Rapid Lentiviral Vector Producer Cell Line Generation Using a Single DNA Construct

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Stable suspension producer cell lines for the production of vesicular stomatitis virus envelope glycoprotein (VSVg)-pseudotyped lentiviral vectors represent an attractive alternative to current widely used production methods based on transient transfection of adherent 293T cells with multiple plasmids. We report here a method to rapidly generate such producer cell lines from 293T cells by stable transfection of a single DNA construct encoding all lentiviral vector components. The resulting suspension cell lines yield titers as high as can be achieved with transient transfection, can be readily scaled up in single-use stirred-tank bioreactors, and are genetically and functionally stable in extended cell culture. By removing the requirement for efficient transient transfection during upstream processing of lentiviral vectors and switching to an inherently scalable suspension cell culture format, we believe that this approach will result in significantly higher batch yields than are possible with current manufacturing processes and enable better patient access to medicines based on lentiviral vectors.

INTRODUCTION

Clinical grade lentiviral vectors are commonly produced by transient transfection of plasmids encoding vector components into adherent HEK293T cells.[1,2] This entails high costs and long timelines to source plasmid DNA and difficulties with scaling adherent cell culture beyond low tens of liters of harvested medium. An attractive alternative would be a suspension-adapted stable producer cell line in which all vector-encoding DNA was stably integrated into the host cell genome. A number of groups have reported attempts to generate stable producer cell lines for lentiviral vector manufacture.[3] These approaches have had limited success, with cell line stability only achieved with low-toxicity envelope glycoproteins rather than the widely used vesicular stomatitis virus envelope glycoprotein (VSVg)[4] or with adherent cell lines.[5] Previous approaches to lentiviral vector producer cell line generation were typically based on sequential stable transfection or transduction of DNA encoding each vector component (transfer vector, gagpol, rev, and VSVg) into host cells at separate genomic loci. This strategy entails lengthy cell line development campaigns and a high risk that genetic or transcriptional instability could occur in at least one locus, resulting in a loss of productivity. To avoid this, we adopted a novel strategy in which all vector components are expressed from a single large DNA construct introduced into host cells in a single stable transfection (Figure 1A).

As a third-generation lentiviral vector system,[6] the vector components in our construct are encoded by four separate transcription units, each with its own promoter and polyadenylation signal to prevent generation of replication-competent lentivirus (RCL) particles (Figure 1B). A single copy of each transcription unit is cloned into the construct. To protect host cells from vector component cytotoxicity, the gagpol, rev, and VSVg cassettes are each inducibly transcribed from a human cytomegalovirus immediate early (CMV) promoter containing two tet operator (tetO) sequences.[7] Transcription from these promoters is blocked by the binding of a codon-optimized *E. coli* tetracycline repressor (TetR) protein and can be activated by the addition of doxycycline to the cell culture medium.[8] Transcription of the transfer vector genome is driven by a constitutive CMV promoter as in pCCL.[6] To prevent promoter interference, transcription units are separated by two copies of the 1.2-kb chicken beta globin HS4 (cHS4) insulator.[9,10] A zeocin selection marker is expressed under the control of an attenuated internal ribosome entry site (IRES).[11] A low-copy bacterial artificial chromosome (BAC) replication origin enables replication of the construct in *E. coli*.[12] The construct is approximately 45 kb in size, depending on the size of the transgene to be delivered. BACs encoding EGFP (BAC-EGFP) or human beta globin[13] (BAC-GLOBE) were cloned as shown in Figure 1B.
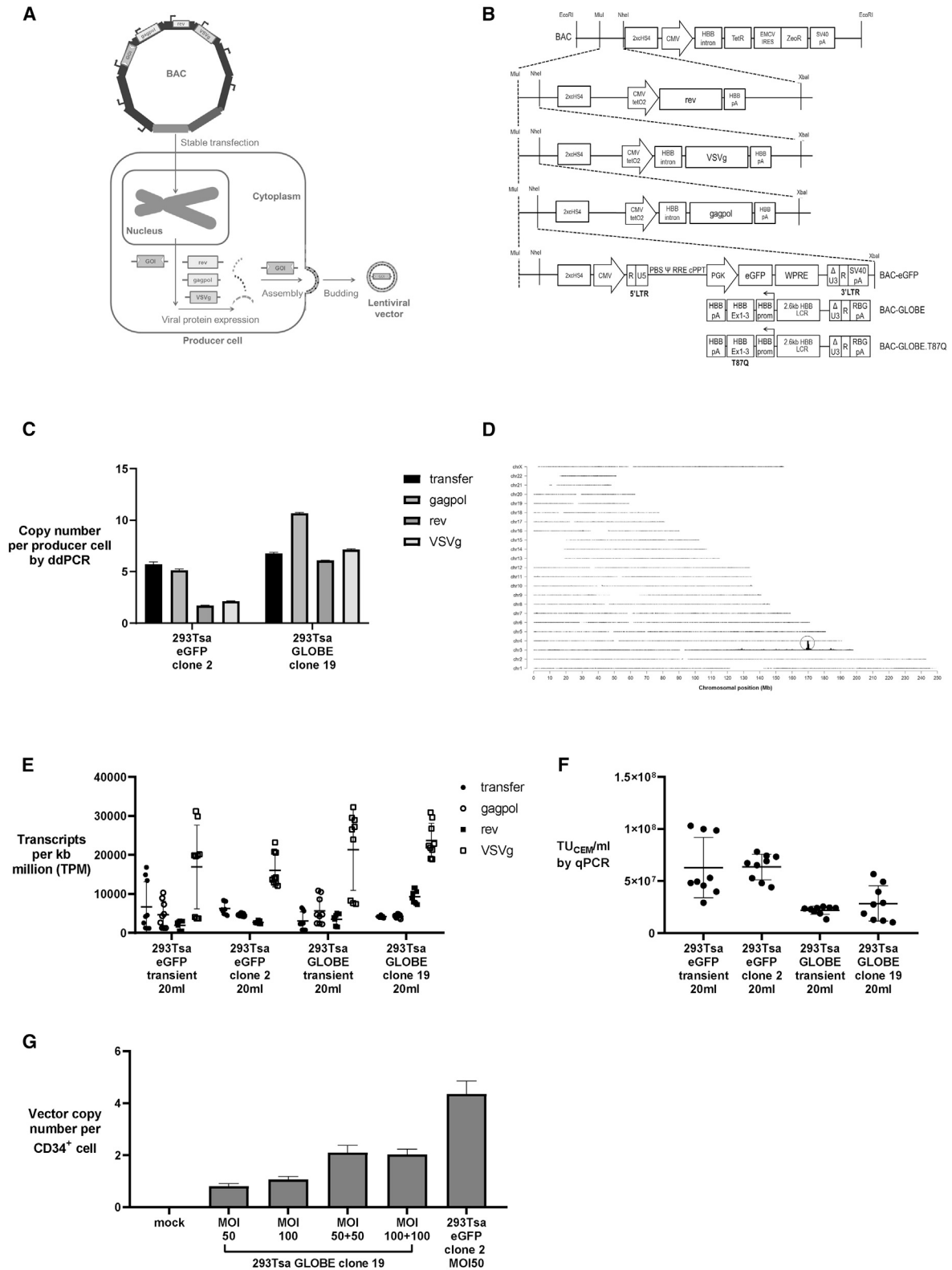
RESULTS

BAC-EGFP was transfected into adherent 293T cells, which were then selected with zeocin to generate polyclonal stable pools. Vector production was induced by the addition of doxycycline, and the media

were harvested 48 h later for titration. The functional titer of the unconcentrated vector was greater than 10^7 TU_{293T}/mL (TU, transducing units; Figure S1A) by EGFP flow cytometry, similar to titers routinely obtained by transient transfection by us (data not shown) and others.[14]

Monoclonal cell lines were generated by stable transfection of suspension-adapted 293T (293Tsa) cells with BAC-EGFP or BAC-GLOBE followed by zeocin selection, single-cell cloning in adherent cell culture, and screening tens of clones to identify those with high yields and fast growth. These clones were suspension adapted by the removal of serum from the cell culture medium to produce the monoclonal cell lines 293Tsa EGFP clone 2 and 293Tsa GLOBE clone 19.

The copy number per producer cell of integrated BAC DNA in these clones was quantified by droplet digital PCR (ddPCR) using primers for each vector component (Figure 1C). The copy numbers of each component varied between clones within the range of 1 to 10 copies per cell. Integration sites were identified by targeted locus amplification (TLA).[15] For 293Tsa EGFP clone 2, a single integration site was identified at chr3:169,967,149–169,967,179 (Figure 1D), whereas two integration sites were detected in 293Tsa GLOBE clone 19 at positions chr1: 146,557,007 and chrX: 127,363,436 (Figure S1B). The integration of multiple copies of BAC DNA at each locus suggests episomal concatemerization of BAC DNA occurred prior to random integration into the host cell genome.[16] In our process, circular BAC DNA was transfected and so is expected to be rearranged by host DNA repair proteins to produce linear integrated DNA. Such a rearrangement process is a likely cause of the 1:1:1:1 transfer:gagpol:rev:VSVg DNA stoichiometry in the BAC prior to transfection not being maintained in integrated BAC DNA in the stable clones.

To compare these stable clones with transient transfection, vector was produced in shake flasks from 293Tsa EGFP clone 2 and 293Tsa GLOBE clone 19 in parallel with transient transfection of 293Tsa cells with a third-generation 4-plasmid system encoding the same transgenes. Producer cell RNA levels of each vector component were quantified by RNA sequencing (RNA-seq). No significant difference between transient or stable expression was observed for the RNA expression levels of each component with the notable exception of elevated rev expression in 293Tsa GLOBE clone 19 (rev, 2.88-fold higher than transient, p = 0.0003) (Figure 1E). Interestingly, the flask-to-flask variability of RNA expression levels was significantly lower with stable expression than transient expression (p < 0.001).

