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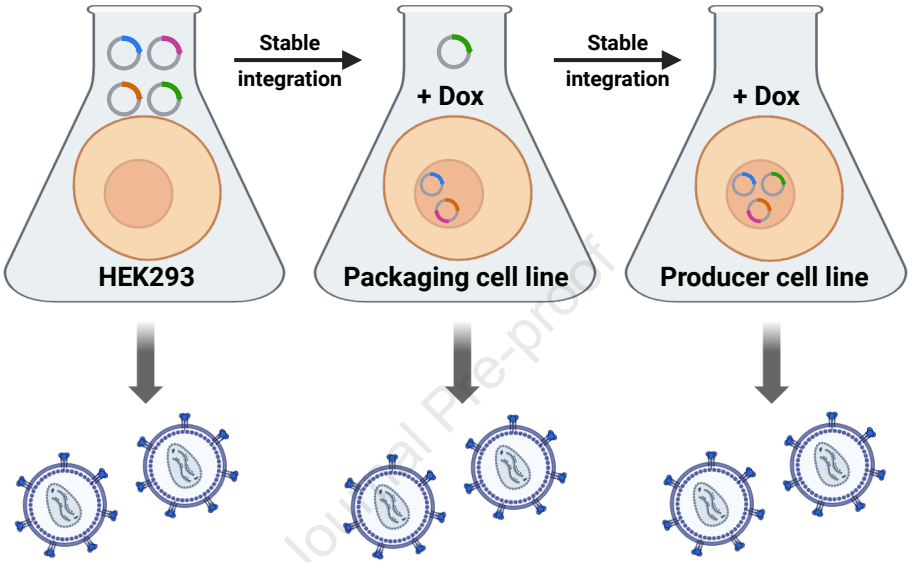

- Equivalent titres
- Simplified production process
- Reduced dependence on plasmids

Lentiviral vector packaging and producer cell lines yield titres equivalent to the industry-standard four-plasmid process

Stable cells for lentiviral vector manufacturing

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Abstract

Lentiviral vector (LVV)-mediated cell and gene therapies have the potential to cure diseases that currently require lifelong intervention. However, the requirement for plasmid transfection hinders large-scale LVV manufacture. Moreover, large-scale plasmid production, testing and transfection all contribute to operational risk and the high cost associated with this therapeutic modality. Thus, we developed LVV packaging and producer cell lines, which reduce or eliminate the need for plasmid transfection during LVV manufacture. To develop a packaging cell line, lentiviral packaging genes were stably integrated by random integration of linearised plasmid DNA. Then, to develop *EGFP*- and anti-CD19 chimeric antigen receptor-encoding producer cell lines, transfer plasmids were integrated by transposase-mediated integration. Single cell isolation and testing were performed to isolate the top-performing clonal packaging and producer cell lines. Production of LVV that encode various cargo genes revealed consistency in the production performance of the packaging and producer cell lines compared to the industry-standard four-plasmid transfection method. By reducing or eliminating the requirement for plasmid transfection, while achieving production performance consistent with the current industry standard, the packaging and producer cell lines developed here can reduce costs and operational risks of LVV manufacture, thus increasing patient access to LVV-mediated cell and gene therapies.

Introduction

Lentiviral vectors (LVV) are an attractive cell line engineering option for ex vivo cell therapies, particularly chimeric antigen receptor (CAR)-T cell therapy, and have shown promise in the development of in vivo gene therapies.[1-4] However, the dependence of LVV production on plasmid transfection is a contributing factor to the high cost of goods associated with this cell therapy modality.[5,6] This is due in part to the operational complexity and risk associated with producing several plasmid batches.[5] In addition, the cost of raw materials associated with plasmid dependence is high: Specifically, both the sourcing of large quantities of transfection reagent, and the production and testing of a number of large-scale, GMP-grade plasmid preparations, are costly.[5,6] Furthermore, the requirement to remove residual plasmid DNA and transfection reagent from LVV preparations contributes to the complexity and thus the cost of downstream processing.[6] Despite these operational and technological drawbacks, the current standard LVV production modality relies on the simultaneous transfection of four plasmids.[7,8] To address these issues, several research groups have attempted to reduce or eliminate plasmid transfection dependence by developing various stable LVV production cell lines, which require production/transfection of only one or no plasmids (reviewed by Ferreira and colleagues).[9]

Stable packaging cell lines have all LVV genetic sequences except the LVV transfer plasmid stably integrated into the cell genome, thus require preparation, testing, and transfection of only one plasmid to produce LVV, as opposed to four for fully transient production. Stable producer cell lines, however, have all LVV-producing elements integrated, thus require no plasmid transfection to produce LVV. This is advantageous as scaling up plasmid transfection to manufacturing scale is a significant challenge.[5,10,11] In both cases, elimination of the requirement to produce, test, and transfect three or four batches of plasmid DNA reduces the cost, complexity, and operational risk of the manufacturing process.

Although stable LVV production cell lines have the potential to solve several issues, there are technical challenges associated with their development. The first is that, unlike transient systems, stable production cell lines require development before they can be used for a given purpose. For instance, in the case of packaging cell lines, if LVV particles are required to be pseudotyped with a glycoprotein other than what was already encoded by the packaging cell line, a new cell line would

require development. In addition, a new producer cell line requires development each time the cargo gene is changed.

