutions of 6.3 and 7.2 pg plasmid per 25-μl reaction were used in combination with human and murine gDNA, respectively. The curves thus represented VCN equivalents of 20, 4, 0.8 and 0.16 for 100 ng gDNA, with circular
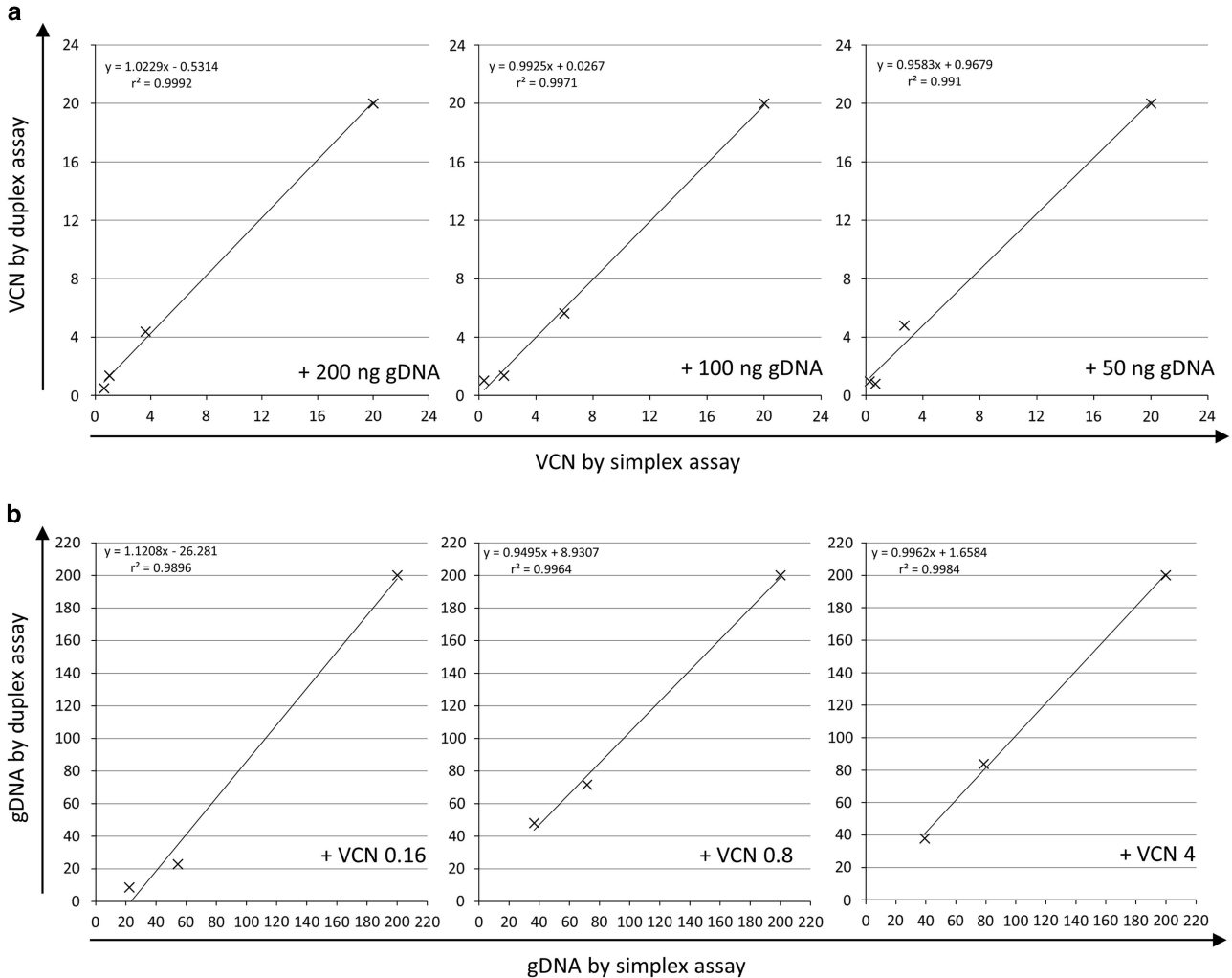

Figure 2. Simplex versus duplex quantification of LV plasmid and gDNA. The scatter plots show the correlation of template amounts determined by simplex assay with those determined by duplex assay in the presence of varying amounts of the respective alternative template, giving the formula of the trend line and the correlation (r^2) between simplex and duplex values for each plot. (**a**) Quantification of LV plasmid DNA (VCN equivalence) in the presence of varying amounts (200, 100 and 50 ng) of murine gDNA. (**b**) Quantification of murine gDNA (ng) in the presence of varying LV plasmid DNA amounts (equivalent to VCN 0.16, 0.8 and 4).