469- to 2,885-fold increases in BAC-derived transcript abundance were observed upon induction with doxycycline (Figures S1C–S1E), similar to previous reports with this promoter.[7] BAC-derived transcripts were among the most abundant in the cell and accounted for 3%–4% of all polyadenylated transcripts (Figures S1F–S1H). By comparison, provirus-derived transcripts were previously reported to account for 0.7% of transcripts in cells infected with replicating HIV-1 in vitro.[17] No significant differences in either functional or physical titer were observed between transient and stable production (Figure 1F). Unlike flask-to-flask RNA expression level variability, titer variability was not significantly different between transient and stable production methods (TU, p = 0.2419; and p24, p > 0.9999). It is possible that the low variability of RNA expression did not transmit through to titer because shake flasks are a relatively uncontrolled environment, so significant variability is reintroduced during the cell culture process. It may be possible to address this by culturing producer cells in more controlled conditions such as a bioreactor that actively controls critical process parameters such as pH, oxygen, and other factors.

To investigate the functionality of vector produced from the stable clones in an ex vivo gene therapy model, GLOBE vector produced in shake flasks was concentrated by ultracentrifugation to a titer of 5.22×10^8 TU_{CEM}/mL and used to transduce primary human CD34+ cells at multiplicities of infection (MOIs) of 50 or 100 TU per target cell in either single (1 hit) or repeated (2 hit) transductions. Vector copy number (VCN) in the transduced cells was determined by qPCR (Figure 1G). The 2-hit transductions resulted in a VCN of 2 copies per cell, indicating that vector produced from these stable cell lines can efficiently transduce this challenging primary cell type.

The T87Q antisickling amino-acid substitution[18] was introduced into the beta globin transgene to generate BAC-GLOBE.T87Q so that the same producer cell line could be used for the clinical supply of lentiviral vector for treatment of both beta thalassemia and sickle cell disease. A cell line development campaign was then performed as shown in Figure S2A. In order to rank the resulting 12 cryobanked clones, vector was produced in 15-mL single-use stirred-tank microbioreactors, and functional and physical titers were assessed (Figure 2A). The functional and genetic stability of the highest titer clones was assessed by continuous shake flask culture over several weeks, with regular subculturing to regulate cell density. At three time points, parallel cultures were split off for doxycycline induction and titration of harvested vector (Figure 2B). In addition, uninduced producer cell genomic DNA was collected at the same three time points for BAC

Figure 1. Cloning and Testing of BAC Constructs
(A) Concept of generating stable producer cell lines by stable transfection of BAC DNA and use of cell lines for lentiviral vector production. GOI, gene of interest. (B) Design and cloning of the BAC constructs. (C) Integrated BAC DNA copy number in two monoclonal stable producer cell lines. Columns and error bars indicate the mean and 1 standard deviation, respectively, from 3 independent ddPCR reactions. (D) Integration site mapping of BAC DNA in the 293Tsa EGFP clone 2 cell line by TLA sequencing. (E) RNA expression levels in suspension adapted 293T cells transiently transfected in shake flasks with a 4-plasmid lentiviral vector system and in stable producer cell lines induced with doxycycline in 3 batches with 3 flasks per condition. Individual points indicate RNA levels for each of the 9 flasks; mean of all 9 flasks ± 1 standard deviation is also plotted. (F) Unconcentrated functional and physical lentiviral vector titers obtained from transient transfection and stable producer cell lines in shake flasks. Individual points indicate titers for each of the 9 flasks; mean of all 9 flasks ± 1 standard deviation is also plotted. (G) VCN in primary human healthy donor CD34+ cells transduced with concentrated lentiviral vector obtained from stable producer cell lines with MOI of 50 or 100 in a 1-hit transduction protocol and with MOI of 50+50 or 100+100 in 2-hit transduction protocol. Columns and error bars indicate the mean and 1 standard deviation, respectively, from 3 independent transductions.
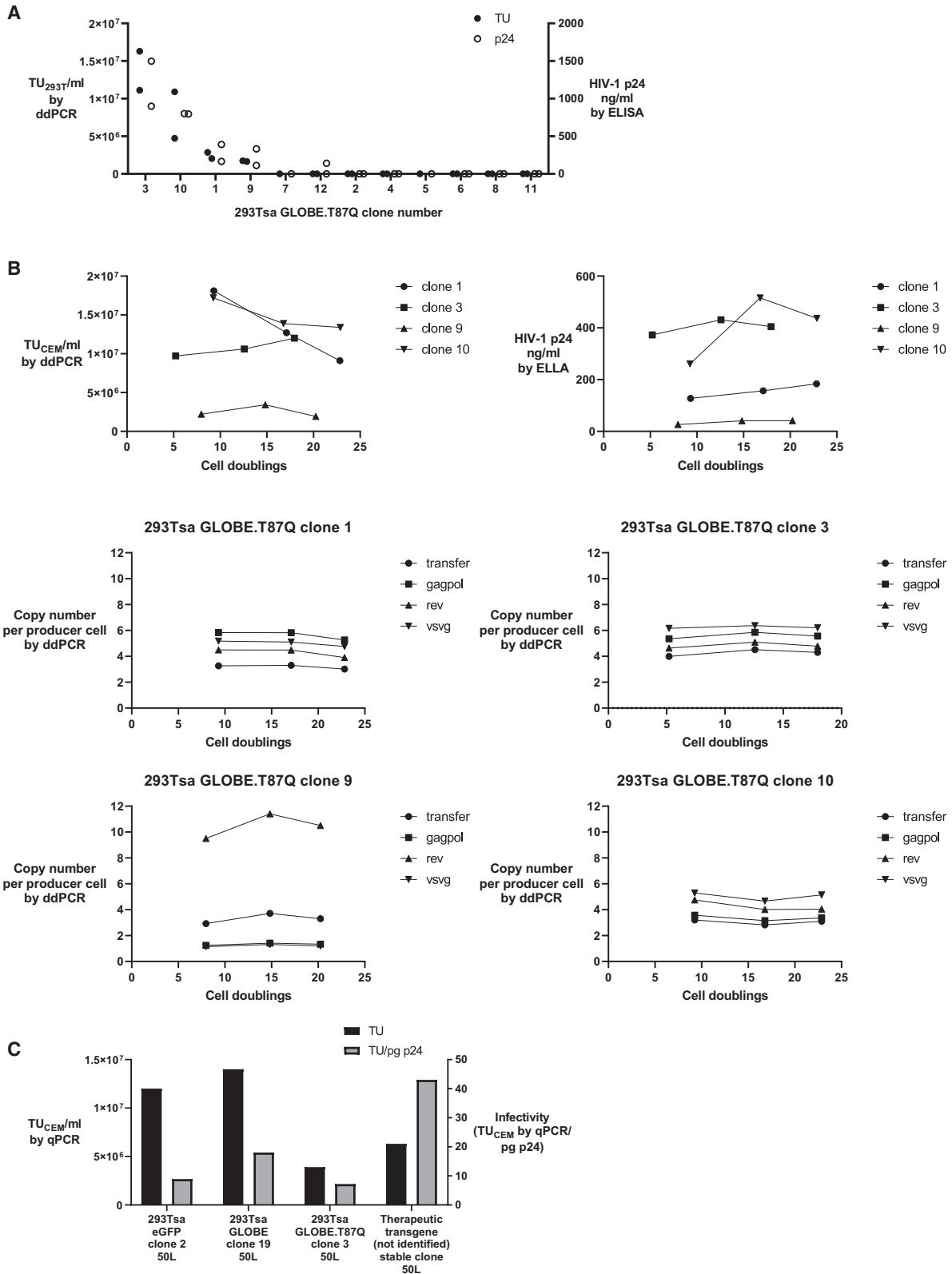
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copy number quantification (Figure 2B). For context, 20 cell doublings starting from a single cryovial containing 10^7 producer cells would be sufficient to fill a 5,000-L bioreactor at a cell density of 2×10^6 cells per milliliter. 293Tsa GLOBE.T87Q clones 3, 9, and 10 underwent practically no significant changes in functional or physical titer throughout the stability assessment, whereas a fall in functional titer was observed with clone 1 (p = 0.0011). All four clones were genetically stable at the level of BAC copy number, suggesting that neither loss of integrated DNA encoding vector components[19] nor amplification of transfer VCN through superinfection[20] were occurring to a significant extent. BAC integration site analysis was also performed for clones 1, 3, and 10, and a single integration site was detected in all cases (Figure S2B).

In parallel with the work described so far, preliminary efforts were made to develop and optimize an upstream process based on single-use stirred-tank bioreactors for the clinical supply of lentiviral vector. During this period of process development, 293Tsa EGFP clone 2, 293Tsa GLOBE clone 19, 293Tsa GLOBE.T87Q clone 3, and a producer cell clone generated for a further therapeutic transgene were seeded into 50-L single-use stirred-tank bioreactors, and unconcentrated vector batches were harvested after 48 h. Results from the 50-L batches, which yielded the highest functional titers, are shown in Figure 2C. Functional titers for unconcentrated vector were in the range of $0.4–1.4 \times 10^7$ TU_{CEM}/mL, and infectivities were in the range of 7–43 TU_{CEM}/pg p24. By comparison, vector batches generated in a good manufacturing practice (GMP) facility for the treatment of metachromatic leukodystrophy were previously reported by Biffi et al.[21] to have functional titers for unconcentrated vector at harvest in the range of $1.0–1.4 \times 10^7$ TU_{CEM}/mL and infectivities in the range of 30–65 TU_{CEM}/pg p24. In the pediatric ex vivo gene therapy protocol described by Biffi et al., treatment of a 10-kg patient with 10^7 CD34$^+$ cells per kilogram transduced at an MOI of 200 TU_{CEM} per cell would require a vector dose of approximately 2×10^{10} TU_{CEM} to treat each patient. Assuming a 30% downstream process recovery similar to that reported by Biffi et al., one 50-L batch generated using a stable producer cell line yielding 1.4×10^7 TU_{CEM}/mL as demonstrated in Figure 2C could supply 2×10^{11} TU_{CEM}, enough to treat 10 patients from a single batch. For comparison, the 25-L adherent cell transient transfection-based process reported by Biffi et al. yielded $0.49–1.1 \times 10^{11}$ TU_{CEM} per batch.