A further technical challenge is that stable LVV production cell lines are often less productive than fully transient systems. When using the four-plasmid transfection method to produce green fluorescence protein (GFP)-encoding LVV, one could expect a titre of ~1 $\times 10^7$ – 1 $\times 10^8$ TU/mL (pre-downstream processing).[12,13] In contrast, when preparing GFP-encoding LVV with a stable production cell line, one could expect a titre of ~1 $\times 10^6$ TU/mL.[14,15] (It must be noted that comparison of titres from different publications is controversial as different titration methods are used in different laboratories. Thus, comparisons must be taken as an approximate guide only). The reason for this disparity between the modalities is not fully understood, as it could depend on the characteristics of the specific production cell line at hand. For example, we have previously been able to increase packaging cell line titres by supplementing cells during production with additional copies of already-integrated LVV plasmids by transient transfection. In this experiment, different plasmids impacted titre to different extents, suggesting that in this case production had been limited by the integrated copy numbers of the various constructs (M. Mulet and M. Raghunath, unpublished data). Another factor that could explain the productivity discrepancy between stable cell lines and the full transient system is the long-term host cell metabolic burden by leaky expression of integrated genes:[16,17] Cytotoxic and/or cytostatic effects in production cells due to expression of VSV-G, Rev, and potentially the cargo gene might be amplified in stable production cell lines compared to the fully transient system. This is expected since there would be more time for accumulation of the gene products when the genes are stably integrated than if plasmids were used transiently. To address this challenge, stable cell lines have been developed with VSV-G and Rev expression controlled by chemically inducible promoters, to limit expression to the production window only. Inducible LVV packaging/producer cell lines have included tetracycline-repressible/inducible, and cumate-inducible expression systems and combinations thereof.[18-24] Other factors limiting the productivity of stable cell lines compared to fully transient systems could include: Instability of integrated constructs;[25,26] Shorter LVV gene expression window in systems that require induction than in fully constitutive, fully transient production systems; Disruption of LVV gene expression by readthrough by host cell factors or – if the construct formed a concatemer before integration – readthrough by neighbouring concatemer subunits;[27,28] Silencing of LVV gene expression by host chromatin remodelling.[29]

In addition to the technical challenges described above, a regulatory challenge associated with developing stable LVV production cell lines is the perceived risk of replication-competent lentivirus (RCL) generation. There remains concern that recombination events between stably integrated lentiviral sequences could result in the generation of RCL. However, when Chen and colleagues developed an LVV producer cell line with all LVV genes encoded by a single construct, neither the Food and Drug Administration nor the European Medicines Agency raised concerns beyond what would be expected for a typical LVV production method.[30] Moreover, assays to detect RCL can be performed during development and manufacturing.[31]

Here we describe two iterations of clonal packaging and producer cell line generation based on suspension HEK293 cells, an easily scalable platform for LVV manufacturing. The first iteration was based on the HEK293BSusp_MCB1 cell line (suspension HEK293 cell line owned by OXGENE), and the second was based on the WXATUS0028 cell line (adherent HEK293 cell line owned by WuXi ATU and adapted to suspension in serum-free media). In both cases, the integrated plasmids encoded several safety features to reduce the theoretical risk of RCL generation (described in detail below). Packaging cell line v1.0 produced on average 3.0×10^7 TU/mL with *EGFP* (enhanced GFP) as the gene of interest (GOI). Producer cell line v1.0 produced on average 9.0×10^7 TU/mL with *EGFP* as the GOI. Our second generation of packaging cell line (v2.0) yielded an average of ~1.5×10^8 TU/mL and the producer cell line v2.0 produced on average 2.5×10^8 TU/mL (both with *EGFP* as the GOI). For comparison, using the WXATUS0028 cell line for fully transient production typically yields ~1.5×10^8 TU/mL. Thus, the v2.0 packaging/producer cell lines are a substantial improvement upon the v1.0 cell lines and are comparable in terms of LVV production yield with the fully transient system. Furthermore, we produced LVV preparations encoding various therapeutically relevant GOIs using the fully transient system and the v2.0 packaging/producer cell lines, demonstrating that the stable systems are consistent with the fully transient system. Finally, we developed a method to screen GOI constructs rapidly in packaging cell lines and to estimate the production titre in an equivalent producer cell line.

Results

Plasmid performance in transient system

The plasmids used to generate stable packaging/producer cell lines were third generation, self-inactivating lentiviral vector production plasmids (Figure 1). They were designed with the following

features: 1) *Gag-Pol* and *VSV-G* gene expression was controlled by Tet-repressible promoters to limit production cell cytotoxicity; 2) *Gag-Pol* and *VSV-G* genes were encoded within separate cassettes on opposing strands to prevent co-transcription and co-packaging of these genes, to reduce the risk of formation of replication-competent lentivirus (RCL); 3) To limit production cell death during LVV production, *Bombyx mori* nucleopolyhedrovirus inhibitor of apoptosis 1 (*IAP1*) gene, and Epstein-Barr virus-encoded nuclear antigen 5 (*EBNA5*) were encoded by the *VSV-G*/*Gag-Pol* plasmid; 4) The *Rev* CDS was codon optimised for expression in human cells; 5) Since a low level of *Rev* expression was required, a beta globin insulator was positioned upstream of the gene to prevent distal activation by host cell factors; 6) Since high levels of *VSV-G* and *Gag-Pol* expression were required (upon induction), beta globin insulators were positioned both upstream and downstream of these genes to limit silencing by heterochromatin.

To limit the risk of transduction of the *IAP1*/*EBNA5* genes, they were encoded within the *VSV-G*/*Gag-Pol* plasmid, rather than on the transfer plasmid. It was thus concluded that the likelihood of IAP1/EBNA5 transduction would be similar to the likelihood of RCL formation, which we have also deemed acceptable. Although we did not test for transduction of these genes here, LVV sequencing and host cell DNA testing against these two genes are part of our release specifications following manufacturing, in line with FDA guidance regarding the control of host cell DNA.[32] Thus, prior to the release of LVV batches, controls would be in place to further diminish the risk of transduction of *IAP1*/*EBNA5* genes.

Since several alterations were made to their sequences, the LVV production performance of the in-house LVV plasmids was assessed in comparison to two commonly used commercial systems in a fully transient production setup using suspension HEK293 cells in the absence of antibiotics. The LVV preparations generated using our in-house plasmids had the highest infectious titre of the three sets tested (mean titre = 2.56×10^7 TU/mL, SD = 7.20×10^4 TU/mL, Figure 2A). In addition, the physical/infectious (P/I) ratio of the LVV preparation generated with our plasmids was lower than that of those generated with sets A and B (In-house = 124 VP/TU (SD = 5 VP/TU), Set A = 250 VP/TU (SD = 14 VP/TU), Set B = 174 VP/TU (SD = 26 VP/TU), Figure 2B). Our plasmids were thus used to develop LVV packaging and producer cell lines.