Figure 1. Sequence alignment of qPCBP2 and validation of qLV and qPCBP2 qPCR assays. (**a**) Cross-species alignment for target sequences of the *PCBP2*-specific qPCR assay *PCBP2* gene sequences were accessed using the UCSC Genome Browser (http://genome.ucsc.edu; Build GRCh37/hg19) and its Vertebrate Multiz Alignment track. Perfect sequence matches for *PCBP2* probe and primer sequences are highlighted. (**b–d**) Duplex standard curves for qLV and qPCBP2, and same-plate amplification of MEL clones with a known VCN of 1, 2, 3 and 4, respectively, for the MA821^{T87Q} LV are shown and analysed. For scatter plots, corresponding trend line formulas and curve fit values (r^2) are shown. All outlier-removed reactions of the duplex assay are shown. (**b**) Fluorescence plot of duplex qPCR for LV plasmid standard curve in the presence of 100 ng MEL gDNA, used for the absolute quantification of LV integration sites in MEL clones. The cause of plateau-phase noise is unknown. (**c**) Fluorescence plot of duplex qPCR for MEL clones with known numbers of viral integration sites. (**d**) Scatter plot of the standard curve shown in **b**. The plot shows the logarithm of expected VCNs against the automatically determined threshold cycle (CT) values and provides average CT values for each data point for qLV and qPCBP2 detection as a table insert. (**e**) Scatter plot for VCN determination of MEL clones analysed in **c**. The plot shows the expected VCN against that determined (observed) using the standard curve in **d**. The expected and observed VCN values and average CT values are additionally shown as a table insert.

and linearised plasmid (using *Sca*I-HF (New England Biolabs, Inc., Ipswich, MA, USA) for linearisation) performing equivalently. *PCBP2* was detected as an endogene in defined amounts of gDNA, both for transgenic samples and for standard curves, which comprised serial dilutions to 200, 100 and 50 ng of wild-type DNA per 25-μl reaction, either neat or laced with the MA821^{T87Q} transfer vector plasmid at the VCN equivalents given above. Quantification of viral DNA and gDNA in simplex reactions was initially performed with the TaqMan 2× PCR Master Mix (Applied Biosystems). Of the quantitative PCR assays (all from Metabion International AG, Planegg/Steinkirchen, Germany), qLV for detection of LV sequences was based on a previous publication by Amendola et al.[9] (see Table 1), and the endogene assay qPCBP2 recognised one of the *PCBP2* regions of perfect cross-species sequence identity long enough to allow primer and probe design, and identical amplicon composition (Vector NTI Advance 11.5, Life Technologies) (see Figure 1a). Additional assays qLV-b (FW 5′-TGAAAGCGAAAGGGAAACCA-3′, RV 5′-*TTG*CCGTGCG CGCTTCAG-3′; Probe *HEX*-5′-AGCTCTCTCGACGCAGGACTCGGC-3′-*TAMRA*, based on Miccio et al.[12]) and qPCBP2-b (FW 5′-CTGCAT AATCGGGCGTCAAG-3′, RV 5′-GCAGCAGATCCAGTGATGGTAACCT -3′; FAM-5′-CGCCAAAAT<u>C</u>AATGAGATCCGTCAGATGTCT-3′-TAMRA, which inadvertently co-amplifies a human pseudogene, *PCBP2P2*, with a single mismatch (underlined) in the probe sequence) were additionally used in simplex reactions and during the optimisation of duplex assays. Attempts to establish a reliable duplex assay for the above range of gDNA and LV amounts included the independent modulation of many PCR parameters, including probe concentration (50, 100, 250 or 375 nM), primer concentration (300, 600 or 900 nM), MgCl$_2$ concentration (3–6 mM), dNTP concentration (200–400 μM), annealing temperature (60, 62 and 64 °C) and the use of alternative qPCR assays qLV-b with qPCBP2-b and qLV with qPCBP2.

All results shown in this study are based on the 2× Multiplex PCR Master Mix (Qiagen), using qPCR assays qLV and qPCBP2 in triplicate 25-μl reactions (1× Master Mix, 250 nM for each probe and 300 nM for each primer), and the 7900HT Fast Real-Time PCR System running SDS v2.4 and the standard PCR programme of 2 min 50 °C, 10 min 95 °C, 40× (15 s 95 °C, 1 min 60 °C) (all from Applied Biosystems). To aid reproduction of the analyses performed here, a simple workflow chart (Supplementary Materials and Methods 1) and Excel (Office 2010, Microsoft, Redmond, WA, USA) spread sheets for the calculation of LV plasmid and gDNA standard curves (Supplementary Materials and Methods 2), for the preparation of reactions for 96-well plates (Supplementary Materials and Methods 3) and for the postrun determination of VCN (Supplementary Materials and Methods 4), respectively, are provided as Supplementary Information. In brief, to avoid bias in the quantification of samples, all threshold cycle (CT) values were determined using automatic calculation of threshold levels in SDS v2.4. All samples were run in triplicate reactions and outliers removed automatically before statistical analyses (all Microsoft Excel), choosing a deviation of 2 s.d. from the triplicate median as a cutoff value. Statistical comparisons (all Microsoft Excel) between expected and observed VCN or gDNA values by duplex assay, and between simplex and duplex results for observed VCN or gDNA values were based on outlier-removed averages of triplicate repeats. For VCN standard curves and quantification of LV insertion sites in cellular clones, relative and absolute VCN values, respectively, were calculated using the ΔΔCT method values with *PCBP2* as the calibrator gene. For analyses of expected versus observed *PCBP2* values, no additional calibrator gene was used and relative *PCBP2* quantities were instead determined by subtracting the smallest *PCBP2* CT value n from all the automatically determined CT values x_i of the same run to determine the relative quantity $2^{(x_i - n)}$ for each sample. (This can be emulated in Supplementary Materials and Methods 4 by filling