Lastly, in order to determine whether the clones were producing RCL, samples of both harvested and final concentrated vector generated from 12 vector batches made with 293Tsa EGFP clone 2, GLOBE clone 19, and GLOBE.T87Q clones 1, 3, 9, and 10 were tested for

RCL using an assay based on inoculation of the permissive C8166-45 cell line for RCL amplification followed by product-enhanced reverse transcriptase (PERT) assay for detection (a summary of test results is given in Table 1; details of individual batches are given in Table S1). No RCL was detected in these samples or in any other sample derived from our stable producer cell lines to date.

DISCUSSION

The stable producer cell line development method we have demonstrated can be performed using conventional plasmid subcloning and mammalian tissue culture techniques and does not require automation for high-throughput clone screening, so it should be applicable in many academic or industrial laboratories. These cell lines could potentially be used in a wide range of cell culture formats such as cell factories or stirred-tank or fixed-bed bioreactors, and upstream process conditions no longer need to be limited to those that permit efficient transient transfection, leaving greater flexibility to optimize processes for higher quality and yield. While the functional titers and infectivities we observed are similar to previous reports of vector used in clinical trials, the titers we observed in shake flasks suggest that it may be possible through further optimization of stirred-tank bioreactor conditions to significantly increase volumetric titers. Stirred-tank bioreactors are a scalable format, and mammalian cell culture up to 10,000- to 20,000-L scale is currently in use in the biopharmaceutical industry.[22]

Further work is required to evaluate this platform for a wider range of transgenes, particularly in highly active areas of clinical investigation such as chimeric antigen receptor T cell (CAR-T) therapies. We anticipate that the platform should be compatible with strategies for transgene suppression in producer cells such as the recently reported TRiP System.[23]

The architecture of this cell line development platform offers a high degree of flexibility that should help it to develop quickly in the future. We are currently generating BAC constructs in which vector components can be replaced in a single cloning step in order to enable alternative pseudotypes or improved versions of gagpol or rev to be introduced. Future BAC constructs could potentially be rapidly generated by multiple fragment assembly[24] or simply gene synthesized, as 45 kb is comfortably within the size range currently offered commercially. Efficiently screening alternative host cell lines, such as other HEK293 derivatives or even non-293-based cell lines, is feasible, given the single transfection approach. The modular design of the BAC should simplify host cell engineering strategies based on overexpression or knockdown of host genes to improve vector quantity or quality.[25]

Figure 2. Cell Line Development and Upstream Process Scaleup

(A) Unconcentrated functional and physical lentiviral vector titers obtained from stable producer cell line clones run in duplicate ambr 15-mL microbioreactors at the end of a cell line development campaign. (B) Unconcentrated functional and physical lentiviral vector titers and integrated BAC DNA copy number obtained from stable producer cell line clones run in single-shake flasks. (C) Unconcentrated functional lentiviral vector titers and infectivities obtained from stable producer cell lines in 50-L, single-use, stirred-tank bioreactors during early stages of process development. Results shown represent the batch with the highest functional titer for each cell line. Total number of process development 50L batches run for each cell line up to time of data cutoff: 293Tsa EGFP clone 2 (n = 2), 293Tsa GLOBE clone 19 (n = 4), 293Tsa GLOBE.T87Q clone 3 (n = 1), therapeutic transgene (not identified) stable clone (n = 1).

Table 1. RCL Test Results

Producer Cell Line	Number of Vector Batches Tested for RCL	Total TU$_{CEM}$ Tested	RCL Test Results
293Tsa EGFP clone 2	2	3.8E+10	not detected
293Tsa GLOBE clone 19	4	1.3E+10	not detected
293Tsa GLOBE.T87Q clone 1	1	4.4E+07	not detected
293Tsa GLOBE.T87Q clone 3	3	2.4E+09	not detected
293Tsa GLOBE.T87Q clone 9	1	<LOD[a]	not detected
293Tsa GLOBE.T87Q clone 10	1	1.1E+08	not detected
TOTAL	12	5.4E+10	not detected

Summary of RCL test results from lentiviral vector batches generated using six monoclonal producer cell lines. Total TU$_{CEM}$ tested was calculated as the sum of the TU$_{CEM}$ present in each test sample across all batches tested for each cell line. Detailed data for each vector batch are given in Table S1.

[a]<LOD indicates that the test result was below the limit of detection of the TU$_{CEM}$ titration assay.

In the BACs reported here, we used a relatively simple design of CMV promoter-driven transcription units arranged head-to-tail in the order transfer-gagpol-VSVg-rev with two copies of the 1.2-kb chicken HS4 insulator between transcription units to prevent promoter interference. We believe that this design has a number of advantages, including the high transcriptional activity of the CMV promoter in 293T cells and the high *in vivo* stability of head-to-tail arrays that has been previously reported.[5] It is conceivable that this design could be improved upon in the future, for example, by altering the strength of the promoters driving each transcription unit to change the relative expression levels of the components; switching to a more compact insulator system or removing it altogether if it is not required, as promoter interference has been reported to be cell line dependent;[9] or changing the order or orientation of the vector component transcription units to attempt to further reduce the theoretical risk of RCL generation. In terms of assessing this risk, no RCL has ever been reported with a third-generation lentiviral vector system, but replication competent retroviruses (RCRs) have been reported in early generation gammaretroviral systems.[26,27] In both cases, the authors reported the mechanism of recombination to be between copackaged RNA molecules during reverse transcription. Similarly, we believe that recombination between copackaged RNA molecules during reverse transcription is the most likely mechanism by which an RCL would be generated in any lentiviral vector production system due to the high frequency of template switching by HIV reverse transcriptase. By contrast, homologous recombination between DNA molecules is relatively inefficient in mammalian cells, particularly in the absence of stimulation such as a double-strand break. If the main risk is, in fact, at the reverse transcription level and integrated BAC constructs express the same four species of RNA molecule as a transient or serially stably transfected system (transfer, gagpol, rev, and env) then cointegration at a single locus would not be expected to significantly alter the risk of RCL formation relative to integration of vector components at multiple loci or episomal expression from separate constructs. While the order and orientation of the transcription units in the BAC construct is likely to have an effect on the configuration of the final integrated BAC, we have observed that multiple BACs are integrated at a single locus and that integrations can be complex with multiple break points and a non-1:1:1:1 transfer:gagpol:-rev:VSVg DNA copy number ratio of the vector component transcription units. It is conceivable that the configuration of the integrated BAC could be used as a screening criterion for clones during cell line development if so desired, for example, by rejecting clones that have a break within the LTR (long terminal repeat)-to-LTR region of the transfer vector to prevent packaging of non-transgene sequences. In addition to the RCL testing described in this paper, we believe that the theoretical risk of RCL generation with this system is extremely low due to design features such as four separate transcription cassettes for the vector components, the self-inactivating LTR, and the absence of HIV-1 tat, env, and accessory genes from the construct. We obtained scientific advice from both the Food and Drug Administration and the European Medicines Agency regarding a product intended for clinical supply generated using this stable producer cell line platform. Neither agency raised additional concerns or requested additional testing for the stable cell line approach beyond that which would be expected for any lentiviral vector manufacturing process.

Although the vector component transcription units in the BAC construct are present in a 1:1:1:1 transfer:gagpol:rev:VSVg DNA copy number ratio, we have observed variation in both DNA copy number and mRNA expression levels of each vector component between different monoclonal cell lines generated using the same construct (data not shown), so it is advisable to screen sufficient clones during cell line development to obtain one with the desired mRNA expression levels for optimal yield and infectivity. It is possible that targeted integration of the BAC into a suitable genomic locus could result in reproducibly high titers and a reduced need for screening large numbers of clones, though we have obtained high-titer cell lines by screening only tens of clones produced by random integration, and targeted integration is not commonly used in the biopharmaceutical industry to generate monoclonal antibody producer cell lines.

In the future, this BAC method could be used to generate a packaging cell line containing all vector components apart from the transfer vector, and we believe it is likely that this would be successful, given the success of the producer cell line approach reported here. We believe there are advantages and disadvantages to the strategy of adding

transfer vector DNA into a packaging cell line to generate a producer cell line compared to generating producer cell lines from host cell lines with a single construct. While generating a packaging cell line in which the packaging components are all present and expressing at a suitable level reduces one possible source of variation during cell line development, there are also potential disadvantages. The additional time in culture required to perform two rounds of stable transfection increases the risk that cell line stability may occur prior to clinical vector manufacturing. Packaging cell line strategies are also inflexible with respect to switching host cell lines when desirable or necessary; for example, if a more productive host cell line is found or the older host cell line is found to have a problem such as the presence of adventitious microorganisms,[28] or if it is later discovered that the packaging cell line was cultured in the presence of cell culture media that contained raw materials with an unacceptable safety profile.[29] In our experience, we are able to generate useful clones from screening only tens of monoclonal producer cell lines, so we believe the inflexibility of the packaging cell line approach may not always justify the potential reduction in variation between clones, which is one of its main proposed advantages.

Clinical supply of lentiviral vector by manufacturing processes based on transient transfection is currently the standard approach in the field of gene therapy. As with packaging and producer cell lines, there are advantages and disadvantages to lentiviral vector supply by transient transfection relative to stable producer cell lines. In order to generate clinical grade lentiviral vector, investigators typically outsource cell banking of *E. coli* containing the plasmids and high-quality or GMP-grade plasmid DNA production to a contract manufacturing organization. This approach is attractive early in a drug development project in terms of the length of time it typically takes to perform cell banking and generate a batch of plasmid DNA, but it introduces added complexity into the supply chain through the need to maintain plasmid and transfection reagent supply throughout the lifetime of the drug. Transient transfection-based processes also limit the range of upstream conditions that can be used during vector manufacture to those that are compatible with efficient transfection and may not be optimal for cell growth, vector yield, and infectivity. By contrast, lentiviral vector supply based on stable producer cell lines involves added complexity at the start of the drug development project through the need to perform a cell line development campaign and establish GMP mammalian cell banks but has benefits later in terms of a simpler supply chain and a simpler upstream process with fewer constraints on optimization of upstream conditions that may be beneficial for maximizing cell growth, vector titer, and infectivity. The balance between these advantages and disadvantages is likely to be specific to each drug development project—for example, whether the quantity of vector needed for clinical and commercial supply is high due to target cell transduction being challenging or whether the drug involves *ex vivo* or *in vivo* transduction—and the balance may shift toward stable producer cell lines if cell line development for lentiviral vector production becomes a standard service offered by contract manufacturing organizations, as is currently available for monoclonal antibodies.