Development of packaging cell line v1.0 – LV001

LVV packaging cell line v1.0 (LV001) was developed by two successive rounds of plasmid integration and clonal cell line isolation. First, linearised Q1850 plasmid (encodes *VSV-G* and *Gag-Pol*; plasmid linearised with PmeI restriction enzyme) was integrated into the genome of HEK293-Ox (HEK293BSusp_MCB1; CD293 media-adapted suspension HEK293 heterogeneous pool acquired from Oxford Clinical Biomanufacturing Facility, University of Oxford). Clonal isolation by FACS, then screening for VSV-G expression by immunostaining after addition of doxycycline, and for *VSV-G* and *Gag-Pol* sequence by PCR, yielded a clonal pre-packaging cell line, hereafter referred to as LVPP001. Next, LVPP001 was adapted to BalanCD HEK293 media, then transfected with linearised Q8890 plasmid (encodes *Rev* gene under the constitutive RSV promoter; plasmid linearised with PmeI restriction enzyme).

Clonal packaging cell lines were isolated by FACS. The selection of the top performers involved 1) cell doubling time and lactate production monitoring and; 2) in E125 flask format, assessment of transfection efficiency (using Q1365 transfer plasmid: encodes SFFV-*EGFP*-WPRE within an LVV genome driven by CMV promoter with a CMV enhancer) and infectious titre by flow cytometry. The top four clonal cell lines had doubling times of 28.7 – 34.1 hours, had (lactate produced) / (glucose consumed) ratios of 0.83 – 0.96, had transfection efficiencies of 43.9 – 63.5 %, and produced 1.14 $\times 10^7$ – 3.67 $\times 10^7$ TU/mL (infectious titre by flow cytometry). These top four cell lines were then tested for stability. Replicate clonal cell lines were passaged both with and without antibiotic supplementation and were tested for LVV production at passage numbers seven and fifteen. Transfection efficiencies varied between 55 – 70 % across all cell lines (Figure 2C). Clone NVC8 had the most consistent transfection efficiency of 68 – 70 %. Production titres varied between 8.35 $\times 10^6$ – 3.19 $\times 10^7$ TU/mL (infectious titre by flow cytometry), with clone NVC2 producing the highest titres most consistently (2.70 $\times 10^7$ – 3.19 $\times 10^7$ TU/mL; infectious titre by flow cytometry; Figure 2C). LVV packaging cell line clone NVC2 was thus cryopreserved for further use and development and was re-named LV001.

Development of producer cell line v1.0 – LVPr001

An initial attempt to develop an LVV producer cell line used random integration of PmeI-linearised Q8887 plasmid (encodes *Rev* and LVV genome with *EGFP* as the GOI) and yielded a clonal cell line that produced 3 $\times 10^6$ TU/mL (infectious titre by flow cytometry; data not shown). This titre was deemed insufficient to be competitive with the industry-standard four-plasmid transfection method. Thus, piggyBac transposon technology was used here with the rationale that it should result in a high

number of integrations per cell,[33] and integrations directed to transcriptionally active loci,[34,35] which thus might result in stable cell lines that produce high LVV titres.

To generate producer cell line pools, LV001 (passage 19) cells were transfected with plasmids R2435 (*EGFP*-encoding LVV genome flanked by transposon inverted terminal repeats; Figure 1 'LVV transfer plasmid') and Q9751 (piggyBac transposase). Next, clonal producer cell lines were isolated by FACS. To select the top two clonal cell lines, LVV production by the top eight clonal cell lines was tested. The highest observed titre in this screen was 2.3×10^8 TU/mL (SD = 3.6×10^7 TU/mL) (infectious titre by flow cytometry; data not shown). The top two clonal cell lines were named LVPr001_3.5 and LVPr001_4.3.

To test the long-term stability of the top two LVPr001 clonal cell lines, the cell lines were subjected to repeated rounds of cryopreservation and revival and subcultured with and without antibiotics, while LVV production was tested (full description in Material and Methods section). Clonal cell line LVPr001_4.3 consistently out-performed LVPr001_3.5, but still lost productivity over the course of the experiment (Figure 2D). Cryopreservation did not have a marked impact on productivity. Subculturing without antibiotics did not have a consistent effect on productivity: in only one instance did the non-antibiotic culture perform worse than the corresponding antibiotic-containing culture (Figure 2D, LVPr001_3.5 P5 after second round of cryopreservation and revival). In summary, over 30 passages a negative trend in LVV titre was observed: with two rounds of cryopreservation and three rounds of revival, clones LVPr001_3.5 and LVPr001_4.3 lost 3.4-4.7-fold productivity. This was not altered by the presence of antibiotics in the cell culture medium during routine subculture between productions.

Development of packaging cell line v2.0 – LVPack13-14

To further improve the characteristics of LV001, a new packaging cell line was developed using the WXATUS0028 cell line (clonal, suspension HEK293 cell line, selected for high growth rate and low aggregation – kindly provided by WuXi Advanced Therapies, Philadelphia, USA). To generate a packaging cell line pool, PmeI-/PacI-linearised Q1850 and PmeI-linearised Q8890 plasmids were simultaneously transfected into WXATUS0028 cells (passage number six). Next, clonal packaging cell lines were isolated by FACS. Fifteen days after sorting, 465 clonal cell lines were transferred to five master 96-well plates. To screen for high-producing clones, clonal cell lines were transfected with Q6974 (*EGFP*-encoding LVV transfer plasmid). LVV supernatants were harvested from transfected cells and used to transduce adherent HEK293T cells. EGFP positivity of the transduced HEK293T

cells was used to select the top 20 clonal packaging cell lines. Sixteen of the 20 chosen clonal cell lines survived the process of expansion to E125 flask format. LVV production in 24-DWP format revealed the top three clonal cell lines, which all produced over 2.15 $\times 10^7$ TU/mL (LVPack13-1, 13-5 and 13-14; data not shown). LVV production in E125 format over several passages revealed the top two clonal cell lines, LVPack13-5 and LVPack13-14, which yielded 2.23 $\times 10^8$ TU/mL and 2.09 $\times 10^8$ TU/mL, respectively (data not shown).