in *PCBP2* values for the test gene (column C)] and setting all CT values for the calibrator (column H) to 0).

Amplification efficiencies and the squared Pearson's correlation coefficient r^2 of the curve fit for the log-linear phase of the qPCR amplification were calculated using the program LinRegPCR (v2015.2),[15] by exporting ΔRN values from the SDS v2.4 acquisition software, dismissing outlier-removed reactions and using at least four data points per reaction for fit calculations.

VCNs of LV clones in cell lines were isolated by twofold limiting dilution and selection of clones by visual confirmation of single-cell status after seeding. Absolute copy numbers were determined using standard curves of gDNA dilutions and LV-plasmid dilutions in simplex reactions based on qLV and qPCPB2, and, for MEL cells, additional LV and *PCBP2* qPCR assays. Clones moreover consistently showed the expected LV quantity ratios in relative qPCR comparisons in three independent experiments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Pluta K, Kacprzak MM. Use of HIV as a gene transfer vector. *Acta Biochim Pol* 2009; **56**: 531–595.

2 Cavazza A, Moiani A, Mavilio F. Mechanisms of retroviral integration and mutagenesis. *Hum Gene Ther* 2013; **24**: 119–131.

3 Schuesler T, Reeves L, Kalle C, Grassman E. Copy number determination of genetically-modified hematopoietic stem cells. *Methods Mol Biol* 2009; **506**: 281–298.

4 Cooper AR, Patel S, Senadheera S, Plath K, Kohn DB, Hollis RP. Highly efficient large-scale lentiviral vector concentration by tandem tangential flow filtration. *J Virol Methods* 2011; **177**: 1–9.

5 Romero Z, Urbinati F, Geiger S, Cooper AR, Wherley J, Kaufman ML et al. beta-globin gene transfer to human bone marrow for sickle cell disease. *J Clin Invest* 2013.

6 Joglekar AV, Hollis RP, Kuftinec G, Senadheera S, Chan R, Kohn DB. Integrase-defective lentiviral vectors as a delivery platform for targeted modification of adenosine deaminase locus. *Mol Ther* 2013; **21**: 1705–1717.

7 Giannoni F, Hardee CL, Wherley J, Gschweng E, Senadheera S, Kaufman ML et al. Allelic exclusion and peripheral reconstitution by TCR transgenic T cells arising from transduced human hematopoietic stem/progenitor cells. *Mol Ther* 2013; **21**: 1044–1054.

8 Karumbayaram S, Lee P, Azghadi SF, Cooper AR, Patterson M, Kohn DB et al. From skin biopsy to neurons through a pluripotent intermediate under Good Manufacturing Practice protocols. *Stem Cells Transl Med* 2012; **1**: 36–43.

9 Amendola M, Passerini L, Pucci F, Gentner B, Bacchetta R, Naldini L. Regulated and multiple miRNA and siRNA delivery into primary cells by a lentiviral platform. *Mol Ther* 2009; **17**: 1039–1052.

10 Makeyev AV, Chkheidze AN, Liebhaber SA. A set of highly conserved RNA-binding proteins, alphaCP-1 and alphaCP-2, implicated in mRNA stabilization, are coexpressed from an intronless gene and its intron-containing paralog. *J Biol Chem* 1999; **274**: 24849–24857.

11 Gifford RJ. Viral evolution in deep time: lentiviruses and mammals. *Trends Genet* 2012; **28**: 89–100.

12 Miccio A, Cesari R, Lotti F, Rossi C, Sanvito F, Ponzoni M et al. In vivo selection of genetically modified erythroblastic progenitors leads to long-term correction of beta-thalassemia. *Proc Natl Acad Sci USA* 2008; **105**: 10547–10552.

13 Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 2000; **25**: 217–222.

118

14 Roselli EA, Mezzadra R, Frittoli MC, Maruggi G, Biral E, Mavilio F *et al.* Correction of beta-thalassemia major by gene transfer in haemato-poietic progenitors of pediatric patients. *EMBO Mol Med* 2010; **2**: 315–328.

15 Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 2003; **339**: 62–66.

Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)