In conclusion, we believe that the simple and effective producer cell line development technique we report here is widely applicable for the industrialization of lentiviral vector manufacture and may make it possible to supply gene therapy medicines to a wider number of patients in the future.

MATERIALS AND METHODS

Parental DNA Constructs

The 277 plasmid is a self-inactivating HIV-1-based lentiviral transfer vector encoding an EGFP expression cassette under the transcriptional control of the human phosphoglycerate kinase (PGK) promoter. The pGLOBE plasmid is a self-inactivating HIV-1-based lentiviral transfer vector encoding a human beta globin expression cassette under the transcriptional control of the human beta globin locus control region.[13] 277 and pGLOBE were originally developed by TIGET in Milan, Italy, and transferred to GlaxoSmithKline (GSK) by TIGET under the rare diseases strategic alliance.

pKLgagpol, pKrev, and pKG are third-generation packaging plasmids[6] encoding HIV-1 gagpol, rev, and VSVg under the control of the CMV immediate early promoter. They were originally developed by Cell Genesys (Foster City, CA, USA) and transferred to GSK by TIGET under the rare diseases strategic alliance.

DNA was assembled in a BAC-based cloning vector derived from the *E. coli* fertility (F) factor.[30]

Cloning of BACpack WTGP

BAC modules were designed *in silico* to enable iterative cloning into the BAC backbone as shown in Figure 1B. The two copies of the chicken beta globin cHS4 insulator are to prevent promoter interference.[9] The CMV-TO2 promoter and TetR protein enable doxycycline-inducible expression of the components as described by Yao et al.[7] The TetR coding sequence was codon optimized for increased expression in mammalian cells.[8] The attentuated IRES-zeocin cassette enables efficient antibiotic selection in HEK293 cells.[11] The coding sequences for the packaging components gagpol, rev, and VSVg were derived from the pK packaging plasmids described earlier, with the exception that rev was codon optimized. All packaging components were placed under the transcriptional control of the CMV-TO2 promoter. BAC modules were gene synthesized by GeneArt (Regensburg, Germany). Modules were cloned into the BAC in the order TetR-IRES-zeocin, rev, VSVg, and gagpol to generate the packaging BAC construct BACpack WTGP.

Cloning of BAC-EGFP

An LTR-to-LTR transfer vector sequence containing a PGK-EGFP-WPRE transgene was amplified from plasmid 277 by PCR and cloned in place of the rev cassette in BAC module format to yield pMA-BAC-mod277. To reduce LTR-to-LTR homology and make the BAC less prone to recombination during cloning and DNA preparation, the 3′ LTR in pMA-BACmod277 was replaced with a gene-synthesized fragment containing the pCCL 3′ LTR from the KpnI site upstream of the 3′ polypurine tract (PPT) to the HindIII site in the 3′ R region

followed by a rabbit beta globin (RBG) polyadenylation signal (ory-Cun2 genome minus strand chromosome [chr]1:146236955–146237049) to yield pMA-BACmod277delU5, a strategy similar to that of Hanawa et al.[31] The BAC module containing the PGK-EGFP-WPRE transfer vector was cloned into BACpack WTGP using the Mlu+NheI/XbaI strategy as for the other modules to yield BAC-EGFP (Figure 1B).

Cloning of BAC-GLOBE

The DNA sequence of pGLOBE (also known as pCCL-GLOBE-Ka-naR) was transferred from TIGET to GSK during acquisition of the beta thalassemia asset. pMK-BACmod-GLOBE delXhoI was synthesized by GeneArt to contain two copies of the cHS4 insulator followed by the pGLOBE transfer vector cassette. An XhoI site near the 3′ LTR that was present in the pGLOBE gene was removed from the sequence prior to gene synthesis. The previous MluI and NheI/XbaI strategy to load the BAC module into the BACpack WTGP could not be used due to the presence of an XbaI site in the pGLOBE transfer vector, so the module was instead flanked with MluI and NheI. To reinsert the XhoI site near the 3′ LTR that is present in pGLOBE, plasmid XhoI-DelU5-IsceI_pMA was designed and gene synthesized so that it would contain a ClaI-NheI partial pGLOBE fragment containing the XhoI site. The designed fragment also removed the R-U5 region of the 3′ LTR downstream of the HindIII site in order to minimize homology with the 5′ LTR and reduce the risk of recombination during cloning. An SV40 early polyadenylation signal was included downstream of the HindIII site. The ClaI-NheI fragment from XhoI-DelU5-IsceI_pMA was cloned into pMK-BACmod-GLOBE delXhoI to produce pMK-BACmod-GLOBE-delU5. To generate BAC-GLOBE (Figure 1B), the MluI-NheI fragment from pMK-BACmod-GLOBE-delU5 was cloned into BACpack WTGP.

Cloning of BAC-GLOBE.T87Q

Pawliuk et al.[18] described the T87Q anti-sickling mutation that would enable the same lentiviral vector to be used to treat both beta thalassemia and sickle cell disease. To introduce the T87Q antisickling mutation, a partial pGLOBE fragment named BG-T87Q NotI-EcoRV containing the T87Q mutation was gene synthesized by GenScript (Piscataway, NJ, USA) and cloned into pMK-BACmod-GLOBE-delU5 with NotI and EcoRV to generate BACmodGLOBE.T87Q (Figure 1B). The transfer vector module from BACmodGLOBE.T87Q was cloned into BACpack WTGP using MluI and NheI to generate BAC-GLOBE.T87Q.

Cell Line Development

The history of the 293T host cell line is described by Biffi et al.[21] Vials of adherent 293T cells stored in the GMP facility at MolMed (Milan, Italy) were transferred to GSK (Stevenage, UK). Research cell banks derived from these cells were established. These adherent cells were adapted to suspension culture by removal of fetal bovine serum (FBS) to generate the 293Tsa cell line.

To generate adherent BAC-EGFP polyclonal pools, 293T cells cultured in 6-well plates were transfected using calcium phosphate in triplicate with 4 μg per well of BAC-EGFP. 3 days after transfection, antibiotic selection was initiated with 500 μg/mL zeocin (Life Technologies). After 18 days of selection, polyclonal pools cultured in 6-well plates were split into parallel cultures for doxycycline induction and vector titration. Uninduced polyclonal pools were maintained in culture in the presence of 500 μg/mL zeocin for a further 4 months with periodic induction, harvesting, and vector titration of parallel cultures.

To generate 293Tsa EGFP clone 2, 2×10^6 293T cells were transfected with 4 μg BAC-EGFP using the Amaxa Nucleofector Kit V (Lonza) and maintained in adherent cell culture in the presence of fetal calf serum. 2 days after transfection, antibiotic selection was initiated with 600 μg/mL zeocin. After 6 days, the zeocin concentration was reduced to 300 μg/mL and maintained for 2 weeks. To obtain single-cell clones, manual limiting dilution cloning was performed into 96-well plates followed by the scale-up of clones to 24-well, 6-well, and then T-75 flask scales. Suspension adaptation of clone 2 was performed by transferring 5×10^6 cells into a shake flask and culturing with shaking for 4 days, replacing the medium, and then shaking for a further 4 days until viability as measured by trypan blue staining on a Vi-CELL cell counter was greater than 90%.

To generate 293Tsa GLOBE clones 19, 3×10^7 293Tsa cells were transfected with 30 μg BAC-GLOBE using 293fectin (GIBCO) in a 30-mL shake flask culture. 2 days after transfection, antibiotic selection was initiated with 600 μg/mL zeocin. 11 days later, fetal calf serum was added, and cells were cultured in an adherent format for a further 4 days. To obtain single-cell clones, manual limiting dilution cloning was performed into 96-well plates followed by sequential scale-up of clones to 24-well, 6-well and T-25 flask scales. Suspension adaptation of clone 19 was performed by transferring cells directly into a 30-mL shake flask culture, culturing with shaking for 4 days, replacing the medium, and then shaking for a further 4 days until viability as measured by trypan blue staining on a Vi-CELL cell counter was greater than 90%.

To generate the 293Tsa GLOBE.T87Q clones, 4×10^7 293Tsa cells were transfected with 30 μg BAC-GLOBE.T87Q using PEIpro (Polyplus) in a 20-mL shake flask culture. 2 days after transfection, cells were plated in adherent format in the presence of fetal calf serum, and antibiotic selection was initiated with zeocin. After 17 days of selection, the polyclonal pool was suspension adapted by transferring into shake flask format and gradually reducing the serum concentration from 10% to 0% over a period of 10 days. Single-cell cloning into 96-well plates was performed using a Cytena single-cell printer. Clones were scaled up over a number of weeks into 24-well plates and then Erlenmeyer shake flasks.

Quantification of BAC Module Copy Numbers in Producer Cell Lines

Genomic DNA was extracted from uninduced producer cell lines using the DNeasy Blood and Tissue Kit (QIAGEN), and copy numbers were determined by ddPCR using a QX200 AutoDG Droplet Digital

PCR System (Bio-Rad) with the primers and probes listed in the following text. Copy number per cell was calculated by dividing the copies per 20-μL reaction detected for the target gene of interest by the copies per 20-μL reaction detected for RNaseP and multiplying by 3 (i.e., assuming that RNaseP is present at 3 copies per cell). The RNaseP reference gene assay location is reported by the supplier to target chr14:20811565, and this genomic locus was reported to be present at 3 copies per 293T cell by next-generation sequencing.[32] Primers and probes were as follows: HIVnew (transfer vector): forward (Fwd), 5′-TGA AAG CGA AAG GGA AAC CA-3′, reverse (Rev), 5′-GCC GTG CGC GCT TCA G-3′, and probe, 5′-FAM-AGC TCT CTC GAC GCA GGA CTC GGC-3′-MGB; codon-optimized HIV rev: Fwd, 5′-CGA CTC TGA CGA GGA TCT G-3′, Rev, 5′-CAC CTC CTC CTG CGG TTC-3′, and probe, 5′-FAM-AGG CCG TGA GGC TGA TCA AGT TC-3′-MGB; VSVg: Fwd, 5′-GAT ATT GCT GCT CCA ATC CTC-3′, Rev, 5′-TTC CAC GTC TTC ATA TGG TGC-3′ , and probe, 5′-FAM-GAA TGG TCG GAA TGA TCA GTG GAA CTA CC-3′-MGB; HIV gagpol: Fwd 5′-CCA GCT GTG ATA AAT GTC AG-3′, Rev, 5′-AAT GTG TAC AAT CTA GCT GC-3′, and probe, 5′-FAM-AGG GGA AGC CAT GCA TGG ACA AGT A-3′-MGB; human beta globin exon 2: Fwd, 5′-ACA GTG CAG CTC ACT CAG TG-3′, Rev, 5′-TGG GCA ACC CTA AGG TGA AG-3′, and probe, 5′-FAM-AGG TGC CCT TGA GGT TGT CCA GGT G-MGB-3′; Human RNaseP Taqman Copy Number Reference Assay RNaseP VIC-TAMRA (Thermo Fisher Scientific, catalog no. 4403328).