LVPack13-5 and LVPack13-14 were then tested for long-term stability. Both cell lines were passaged with and without antibiotics until passage number 27 (~90 generations), with production tests being performed at passage numbers 5, 11, 19 and 27 (Figure 3). When antibiotics were excluded, this was to assess the stability of the cell lines in conditions that mimic typical manufacturing practice. In the presence of antibiotics, LVPack13-5 produced 1.60 $\times 10^8$ – 2.17 $\times 10^8$ TU/mL and had 65 – 82 % transfection efficiency; in the absence of antibiotics, LVPack13-5 produced 7.61 $\times 10^7$ – 1.74 $\times 10^8$ TU/mL and had 35-80 % transfection efficiency (Figure 3A). Non-induced LVPack13-5 produced 4.71 $\times 10^4$ – 5.36 $\times 10^5$ TU/mL and had 26-77 % transfection efficiency (Figure 3A). In the presence of antibiotics, LVPack13-14 produced 1.07 $\times 10^8$ – 1.95 $\times 10^8$ TU/mL and had 71-79 % transfection efficiency; in the absence of antibiotics, LVPack13-14 produced 1.40 $\times 10^8$ – 1.63 $\times 10^8$ TU/mL and had 82-89 % transfection efficiency (Figure 3A). Non-induced LVPack13-14 did not produce LVV at titres above the lower limit of detection of the titration assay (5.0 $\times 10^4$ TU/mL) throughout stability testing and had 45-89 % transfection efficiency (Figure 3A).

To assess P/I titre ratios, viral vector supernatants from stability testing were additionally titrated by p24 ELISA (enzyme-linked immunosorbent assay). In the presence of antibiotics, LVPack13-5 produced 210.1 LVV particles/TU (SD = 94.2 LVV particles/TU); in the absence of antibiotics, LVPack13-5 produced 273.6 LVV particles/TU (SD = 152.7 LVV particles/TU) (Figure 3B). In the presence of antibiotics, LVVPack13-14 produced 141.4 LVV particles/TU (SD = 63.7 LVV particles/TU); in the absence of antibiotics, LVPack13-14 produced 114.2 LVV particles/TU (SD = 20.5 LVV particles/TU) (Figure 3B).

To further assess stability, at passage numbers 11, 19 and 27, copy numbers of *VSV-G*, *Gag-Pol*, and *Rev* were measured by droplet digital PCR (ddPCR). This analysis indicated no change in copy number of all integrated lentiviral vector constructs throughout the stability testing, regardless of the inclusion of antibiotics in the cell culture media (Figure S1). We did, however, observe a small

discrepancy between the copy numbers of *VSV-G* and *Gag-Pol* genes despite the fact that they were co-encoded by the same plasmid (Q1850, Figure 1). We thus cannot exclude the possibility of the presence of partial copies of the Q1850 plasmid in the LVPack13-14 cell line. In addition, it must be noted that instability in production cell lines can be the result of silencing rather than chromosomal deletion, hence cell line productivity and/or gene expression level should also be considered when assessing cell line stability. Since only minimal differences were observed in LVV production and copy number retention in the presence or absence of antibiotics, it was concluded that antibiotic supplementation during routine cell line subculture did not impact packaging cell line stability. In addition, it was concluded that antibiotic selection can be removed during manufacturing of clinical LVV batches, in line with regulatory guidance.

Although LVPack13-5 produced the highest titre overall, LVPack13-14 was selected as the top clonal cell line as it produced LVV with the least variability during the long-term stability test, produced the least LVV when not induced (indicating tight repression of packaging genes in the absence of doxycycline), and produced LVV with the lowest P/I ratio (Figures 3A and 3B).

Development of *EGFP*-/anti-CD19 CAR-encoding producer cell line v2.0

To generate a producer cell line pool, R2435 (Figure 1 'LVV transfer plasmid') or R3124 (anti-CD19 CAR-encoding LVV genome flanked by piggyBac inverted terminal repeats; Figure 1 'LVV transfer plasmid') and Q9751 (piggyBac transposase) plasmids were simultaneously transfected into LVPack13-14 cells (passage number five). Next, clonal producer cell lines were isolated by single cell printing. Fifteen days after single cell isolation, to screen for high-producing clonal cell lines, LVV production was induced with doxycycline, then viral vector supernatants were harvested. LVV production was ranked by RT-qPCR probing for Psi packaging element. This screen – alongside selection of clonal cell lines based on rapid growth in 96WP – identified the top 12 EGFP-encoding clonal cell lines, and the top 12 anti-CD19 CAR-encoding clonal cell lines, which were then expanded to E125 flask scale. Seven fast-growing *EGFP*-encoding clonal cell lines were expanded and screened for LVV production in 24-DWP format. *EGFP*-encoding LVV production yield varied from 1.82×10^8 TU/mL (SD = 5.39×10^6 TU/mL) to 4.56×10^8 TU/mL (SD = 2.71×10^7 TU/mL) (data not shown). Five anti-CD19 CAR-encoding clonal cell lines survived the expansion process and were screened for LVV production in E125 flask format. Anti-CD19 CAR-encoding LVV production varied from 2.94×10^7 TU/mL (SD = 2.83×10^6 TU/mL) to 9.95×10^7 TU/mL (SD = 1.23×10^7 TU/mL) (data not

shown). All clonal cell lines were next tested for growth characteristics (Figures S2 and S3), then cryopreserved.

To examine their growth profiles, the top seven *EGFP*-encoding LVV producer clonal cell lines were overgrown while cell growth, viability and metabolites were monitored. This study revealed little difference in growth characteristics between the clonal cell lines (Figure S2) with the following exceptions: clonal cell line EGFP_05 slowed in growth after day three when all others continued at the rate observed prior to day three (Figure S2, A); clonal cell lines EGFP_05 and EGFP_07 in culture exhibited a substantial decrease in lactate between days two and three, indicating that the cells had entered stationary phase (Figure S2, D; as reported by Chandra and colleagues);[36] clonal cell line EGFP_04 accumulated a higher concentration of ammonium than the other clonal cell lines (Figure S2, E), which can increase death rate in ammonium-sensitive cell lines.[37] Finally, to screen for residual transposase in the top seven *EGFP*-encoding LVV producer clonal cell lines, genomic DNA (gDNA) was extracted and probed with two primer pairs, targeted to the transposase and G418 resistance genes (PGB_F/R and G418_F/R). Gel electrophoresis of PCR products revealed that all samples were positive for the G418 resistance gene (890-bp band), confirming successful gDNA extraction and PCR. Clones EGFP_03 and EGFP_07 were positive for the transposase gene (1770-bp band) and were thus excluded from further experiments (Figure S4). Based on the outcome of the overgrowth test, the production test in 24DWP, and the screen for transposase gene integration, clonal cell lines EGFP_01 and EGFP_06 were selected for stability testing.