Vector Production

Vector production from stable producer cells in shake flasks was performed using 100–120 mL cell culture medium in a 500-mL Erlenmeyer flask or 500 mL cell culture medium in a 2-L Erlenmeyer flask cultured in a shaking incubator at 37°C with 5% CO2. Cells growing in suspension were seeded so that they reached a density of 2–3×10^6 cells per milliliter on the day of induction. On the day of induction, doxycycline was added to a final concentration of 2 μg/mL, and sodium butyrate was added to a final concentration of 5 mM. 2 days after induction, the culture supernatant was harvested by centrifugation and filtration to remove cells and cell debris followed by storage of filtered supernatant at −80°C.

Vector production from adherent stable polyclonal pools was performed by inducing 10^6 cells per well cultured in 6-well plates in cell culture medium containing 10% fetal calf serum and doxycyclin. Vector was harvested 2 days after induction.

Vector production by transient transfection in shake flasks was performed using 20 mL cell culture medium in a 125-mL Erlenmeyer flask cultured in a shaking incubator at 37°C with 5% CO2. Cells growing in suspension were seeded at a density of 2×10^6 cells per milliliter on the day of transfection. Transfection complex was prepared using transfer vector plasmid, pKLgagpol, pKrev, pKG, and polyethylenimine (PEI) and added to the flask. After 24 h, sodium butyrate was added to a final concentration of 5 mM. 2 days after transfection, the culture supernatant was harvested by centrifugation and filtration to remove cells and cell debris followed by storage of filtered supernatant at −80°C. For CD34$^+$ cell transduction tests, vector supernatant was further concentrated by ultracentrifugation. Briefly, clarified vector supernatant was layered above a 20% sucrose cushion and ultracentrifuged at $75,000 \times g$ RCF (relative centrifugal force) for 2 h at 4°C. The vector pellet was resuspended with CellGro SCGM medium (Cellgenix) to obtain a 200× concentration factor.

Vector Titration

TU$_{CEM}$ per milliliter by qPCR was determined by transduction of a 4-fold dilution curve of harvested vector into 5×10^5 CEM A3.01 cells per well in 24-well plates in cell culture medium containing 10% v/v protamine sulfate followed by genomic DNA extraction 5 days posttransduction and qPCR using primers and probes against HIV-1 and human telomerase, assuming 2 copies of the human telomerase gene per CEM A3.01 cell as previously described[21,33] Absolute quantifications were plotted on standard curves prepared with serial dilutions of cell lysate from a CEM A3.01 clone containing 1 copy of HIV target per cell.[21] Primers and probes were as follows: human telomerase: Fwd 5′-GGC ACA CGT GGC TTT TCG-3′, Rev, 5′-GGT GAA CCT CGT AAG TTT ATG CAA-3′, and probe, FAM-5′−TCA GGA CGT CGA GTG GAC ACG GTG-3′-MGB; and HIV (transfer vector): Fwd, 5′-TAC TGA CGC TCT CGC ACC-3′, Rev, 5′-TCT CGA CGC AGG ACT CG-3′, and probe, FAM-5′-ATC TCT CTC CTT CTA GCC TC-3′ MGB.

TU$_{CEM}$ per milliliter by EGFP flow cytometry was determined by transduction of a 4-fold dilution curve of harvested vector into 5×10^5 CEM A3.01 cells per well in 24-well plates in cell culture medium containing 10% v/v protamine sulfate followed by flow cytometry to detect EGFP expression.

TU$_{CEM}$ per milliliter by ddPCR was determined by transduction of a 4-fold dilution curve of harvested vector into 5×10^5 CEM A3.01 cells per well in 24-well plates in cell culture medium containing 10% v/v protamine sulfate followed by genomic DNA extraction 5 days posttransduction and measurement of VCN by ddPCR using the QX200 AutoDG Droplet Digital PCR System (Bio-Rad) with the primers and probes listed in the following text. VCN per cell was calculated by dividing the copies per 20-μL reaction detected for the target gene of interest by the copies per 20-μL reaction detected for RNaseP and multiplying by 3 (i.e., assuming that RNaseP is present at 2 copies per CEM A3.01 cell). Primers and probes were as follows: HIVnew (transfer): Fwd, 5′-TGA AAG CGA AAG GGA AAC CA-3′, Rev, 5′-GCC GTG CGC GCT TCA G-3′, and probe, 5′-FAM-AGC TCT CTC GAC GCA GGA CTC GGC-3′-MGB; Human RNaseP Taqman Copy Number Reference Assay RNaseP VIC-MGB (Applied Biosystems, catalog no. 4401631).

HIV-1 p24 by Ella was determined by preparation of a 4-fold dilution curve of harvested vector using Dulbecco's PBS (D-PBS) and 0.5% Triton X-100 as the diluent followed by quantification using an Ella microfluidics device and the HIV-1 Gag p24 kit (ProteinSimple).

HIV-1 p24 by ELISA was determined by preparation of a 4-fold dilution curve of harvested vector followed by quantification using the Lenti-X p24 Rapid Titer Kit (Takara Bio, catalog no. 632200).

TU_{293T} per milliliter by EGFP flow cytometry was determined by transduction of a dilution curve of vector into 293T cells in 24-well plates in cell culture medium containing 8 μg/mL polybrene followed by flow cytometry to detect EGFP expression as described in Milani et al.[14]

TU_{293T} per milliliter by ddPCR was determined by transduction of a dilution curve of vector into 293T cells in 24-well plates in cell culture medium containing 8 μg/mL polybrene followed by genomic DNA extraction 4 days post-transduction and measurement of VCN by ddPCR using the QX200 AutoDG Droplet Digital PCR System (Bio-Rad) with the primers and probes listed in the following text. VCN per cell was calculated by dividing the copies per 20-μL reaction detected for the target gene of interest by the copies per 20-μL reaction detected for RNaseP and multiplying by 3 (i.e., assuming that RNaseP is present at 3 copies per cell). Primers and probes were as follows: HIVnew (transfer): Fwd, 5′-TGA AAG CGA AAG GGA AAC CA-3′, Rev, 5′-GCC GTG CGC GCT TCA G-3′, and probe 5′-FAM-AGC TCT CTC GAC GCA GGA CTC GGC-3′-MGB; Human RNaseP Taqman Copy Number Reference Assay RNaseP VIC-TAMRA (Thermo Fisher Scientific, catalog no. 4403328).

CD34 Isolation and Transduction

$CD34^+$ cells from bone marrow or mobilized peripheral blood were enriched by magnetic-activated cell sorting (MACS). Twenty hours before transduction, $CD34^+$ cells were seeded at 1×10^6 cells per milliliter in CellGro SCGM medium (Cellgenix) supplemented with 300 ng/mL stem cell factor, 300 ng/mL Flt3-L, 100 ng/mL thrombopoietin, and 60 ng/mL interleukin-3 (IL-3) (all from Cellgenix or Miltenyi Biotec). $CD34^+$ cells were transduced at multiplicities of infection (MOIs) of 50 or 100 TU per target cell in 1-hit or 2-hit transduction protocols with the following lentiviral vectors: 293Tsa EGFP clone 2 and 293Tsa GLOBE clone 19. For both transduction protocols, cells were washed 14 h after adding the vector, and for the 2-hit transduction protocol, cells were allowed to rest in culture media 10–12 h before adding the second dose of vector for another 14 h of transduction. Cells were cultured for 14 days, and the number of provirus copies integrated per cell was evaluated by qPCR. After genomic DNA isolation, qPCR was performed using the previously described primer and probes for HIV and human telomerase. A reference standard was obtained from a serially diluted transduced CEM A3.01 cell line carrying one copy of integrated provirus.[21] Results of integrated vector copies were normalized by the number of evaluated genomes. As negative control, samples of untransduced cells were used.

Gene Expression Analysis

Total RNA was extracted using the RNeasy Plus Kit (catalog no. 74134, QIAGEN) according to the manufacturer's instructions. RNA samples were treated with Turbo DNase (catalog no. AM1907, Thermo Fisher Scientific) followed by quality assessment using the Agilent TapeStation High Sensitivity RNA Kit (catalog no. 5067-5576, Agilent). Sequencing libraries were constructed using the NEBNext Ultra II RNA Library Kit (catalog no. E7760S, New England Biolabs) together with the NEBNext rRNA Depletion Kit (catalog no. E6310L, New England Biolabs) according to the manufacturer's instructions. Libraries were quantified using the Kapa Library Quantification Kit (catalog no. KK4835, Roche) and sequenced using Illumina HiSEQ SBS Kit, v.4 (catalog no. FC-401-4002, Illumina). Sequencing reads were aligned using STAR (v.STAR_2.6.1c).[30] Alignment data were visualized using the Gviz R package.[34] Expression quantification was performed using Salmon (v.0.13.1).[35] RNA abundance was expressed as transcripts per kilobase million (TPM), which is the number of RNA molecules corresponding to a given gene/transcript per 1 million RNA molecules in the sample.

TLA[15] was performed on 10^7 cells of the different clones by Cergentis (Utrecht, the Netherlands). Sequencing reads were aligned to a reference sequence consisting of the human genome (hg19) and the BAC reference sequence. BAC integration sites and BAC-BAC fusions were identified by the presence of reads spanning the junctions between BAC and genomic sequences or between different locations on the BAC, respectively.

RCL testing was performed by BioReliance (assay number 009130GMP) using culturing of the test sample with C8166-45 cells for 2–5 days and a further 8 passages. During this time, the cell culture is observed by microscopy to detect cytopathic effects. At the assay endpoint, the presence of lentivirus is scored using a quantitative fluorescent PERT (QF-PERT) assay to detect reverse transcriptase activity in the cell culture supernatant. The spiked test sample control and positive control involve the addition of 10 tissue culture infectious dose 50 ($TCID_{50}$)/mL of a replicating wild-type strain of HIV-1 to C8166-45 cells at the start of the assay.

Statistics

See the Supplemental Information for details of statistical analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.08.011.

AUTHOR CONTRIBUTIONS

REFERENCES

1. Segura, M.M., Mangion, M., Gaillet, B., and Garnier, A. (2013). New developments in lentiviral vector design, production and purification. Expert Opin. Biol. Ther. 13, 987–1011.

2. McCarron, A., Donnelley, M., McIntyre, C., and Parsons, D. (2016). Challenges of up-scaling lentivirus production and processing. J. Biotechnol. 240, 23–30.

3. Merten, O.W., Hebben, M., and Bovolenta, C. (2016). Production of lentiviral vectors. Mol. Ther. Methods Clin. Dev. 3, 16017.

4. Ikeda, Y., Takeuchi, Y., Martin, F., Cosset, F.L., Mitrophanous, K., and Collins, M. (2003). Continuous high-titer HIV-1 vector production. Nat. Biotechnol. 21, 569–572.

5. Throm, R.E., Ouma, A.A., Zhou, S., Chandrasekaran, A., Lockey, T., Greene, M., De Ravin, S.S., Moayeri, M., Malech, H.L., Sorrentino, B.P., and Gray, J.T. (2009). Efficient construction of producer cell lines for a SIN lentiviral vector for SCID-X1 gene therapy by concatemeric array transfection. Blood 113, 5104–5110.

6. Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. J. Virol. 72, 8463–8471.

7. Yao, F., Svensjö, T., Winkler, T., Lu, M., Eriksson, C., and Eriksson, E. (1998). Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Hum. Gene Ther. 9, 1939–1950.

8. Stewart, H.J., Leroux-Carlucci, M.A., Sion, C.J., Mitrophanous, K.A., and Radcliffe, P.A. (2009). Development of inducible EIAV-based lentiviral vector packaging and producer cell lines. Gene Ther. 16, 805–814.

9. Moriarity, B.S., Rahrmann, E.P., Keng, V.W., Manlove, L.S., Beckmann, D.A., Wolf, N.K., Khurshid, T., Bell, J.B., and Largaespada, D.A. (2013). Modular assembly of transposon integratable multigene vectors using RecWay assembly. Nucleic Acids Res. 41, e92.

10. Yahata, K., Maeshima, K., Sone, T., Ando, T., Okabe, M., Imamoto, N., and Imamoto, F. (2007). cHS4 insulator-mediated alleviation of promoter interference during cell-based expression of tandemly associated transgenes. J. Mol. Biol. 374, 580–590.

11. Lanza, A.M., Kim, D.S., and Alper, H.S. (2013). Evaluating the influence of selection markers on obtaining selected pools and stable cell lines in human cells. Biotechnol. J. 8, 811–821.

12. Wild, J., Hradecna, Z., and Szybalski, W. (2002). Conditionally amplifiable BACs: switching from single-copy to high-copy vectors and genomic clones. Genome Res. 12, 1434–1444.

13. Miccio, A., Cesari, R., Lotti, F., Rossi, C., Sanvito, F., Ponzoni, M., Routledge, S.J., Chow, C.M., Antoniou, M.N., and Ferrari, G. (2008). In vivo selection of genetically modified erythroblastic progenitors leads to long-term correction of beta-thalassemia. Proc. Natl. Acad. Sci. USA 105, 10547–10552.

14. Milani, M., Annoni, A., Bartolaccini, S., Biffi, M., Russo, F., Di Tomaso, T., Raimondi, A., Lengler, J., Holmes, M.C., Scheiflinger, F., et al. (2017). Genome editing for scalable production of alloantigen-free lentiviral vectors for in vivo gene therapy. EMBO Mol. Med. 9, 1558–1573.

15. de Vree, P.J., de Wit, E., Yilmaz, M., van de Heijning, M., Klous, P., Verstegen, M.J., Wan, Y., Teunissen, H., Krijger, P.H., Geeven, G., et al. (2014). Targeted sequencing by proximity ligation for comprehensive variant detection and local haplotyping. Nat. Biotechnol. 32, 1019–1025.

16. Robins, D.M., Ripley, S., Henderson, A.S., and Axel, R. (1981). Transforming DNA integrates into the host chromosome. Cell 23, 29–39.

17. Lefebvre, G., Desfarges, S., Uyttebroeck, F., Muñoz, M., Beerenwinkel, N., Rougemont, J., Telenti, A., and Ciuffi, A. (2011). Analysis of HIV-1 expression level and sense of transcription by high-throughput sequencing of the infected cell. J. Virol. 85, 6205–6211.

18. Pawliuk, R., Westerman, K.A., Fabry, M.E., Payen, E., Tighe, R., Bouhassira, E.E., Acharya, S.A., Ellis, J., London, I.M., Eaves, C.J., et al. (2001). Correction of sickle cell disease in transgenic mouse models by gene therapy. Science 294, 2368–2371.

19. Stewart, H.J., Fong-Wong, L., Strickland, I., Chipchase, D., Kelleher, M., Stevenson, L., Thoree, V., McCarthy, J., Ralph, G.S., Mitrophanous, K.A., and Radcliffe, P.A. (2011). A stable producer cell line for the manufacture of a lentiviral vector for gene therapy of Parkinson's disease. Hum. Gene Ther. 22, 357–369.

20. Vogt, B., Roscher, S., Abel, B., Hildinger, M., Lamarre, A., Baum, C., and von Laer, D. (2001). Lack of superinfection interference in retroviral vector producer cells. Hum. Gene Ther. 12, 359–365.

21. Biffi, A., Montini, E., Lorioli, L., Cesani, M., Fumagalli, F., Plati, T., Baldoli, C., Martino, S., Calabria, A., Canale, S., et al. (2013). Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 341, 1233158.

22. Nienow, A.W. (2006). Reactor engineering in large scale animal cell culture. Cytotechnology 50, 9–33.

23. Maunder, H.E., Wright, J., Kolli, B.R., Vieira, C.R., Mkandawire, T.T., Tatoris, S., Kennedy, V., Iqball, S., Devarajan, G., Ellis, S., et al. (2017). Enhancing titres of therapeutic viral vectors using the transgene repression in vector production (TRiP) system. Nat. Commun. 8, 14834.

24. Engler, C., Kandzia, R., and Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. PLoS ONE 3, e3647.

25. Fischer, S., Handrick, R., and Otte, K. (2015). The art of CHO cell engineering: A comprehensive retrospect and future perspectives. Biotechnol. Adv. 33, 1878–1896.

26. Garrett, E., Miller, A.R., Goldman, J.M., Apperley, J.F., and Melo, J.V. (2000). Characterization of recombination events leading to the production of an ecotropic replication-competent retrovirus in a GP+envAM12-derived producer cell line. Virology 266, 170–179.

27. Chong, H., Starkey, W., and Vile, R.G. (1998). A replication-competent retrovirus arising from a split-function packaging cell line was generated by recombination events between the vector, one of the packaging constructs, and endogenous retroviral sequences. J. Virol. 72, 2663–2670.

28. Dubin, G., Toussaint, J.F., Cassart, J.P., Howe, B., Boyce, D., Friedland, L., Abu-Elyazeed, R., Poncelet, S., Han, H.H., and Debrus, S. (2013). Investigation of a regulatory agency enquiry into potential porcine circovirus type 1 contamination of the human rotavirus vaccine, Rotarix: approach and outcome. Hum. Vaccin. Immunother. 9, 2398–2408.

29. Gstraunthaler, G., Lindl, T., and van der Valk, J. (2013). A plea to reduce or replace fetal bovine serum in cell culture media. Cytotechnology 65, 791–793.

30. O'Connor, M., Peifer, M., and Bender, W. (1989). Construction of large DNA segments in Escherichia coli. Science 244, 1307–1312.

31. Hanawa, H., Kelly, P.F., Nathwani, A.C., Persons, D.A., Vandergriff, J.A., Hargrove, P., Vanin, E.F., and Nienhuis, A.W. (2002). Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. Mol. Ther. 5, 242–251.

32. Lin, Y.C., Boone, M., Meuris, L., Lemmens, I., Van Roy, N., Soete, A., Reumers, J., Moisse, M., Plaisance, S., Drmanac, R., et al. (2014). Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. Nat. Commun. 5, 4767.

33. Marin, V., Stornaiuolo, A., Piovan, C., Corna, S., Bossi, S., Pema, M., Giuliani, E., Scavullo, C., Zucchelli, E., Bordignon, C., et al. (2016). RD-MolPack technology for the constitutive production of self-inactivating lentiviral vectors pseudotyped with the nontoxic RD114-TR envelope. Mol. Ther. Methods Clin. Dev. 3, 16033.

34. Hahne, F., and Ivanek, R. (2016). Visualizing Genomic Data Using Gviz and Bioconductor. Methods Mol. Biol. 1418, 335–351.

35. Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. Nat. Methods 14, 417–419.

Supplemental Information

Rapid Lentiviral Vector Producer Cell Line

Generation Using a Single DNA Construct

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Title: Rapid lentiviral vector producer cell line generation using a single DNA construct

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<u>**Supplemental Text**</u>

Supplemental statistics

Fig 1e: For comparison of RNA expression levels, transcripts per kb million (TPM) were compared between transient and stable producer cells for each transgene and vector component; e.g. transient and stable transfer vector RNA in eGFP producers, transient and stable gagpol RNA in GLOBE producers etc. Data was analysed using a three-way mixed-model approach, with factors Component, Transgene and Production method. Additionally, when the variability was statistically significantly different between production methods (assessed using a likelihood ratio test), the residual variability was modelled separately for each production method. This was followed by two-sided Planned Comparisons of the predicted means to compare the TPM values resulting from the Production methods separately for each component/transgene combination. Parametric assumptions were assessed graphically using normal probability plots and residuals vs. predicted plots. The data was log-transformed prior to analysis to stabilise the variance. Statistical significance was declared at the 5% acceptance level.