Next, to examine their growth profiles, the top five anti-CD19 CAR-encoding LVV producer clonal cell lines were overgrown while cell growth, viability and metabolites were monitored. This study revealed little difference in growth characteristics between the clonal cell lines (Figure S3) with the following exception: clonal cell line CAR_05 in culture had higher lactate concentration than all other cultures throughout the experiment (Figure S3, D). However, lactate concentration in CAR_05 cultures decreased a day later than all other cell lines. It was thus concluded that this cell line might have the desirable characteristic of remaining in exponential growth for longer than the other clones, which might be beneficial for extended viral vector production. Finally, to screen for residual transposase in the top five anti-CD19 CAR-encoding LVV producer clonal cell lines, gDNA was extracted and probed with two primer pairs, targeted to the transposase and G418 resistance genes (PGB_F/R and G418_F/R). Gel electrophoresis of PCR products revealed that all samples were positive for the G418

resistance gene (890-bp band), confirming successful gDNA extraction and PCR. All anti-CD19 CAR-encoding clones were negative for the transposase gene (Figure S4). Based on the outcome of the overgrowth test and the production test in E125 flask format, clonal cell lines CAR_02 and CAR_05 were selected for stability testing.

Next, all clonal producer cell lines were tested for stability of LVV production and stability of genomically-integrated LVV genes. Clonal producer cell lines EGFP_01 and EGFP_06 were revived, cultured to passage number five in the presence of antibiotics, and tested for LVV production in E125 flask format. Cultures were then split and passaged with and without antibiotics until passage number 27, testing LVV production in E125 flask format at passage numbers 10, 20 and 27. This analysis revealed the following: No correlation between passage number and titre when clone EGFP_01 was subcultured with or without antibiotics (R^2 = 0.0246 and 0.012, respectively; Figure 4A); A strong and negative correlation between passage number and LVV production titre when clone EGFP_06 was cultured with or without antibiotics (R^2 = 0.8858 and 0.9624, respectively; Figure 4A). Although LVV production by clone EGFP_06 declined over time, it remained at approximately 5 ×10^7 TU/mL at passage 27. To further assess stability, at passage numbers 5 and 29, copy numbers of *VSV-G*, *Gag-Pol*, *Rev* and WPRE were measured by ddPCR. This analysis indicated no change in copy number of all integrated lentiviral vector constructs throughout the stability testing, regardless of the inclusion of antibiotics in the cell culture media (Figure S5). We again observed a small discrepancy between the copy numbers of *VSV-G* and *Gag-Pol* genes despite the fact that they were co-encoded by the same plasmid (Q1850, Figure 1). We thus cannot exclude the possibility of the presence of partial copies of the Q1850 plasmid in cell lines derived from the LVPack13-14 cell line. It must again be noted that instability in production cell lines can be the result of silencing rather than chromosomal deletion, hence cell line productivity and/or gene expression level should also be considered when assessing cell line stability. Since only minimal differences were observed in LVV production and copy number retention in the presence or absence of antibiotics, it was concluded that antibiotic supplementation during routine cell line subculture did not impact *EGFP*-encoding producer cell line stability.

Considering the outcomes of LVV production in 24-DWP format (data not shown), the overgrowth study (Figure S2) and the stability testing (Figure 4A), clone EGFP_01 was selected as the top *EGFP*-encoding LVV producer clonal cell line.

Stability testing was next performed with anti-CD19 CAR-encoding clonal producer cell lines CAR_02 and CAR_05. Passaging and testing were performed exactly as with the *EGFP*-encoding producer cell lines EGFP_01 and EGFP_06. This revealed a strong and negative correlation between passage number and titre for both clones, with and without antibiotics (R^2 = 0.8954 for clone CAR_02 with antibiotics; 0.9297 for clone CAR_02 without antibiotics; 0.8716 for clone CAR_05 with antibiotics; and 0.8864 for clone CAR_05 without antibiotics; Figure 4B). Again, to further assess cell line stability, at passage numbers 10 and 29, copy numbers of *VSV-G*, *Gag-Pol*, *Rev* and WPRE were measured by ddPCR. This analysis indicated no change in copy number of all integrated lentiviral vector constructs between passage numbers 10 and 29, regardless of the inclusion of antibiotics in the cell culture media (Figure S6). Since no difference was observed in LVV production or copy number retention in the presence or absence of antibiotics, it was concluded that antibiotic supplementation during routine cell line subculture did not impact anti-CD19 CAR-encoding producer cell line stability.

Since clones EGFP_01 and EGFP_06 performed differently from each other in the production stability test (Figure 4A), it could not be concluded that the negative correlation between anti-CD19 CAR-encoding producer cell line passage number and production titre (Figure 4B) was due to the identity of the GOI. It is conceivable that increasing the number of clones screened through stability testing could increase the likelihood of identifying an anti-CD19 CAR-encoding producer cell line clone with high production stability.

Considering the outcomes of LVV production in E125 flasks, the overgrowth study (Figure S3) and the stability testing (Figure 4B), clone CAR_02 was selected as the top anti-CD19 CAR-encoding LVV producer clonal cell line.

Lentiviral vector production in stirred-tank bioreactor – Producer cell line clones

We next tested LVV production by the v2.0 producer clonal cell lines in one-litre stirred-tank bioreactor. The aim was to assess the production performance of the cell lines when cultured in conditions consistent with what would be expected in a manufacturing setting. Producer clonal cell line EGFP_01 produced 3.25 $\times 10^8$ TU/mL of *EGFP*-encoding LVV post-clarification, and 1.95 $\times 10^{11}$ TU were recovered post-purification by anion exchange chromatography (AEX) and tangential flow filtration (TFF) (Figure 5, A and B). Producer clonal cell line EGFP_06 produced 1.21 $\times 10^8$ TU/mL of *EGFP*-encoding LVV post-clarification, and 7.26 $\times 10^{10}$ TU were recovered post-purification by AEX

and TFF (Figure 5, A and B). Producer clonal cell line CAR_02 produced 1.11 $\times 10^8$ TU/mL of anti-CD19 CAR-encoding LVV post-clarification, and 6.66 $\times 10^{10}$ TU were recovered post- purification by AEX and TFF (Figure 5, A and B). Producer clonal cell line CAR_05 produced 3.50 $\times 10^7$ TU/mL of anti-CD19 CAR-encoding LVV post-clarification, and 2.10 $\times 10^{10}$ TU were recovered post- purification by AEX and TFF (Figure 5, A and B). Production in stirred-tank bioreactor was thus consistent with production in E125 flask format (post-clarification titres; Development of *EGFP*-/anti-CD19 CAR-encoding producer cell line v2.0 section). Downstream recovery was 37.71 – 42.23 % [(post-downstream processing titre / post-clarification titre) × 100 %].

Primary T cell transduction by lentiviral vector from different production platforms

Following the development of the packaging and producer cell line clones, it was deemed important to compare the function of the LVV produced by these platforms and the fully transient system. Specifically, to assess GOI expression in target cells. First, HEK293T cells and primary T cells (Donor 8: 75 % CD4+, 25 % CD8+; Donor 10: 70 % CD4+, 30 % CD8+) were transduced with *EGFP*-encoding and anti-CD19 CAR-encoding LVV preparations from the three different production platforms at MOI = 0.5. All *EGFP*-LVV-transduced cell populations were positive for fluorescence signal when inspected by microscope (Figure 6A). Untransduced cell populations were not fluorescent, confirming that fluorescent signal was due to transduction by *EGFP*-encoding LVV (Figure 6A). Next, GOI expression in primary T cells was quantitatively compared. T cell activation was measured by CD25/CD69 staining, which revealed 99 % CD25-positivity and 78-92 % CD69 positivity (data not shown). EGFP expression was measured directly by flow cytometry analysis of transduced cells. Anti-CD19 CAR expression was measured by flow cytometry analysis of transduced cells stained with biotinylated protein L and streptavidin-phycoerythrin. In all cases, transduced cells were significantly more fluorescent than untransduced cells (untransduced-and-stained cells in the case of the anti-CD19 CAR experiment; all p values < 0.05 following a two-tailed t test), confirming cargo gene expression in primary T cells (Figure S7). To determine gene expression per integrated vector copy (IVC), first ddPCR was performed to determine the IVC number (IVCN) per cell, then median fluorescence intensity (MFI; of the fluorescence-positive cell population) was divided by IVCN. One-way analysis of variance (ANOVA) revealed that – for a T cell population from a given donor transduced by LVV that encodes a given GOI – there was no significant difference in the GOI expression level per IVC regardless of the production platform that produced the LVV: p = 0.77 with

Donor 8 and *EGFP* (Figure 6B); p = 0.16 with Donor 10 and *EGFP* (Figure 6B); p = 0.07 with Donor 8 and anti-CD19 CAR (Figure 6C); p = 0.98 with Donor 10 and anti-CD19 CAR (Figure 6C). Furthermore, linear regression analysis revealed that variation in IVCN accounted for most of the variation in GOI expression level in primary T cells, indicating that the influence of the production system on GOI expression was minimal: R^2 = 0.90 for EGFP (Figure 6D); R^2 = 0.83 for anti-CD19 CAR (Figure 6E). It was thus concluded that the LVV preparations from the different production platforms were consistent with each other in terms of GOI expression level in transduced primary T cells.

Lentiviral vector production with therapeutic cargo genes

We next sought to address two challenges: 1) As EGFP expression is widely known to be well tolerated in HEK293-based cell lines, it would be a more realistic challenge to the cell lines developed here to encode therapeutically relevant GOIs while producing LVV; and 2) During the development of a production process, one would be required to select a production platform modality. This decision might be informed by cost and the performance of the different platforms when producing LVV that encodes the user's GOI. However, testing the encoding of a new GOI in a producer cell line would require development of a producer cell line, which requires commitment of time and resources. We thus tested LVV production by the three production platforms while encoding several different GOIs for two reasons: 1) To examine how effective the platforms are when they encode therapeutically relevant GOIs; and 2) to assess the feasibility of a model whereby a GOI could be tested in the packaging cell line to estimate the expected titre in an equivalent producer cell line.

LVV transfer plasmids encoding several therapeutically relevant cargo genes were assembled (Table 1). The various cargo genes were selected to cover a range of packageable sizes and to be representative of various approaches in cell and gene therapy.

To compare the performance of the production platforms when encoding various GOIs compared to when they encode *EGFP*, LVV productions were performed in 24-DWP format with all cargo gene-encoding LVV transfer plasmid variants as per the Material and Methods section.

WXATUS0028 four-plasmid transient system produced 3.08 $\times 10^7$ TU/mL (SD = 9.49 $\times 10^6$ TU/mL) of *EGFP*-encoding LVV (Figure 7A). Production titre was not significantly impacted when shRNA (p = 0.9306), anti-CD19 CAR (p = 0.3052), anti-BCMA CAR (p = 0.5776), or *PKLR* (p = 0.0513) were encoded (two-tailed T test). However, production titre was significantly lower than the *EGFP* titre

when *Cas9* (p = 0.0234), *STAG2Cas9* (p = 0.0187), *FANCA* (p = 0.0297), or Factor VIII (p = 0.0227) were encoded. The average non-EGFP titre was 59 % of the corresponding EGFP titre (Figure 7A). LVPack13-14 and single transfer plasmid transfection system produced 5.71 $\times 10^7$ TU/mL (SD = 2.16 $\times 10^7$ TU/mL) of *EGFP*-encoding LVV (Figure 7A). Encoding the various GOIs had no significant impact on production titre compared to when *EGFP* was encoded (p values: shRNA = 0.9465; anti-CD19 CAR = 0.6041; anti-BCMA CAR = 0.6378; *PKLR* = 0.6874; *Cas9* = 0.1169; *STAG2Cas9* = 0.0979; *FANCA* = 0.2625; Factor VIII = 0.0900; Figure 7A). The average non-*EGFP* titre was 84 % of the corresponding *EGFP* titre.