Component	Transgene	Production method vs. production method		comparison (as a fold change)	95% Confidence interval around the fold change		t-value	p-value[*]
					Lower bound	Upper bound		
Transfer	GLOBE	Clone 19	Transient	1.89	1.08	3.31	2.28	0.0260
	eGFP	Clone 2	Transient	1.50	0.86	2.61	1.44	0.1549
VSVg	GLOBE	Clone 19	Transient	1.28	0.73	2.24	0.89	0.3785
	eGFP	Clone 2	Transient	1.23	0.71	2.16	0.75	0.4566
gagpol	GLOBE	Clone 19	Transient	0.90	0.51	1.57	-0.38	0.7030
	eGFP	Clone 2	Transient	1.34	0.77	2.34	1.05	0.2981
rev	GLOBE	Clone 19	Transient	2.88	1.65	5.03	3.78	0.0003
	eGFP	Clone 2	Transient	1.77	1.01	3.10	2.05	0.0445

* p-values based on 69 degrees of freedom

Three comparisons between transient and stable production of the mean RNA expression level were statistically significant, namely the RNA level of the rev component in transient

and stable production of GLOBE and eGFP lentiviral vector (p=0.0003 and p=0.0445 respectively) and also the transfer component of GLOBE (p=0.0260), although the latter two could be false positives as the p-values were only marginally significant. The variability was statistically significantly different between production methods (p<0.001).

Fig 1f: TU and p24 yields in were compared between transient and stable producer cells where transgenes were the same e.g. transient and stable TU in eGFP producers, transient and stable p24 in GLOBE producers etc. TU and p24 yields were analysed separately using a two-way mixed-model approach, with factors Transgene and Production method. Additionally, when the variability was statistically significantly different between production methods (assessed using a likelihood ratio test), the residual variability was modelled separately for each production method. This was followed by two-sided Planned Comparisons to compare the TU and p24 titres resulting from the Production methods separately for each transgene. Parametric assumptions were assessed using normal probability plots and residuals vs. predicted plots. The data were log-transformed prior to analysis to stabilise the variance.

Titre	Transgene	Production method vs. production method		comparison (as a fold change)	95% Confidence interval around the fold change		t-value	p-value*
					Lower bound	Upper bound		
TU	GLOBE	Clone 19	Transient	1.11	0.75	1.66	0.54	0.5896
	eGFP	Clone 2	Transient	1.09	0.73	1.62	0.42	0.6753
p24	GLOBE	Clone 19	Transient	1.42	0.85	2.37	1.39	0.1736
	eGFP	Clone 2	Transient	1.19	0.71	1.98	0.68	0.5020

* p-values based on 32 degrees of freedom

None of the comparisons between transient and stable production for the TU or p24 yields were statistically significant (p>0.05). For both titre TU and p24 the variability was the same for the production methods (p=0.2419 and >0.9999 respectively).

Fig 2b:

Functional stability

TU and p24 data were assessed by performing a two-way ANOVA analysis with Clone as a categorical factor and Cell Doubling as a continuous factor. Also included in the statistical model was the interaction between Clone and Cell Doubling to model any differences between the trends for each clone. For each clone the slope coefficient (a measure of the trend in TU or p24 over cell doubling time) was compared to zero. Statistical significance was determined with a p<0.05 acceptance value.

Across the four clones only two slope coefficients were significantly different from zero, namely clone 1 TU and clone 10 TU (p=0.0011 and 0.0234). The data therefore suggest that clone 1 TU, and possibly clone 10 TU are not stable over time.

Data type	GLOBE.T87Q Clone	Slope (TU$_{CEM}$ or ng p24.ml^{-1}.cell doubling^{-1})	95% Confidence interval around the slope estimate		t-value	p-value*
			Lower bound	Upper bound		
TU	1	-667244	-890367	-444120	-8.3	0.0011
	3	175437	-61328	412202	2.06	0.1088
	9	-14802	-261051	231448	-0.17	0.8756
	10	-285362	-507474	-63249	-3.57	0.0234
p24	1	4.14	-15.07	23.34	0.6	0.5821
	3	2.82	-17.56	23.2	0.38	0.7202
	9	1.25	-19.94	22.45	0.16	0.8775
	10	13.76	-5.36	32.88	2	0.1163

* p-values for comparing slope estimates to zero based on 4 degrees of freedom

Genetic stability

The genetic stability data were assessed by performing a three-way ANOVA analysis with Data Type and Clone as categorical factors and Cell Doubling as a continuous factor. Also included in the statistical model were the two-factor interactions between the three factors to model any differences between the trends for each clone and datatype. For each clone the slope coefficient (a measure of the trend over cell doubling time) was compared to zero. Statistical significance was determined with a $p<0.05$ acceptance value.

None of the slope coefficients were statistically significantly different from zero.

| Data type | GLOBE.T87Q Clone | Slope (Copies.cell^{-1}. cell doubling^{-1}) | 95% Confidence interval around the slope estimate | | t-value | p-value* |
			Lower bound	Upper bound		
VSVg ddPCR	1	-0.03	-0.11	0.06	-0.72	0.4791
	3	0.00	-0.08	0.09	0.11	0.9168
	9	0.00	-0.09	0.10	0.11	0.9124
	10	-0.01	-0.10	0.07	-0.35	0.7304
gagpol ddPCR	1	-0.04	-0.12	0.04	-1.00	0.3307
	3	0.02	-0.07	0.11	0.46	0.6490
	9	0.01	-0.08	0.10	0.19	0.8553
	10	-0.02	-0.10	0.07	-0.43	0.6730
rev ddPCR	1	-0.04	-0.13	0.04	-1.04	0.3125
	3	0.01	-0.07	0.10	0.35	0.7300
	9	0.09	0.00	0.18	2.06	0.0565
	10	-0.05	-0.14	0.03	-1.37	0.1887
transfer ddPCR	1	-0.02	-0.10	0.07	-0.43	0.6755
	3	0.03	-0.06	0.12	0.64	0.5316
	9	0.03	-0.06	0.13	0.78	0.4447
	10	-0.01	-0.09	0.07	-0.22	0.8253

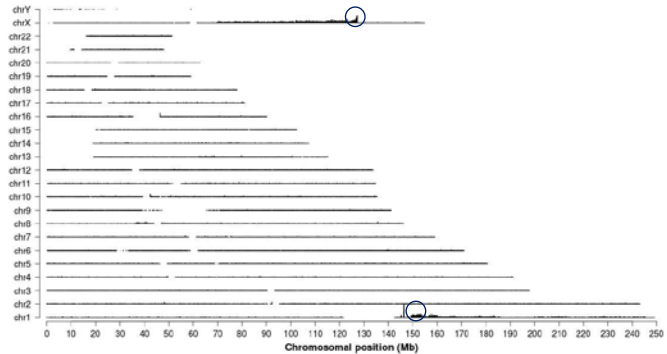* p-values for comparing slope coefficients to 0 based on 16 degrees of freedom
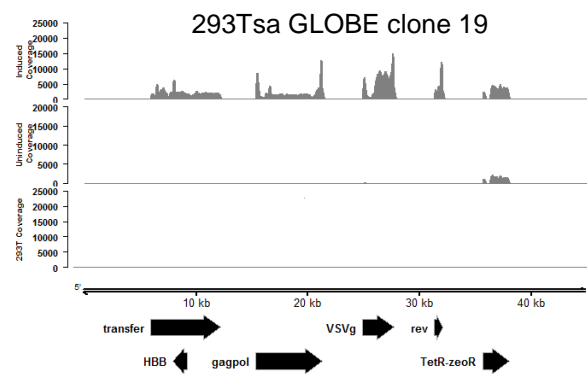
Supplemental Figures and Tables

Figure S1

a
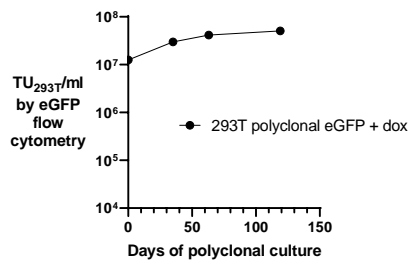
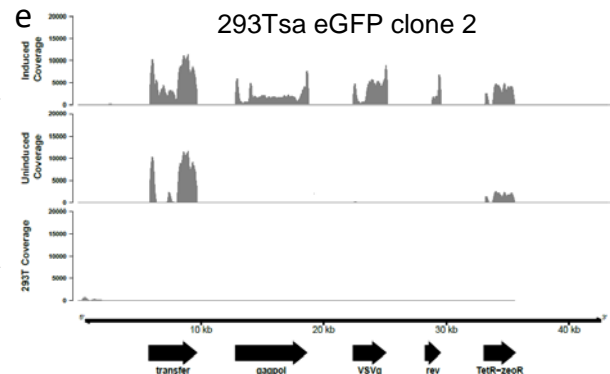
b   293Tsa GLOBE clone 19 TLA sequencing

c

	Fold increase in RNA level upon induction	
	293Tsa GLOBE clone 19	293Tsa eGFP clone 2
gagpol	1069 ± 54	2885 ± 452
rev	1063 ± 316	531 ± 125
VSVg	469 ± 21	1329 ± 122

d   293Tsa GLOBE clone 19

e   293Tsa eGFP clone 2

f

	Transcript abundance relative to all polyadenylated transcripts			
	GLOBE		eGFP	
	Transient	Stable	Transient	Stable
transfer	0.31% ± 0.22	0.42% ± 0.03	0.67% ± 0.61	0.63% ± 0.15
gagpol	0.56% ± 0.33	0.43% ± 0.04	0.47% ± 0.33	0.47% ± 0.02
rev	0.35% ± 0.14	0.90% ± 0.15	0.20% ± 0.11	0.28% ± 0.03
VSVg	2.13% ± 0.98	2.39% ± 0.43	1.69% ± 1.01	1.60% ± 0.40