LVV production by therapeutically relevant GOI-encoding producer cell lines (heterogeneous populations, also referred to as pools) was performed three times, each with two integration replicates and three production replicates. Thus, producer cell line data is based on six integration replicates and 18 production replicates. Producer pools produced 2.60 $\times 10^8$ TU/mL (SD = 6.01 $\times 10^7$ TU/mL) of *EGFP*-encoding LVV (Figure 7A). A significant decrease in production was observed when any of the non-*EGFP* GOIs were encoded by producer cell line pools (p values: shRNA = 0.0002; anti-CD19 CAR = 0.0005; anti-BCMA CAR = 0.0010; *PKLR* = 0.0028; *Cas9* = 0.0005; *STAG2Cas9* = 0.0002; *FANCA* = 0.0005; Factor VIII = 0.0003; Figure 7A). The average non-*EGFP* titre was 27 % of the corresponding *EGFP* titre.

Overall, producer cell line LVV production was more heavily impacted by the non-*EGFP* GOIs than was production by the WXATUS0028 and LVPack13-14 production systems (Figures 7A). The average WXATUS0028 non-*EGFP* titre was 59 % of the corresponding *EGFP* titre. For LVPack13-14, this number was 84 %. For the producer cell lines, it was 27 %. It is conceivable that in the producer cell lines the constitutively expressed cargo gene products – and thus their impacts on cell health and LVV production – have more time to accumulate than in either of the transfection-dependent cell lines. To test this reasoning, linear regression was performed between infectious titre and cargo gene size. This analysis revealed that: 85.3 % of the variance in WXATUS0028 infectious titre was due to cargo gene size (R^2 = 0.853); 73.3 % of the variance in LVPack13-14 infectious titre was due to cargo gene size (R^2 = 0.733); and only 15.7 % of the variance in the producer cell line infectious titre was due to cargo gene size (R^2 = 0.157). It was thus concluded that most of the variance in the producer cell line titre originated from factors other than cargo gene size, presumably the intracellular function and/or physicochemical properties of the gene products.

To assess the quality of production by the different cell lines, LVV supernatants that encode *EGFP*, shRNA, anti-CD19 CAR and Factor VIII were produced in E125 flask format. This panel of cargo genes was selected to be representative of the range of cargo gene sizes used throughout this study. LVV preparations were then titrated by PCR (titration by PCR analysis of integrated vector copy number in transduced HEK293T cells), and p24 ELISA to enable calculation of P/I ratio. This analysis revealed no consistent trend or difference in the P/I ratio of the preparations from the different cell lines when encoding different cargo genes (Figure 7B).

We next tested if the LVV titre of producer cell line with a novel GOI could be predicted by testing said GOI in a packaging cell line, using the data generated above. First, since *EGFP* and shRNA had disproportionate effects on producer cell line LVV titre compared to all other GOIs tested (Figure 7A), these data points were excluded. Next, linear regression was performed between LVPack13-14 titre and producer cell line titre when encoding the remaining GOIs. The data points plotted were the averages of the LVV titres with each of the GOIs, thus there were seven points. If biological/technical replicate data points had been plotted, they could have arbitrarily been rearranged. By arranging the data according to GOI, each point can only have one X/Y value. This analysis revealed a positive and moderately strong correlation (R = 0.57). In addition, the lower bound of the 95 % confidence interval resulted in a positive gradient, indicating a statistically significant correlation between the titres of the LVPack13-14 and producer cell lines (i.e. the 95 % confidence interval does not contain a zero-gradient line). The linear regression equation is $y = 1.000x + 2.93 \times 10^7$ and the 95 % confidence interval is 0.5324 to 1.468 (Figure 7C). In the selection of a production platform for LVV manufacturing, this predictive model could act as an additional tool to facilitate the selection of either packaging cell line or producer cell line as follows: The user could rapidly test the production of LVV that encodes their GOI using LVPack13-14, then use the model to estimate what titre could be expected if a producer cell line were to be developed, thus enabling an informed decision to be made before commitment of time and resources to a producer cell line development campaign.

LVV safety

Since concerns regarding the formation of RCL within LVV producer cell lines are often raised, LVV supernatants prepared using LVPr001_4.3 and LVProEGFP_01 were tested for the presence of RCL (WuXi Advanced Therapies, Philadelphia, USA). LVPr001_4.3 was cultured to passage number 20, then 310 mL of LVV supernatant (3.07 ×10^7 TU/mL) were prepared according to the experimental

section. LVPr001_4.3-derived LVV supernatant was tested at WuXi Advanced Therapies, PHL, USA, and 270 mL were assayed for the presence of RCL by amplification with C8166 cells, then detection of the VSV-G gene by qPCR. No RCL was detected in the LVV preparation derived from LVPr001_4.3 cells.

LVProEGFP_01 was cultured to passage number 26, then 310 mL of LVV supernatant (1.57 $\times 10^8$ TU/mL) were prepared as above. The LVV preparation (310 mL) was assayed for RCL as above. No RCL was detected in the LVV preparation derived from LVProEGFP_01 cells.

Discussion

We developed lentiviral vector packaging and producer cell lines, which reduce or eliminate the need for plasmid transfection during lentiviral vector manufacture. First, we developed a clonal LVV packaging cell line that, in E125 flask format, consistently produced 1-2 $\times 10^8$ TU/mL, with or without selective antibiotics over 27 passages (~90 generations; Figure 3A; *EGFP* as cargo gene). This platform cell line can either be used directly as a packaging cell line or be developed into a producer cell line by integration of a cargo gene-encoding LVV transfer plasmid. We next developed *EGFP*- and anti-CD19 CAR-encoding clonal LVV producer cell lines that, in E125 flask, produced up to 1-3 $\times 10^8$ TU/mL, with no dependence on selective antibiotics over 27 passages (Figures 4A and 4B, respectively). Finally, we developed a model whereby the LVV production performance of a producer cell line developed with LVPack13-14 and a new cargo gene can be estimated by transiently testing performance using LVPack13-14 as a packaging cell line (Figure 7C). This has the advantage of enabling rapid screening of new cargo genes for optimal LVV production.