g   293Tsa GLOBE clone 19

h   293Tsa eGFP clone 2
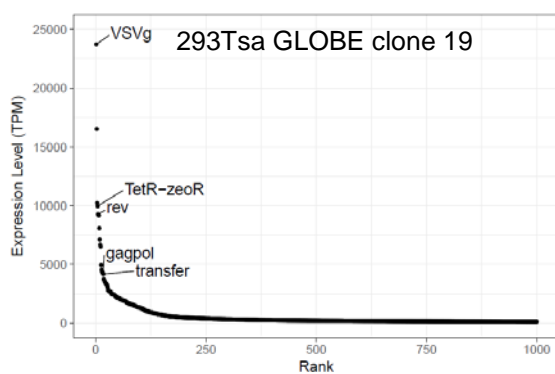
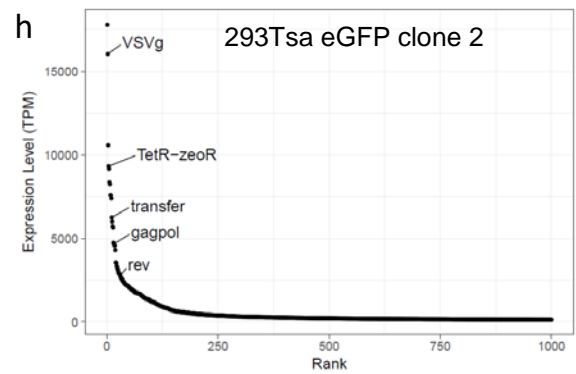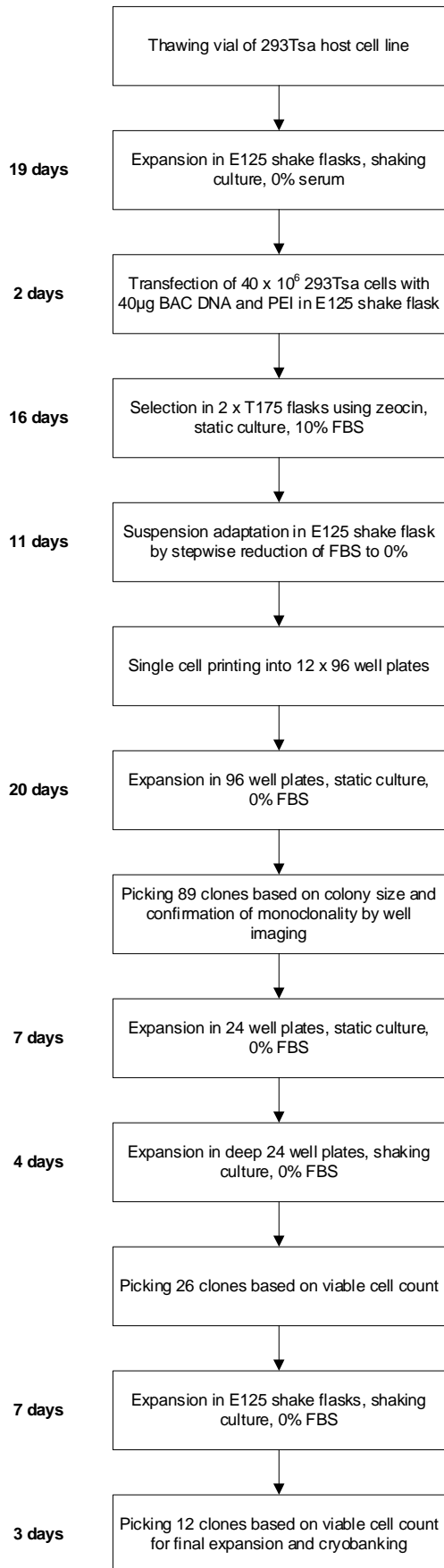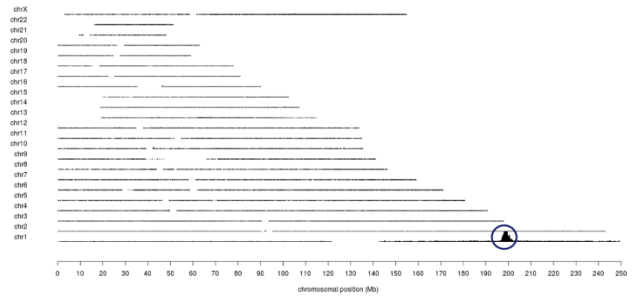**Figure S1.** (a) 293Tsa eGFP polyclonal stable pool functional stability determined by continuous culture of a polyclonal pool in the absence of doxycycline with periodic doxycycline induction and vector titration of a parallel culture. (b) Integration site mapping of BAC DNA in the 293Tsa GLOBE clone 19 cell line by TLA sequencing. (c) Relative increase in transcript abundance in stable producer cell lines following induction with doxycycline. Mean ± standard deviation, n=3. (d) RNAseq read coverage from 293Tsa GLOBE clone 19 mapped onto the BAC-GLOBE construct. (e) RNAseq read coverage from 293Tsa eGFP clone 2 mapped onto the BAC-eGFP construct. (f) Transient plasmid and stable BAC-derived transcript abundance relative to all polyadenylated transcripts in producer cells. Mean ± standard deviation, n=3. (g) RNA expression level of 1000 most abundant transcripts in 293Tsa GLOBE clone 19. (h) RNA expression level of 1000 most abundant transcripts in 293Tsa eGFP clone 2.

Figure S2

a

b 293Tsa GLOBE T8Q clone 1 TLA sequencing

Thawing vial of 293Tsa host cell line

↓

19 days Expansion in E125 shake flasks, shaking culture, 0% serum

↓

2 days Transfection of 40 x 10^6 293Tsa cells with 40µg BAC DNA and PEI in E125 shake flask

↓

16 days Selection in 2 x T175 flasks using zeocin, static culture, 10% FBS

↓

11 days Suspension adaptation in E125 shake flask by stepwise reduction of FBS to 0%

↓

Single cell printing into 12 x 96 well plates

↓

20 days Expansion in 96 well plates, static culture, 0% FBS

↓

Picking 89 clones based on colony size and confirmation of monoclonality by well imaging

↓

7 days Expansion in 24 well plates, static culture, 0% FBS

↓

4 days Expansion in deep 24 well plates, shaking culture, 0% FBS

↓

Picking 26 clones based on viable cell count

↓

7 days Expansion in E125 shake flasks, shaking culture, 0% FBS

↓

3 days Picking 12 clones based on viable cell count for final expansion and cryobanking

293Tsa GLOBE T8Q clone 3 TLA sequencing
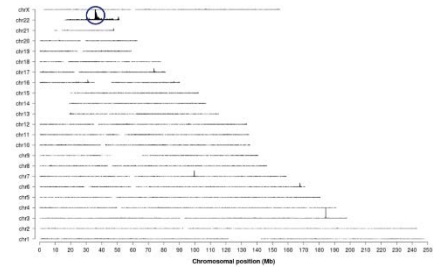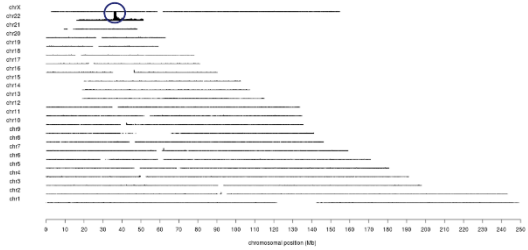
293Tsa GLOBE T8Q clone 10 TLA sequencing

Figure S2. (a) Process flow diagram for the GLOBE.T87Q cell line development campaign. PEI, polyethylenimine. (b) Integration site mapping of BAC DNA in the 293Tsa GLOBE clone 1, 3, and 10 cell lines by TLA sequencing.

Table S1. Details of RCL test results from lentiviral vector batches generated using six monoclonal producer cell lines including internal

controls. Summary data are given in Table 1. n.d. not done, below LOD indicates that the test result was below the limit of detection of the

TU$_{CEM}$ titration assay.

Vector batch production process		Vector quantity in RCL test sample (TUCEM by qPCR)		Cytopathic effect observed				QFPERT by qPCR				RCL test result	
Producer cell line	Bioreactor scale	Harvested vector	Final vector	Harvested vector	Final vector	Spiked test samples	Positive controls	Harvested vector	Final vector	Spiked test samples	Positive controls	Harvested vector	Final vector
293Tsa eGFP clone 2	50L	n.d.	7.4E+09	n.d.	-	+	+	n.d.	-	+	+	n.d.	Not detected
293Tsa eGFP clone 2	50L	3.0E+09	2.8E+10	-	-	+	+	-	-	+	+	Not detected	Not detected
293Tsa GLOBE clone 19	50L	9.3E+08	1.7E+09	-	-	+	+	-	-	+	+	Not detected	Not detected
293Tsa GLOBE clone 19	50L	1.2E+08	3.0E+08	-	-	+	+	-	-	+	+	Not detected	Not detected
293Tsa GLOBE clone 19	50L	7.3E+08	6.0E+09	-	-	+	+	-	-	+	+	Not detected	Not detected
293Tsa GLOBE clone 19	50L	1.9E+09	1.1E+09	-	-	+	+	-	-	+	+	Not detected	Not detected
293Tsa GLOBE.T87Q clone 1	2L	n.d.	4.4E+07	n.d.	-	+	+	n.d.	-	+	+	n.d.	Not detected
293Tsa GLOBE.T87Q clone 3	2L	n.d.	1.4E+08	n.d.	-	+	+	n.d.	-	+	+	n.d.	Not detected
293Tsa GLOBE.T87Q clone 9	2L	n.d.	below LOD	n.d.	-	+	+	n.d.	-	+	+	n.d.	Not detected
293Tsa GLOBE.T87Q clone 10	2L	n.d.	1.1E+08	n.d.	-	+	+	n.d.	-	+	+	n.d.	Not detected
293Tsa GLOBE.T87Q clone 3	50L	9.8E+08	4.3E+08	-	-	+	+	-	-	+	+	Not detected	Not detected
293Tsa GLOBE.T87Q clone 3	200L	5.5E+08	2.6E+08	-	-	+	+	-	-	+	+	Not detected	Not detected