Using the titre prediction model, it will now be possible when screening new cargo genes in the packaging cell line to estimate the expected LVV titre from producer cell lines. The model assumes that the new cargo gene does not have a highly toxic impact on the producer cell lines but has at least some negative impact on the production performance of the cell line that encodes it (in contrast to *EGFP*, which was well tolerated by all cell lines here). In addition, the titre prediction model assumes that the packaging cell line titre with the new cargo gene is within the bounds of the model (2.7 $\times 10^7$ – 7.2 $\times 10^7$ TU/mL). Finally, the model assumes that the packaging cell line production is tested in 24-DWP format.

In contrast to the observed trend that stable production cells are less productive than transient production systems,[9] the packaging and producer cell lines presented here were as productive as the

transient systems from which they were derived. Moreover, encoding several different cargo genes had little impact on the productivity of the stable cell lines, a finding that is often absent in similar publications.[14,15,18,20,21,23,30,38,39]

By transduction of primary T cells, we have demonstrated that LVV produced by the different production platforms are consistent in their ability to express cargo genes in target cells. Combined with the observation that the infectious titres of the LVV produced by the different production platforms are consistent with each other, we conclude that transition from the traditional four-plasmid method to a stable cell line for LVV manufacturing would not alter vector efficacy, thus would be an operationally low-risk undertaking.

In summary, the cell lines developed here produce LVV at titres comparable to the industry-standard four-plasmid transfection method. The advantage is that the requirement for transfection of only one or no plasmids means fewer or no large-scale GMP-grade plasmid preparations would be required for LVV production, reducing operational complexity, variability, and costs, and thereby improving patient access to LVV-based cell and gene therapies.

Materials and Methods

Plasmid construction

A guide to the IDs of all plasmids used in this study in presented in Table S1.

The Q1850 *VSV-G*/*Gag-Pol*-encoding plasmid was assembled in four restriction cloning stages as follows: Vector = OG10 (pUC ori, KanR, and MCS); Stage 1 – TetR and *VSV-G* inserted by AsiSI and XbaI restriction sites; Stage 2 – *Gag*, and *Pol* fragment inserted by SbfI and PacI restriction sites; Stage 3 – *Pol* fragment and *PuroR* gene inserted by XbaI and SbfI restriction sites; Stage 4 – *IAP1* and *EBNA5* genes inserted by BspEI/XmaI and PacI restriction sites.

The Q8890 *Rev*-encoding plasmid was assembled in three restriction cloning stages as follows: Vector = OG1 (pUC ori, AmpR, and MCS); Stage 1: *Rev* and *HygroR* genes inserted by SbfI and PacI restriction sites; Stage 2: AmpR replaced by KanR by PmeI restriction sites; Stage 3: *HygroR* replaced by *BlastR* by AvrII and PacI restriction sites.

The R2435 *EGFP*-encoding LVV transfer plasmid was assembled in four restriction cloning stages as follows: Vector = R1845 (p15A ori, KanR, and piggyBac inverted terminal repeats); Stage 1: p15A ori replaced by pUC ori by SwaI restriction sites; Stage 2: *G418R* inserted by BglII and NheI restriction

sites; Stage 3: *EGFP*-encoding LVV genome inserted by SbfI restriction sites; Stage 4: Reverse orientation of LVV genome by SbfI restriction sites.

The R3124 anti-CD19 CAR-encoding LVV transfer plasmid was assembled in four restriction cloning stages as follows: Vector = Stage 3 product in R2435 assembly; Stage 1: Replace SFFV promoter of stage 3 product with TetO-repressible CMV promoter (= R2440); Stage 2: Reverse orientation of LVV genome by SbfI restriction sites (= R2439); Stage 3: Replace *EGFP* CDS with anti-CD19 CAR CDS by EcoRI and BamHI restriction sites (= R2712); Stage 4: Replace TetO-repressible CMV promoter with SFFV promoter by BstBI and EcoRI restriction sites.

The therapeutic GOI LVV transfer plasmids (R3939, R3941, R3943, R3949, R4132, R3947, R3945) were assembled in one Gibson assembly cloning stage as follows: Vector = R2435; All GOI-encoding inserts except Factor VIII, *Cas9*, and *Cas9_STAG2* were synthesised externally; Stage 1: Replace *EGFP* CDS with various GOI CDSs. In the case of R3939, the entire *EGFP* cassette was replaced with an shRNA cassette (U6 promoter).

The Q9751 piggyBac transposase-encoding plasmid was assembled in one Gibson assembly cloning stage as follows: Vector = OG10 (pUC ori, KanR, and MCS); Codon-optimised piggyBac transposase CDS was synthesised externally; Stage 1: Insert piggyBac CDS by NotI and NheI restriction sites.

Cell line revival

Cryovials containing 1 mL of 2 $\times 10^7$ viable cells/mL in cell culture media supplemented with 10 % (v/v) glycerol were rapidly thawed on a bead bath. Cryovials were transferred aseptically to a biological safety cabinet once only a small amount of ice remained within the cryovials. Cryovial contents were transferred to 24 mL of BalanCD HEK293 media (FUJIFILM Irvine Scientific, Santa Ana, California) supplemented with 4 mM glutamine in an E125 flask. Cells were counted then transferred to an incubator (settings as per Subculture) for 96 hours before subculture as per routine). Antibiotics were reapplied once cell viability reached ≥90 %.

Transfection

When random integration was used (development of packaging cell lines), HEK293-Ox or WXATUS0028 cell lines were seeded at 3 $\times 10^6$ viable cells/mL in 50 % of the final intended volume of antibiotic-free cell culture media. After 30 minutes of incubation as per the Subculture section, 75 µL of 500-ng/µL linearised plasmid DNA was added to the cell cultures. After five minutes of incubation as per the Subculture section, linear-PEI 25 kDa (Polysciences, Warrington, Pennsylvania) was

596 added to a final concentration of 3 µL per 1 µg of DNA. After 24 hours of incubation as per the
597 Subculture section, the final 50 % of cell culture media was added. After 24 hours of incubation as per
598 the Subculture section, transfection efficiency was measured by flow cytometry and antibiotic
599 selection was applied as per the Subculture section.
600 When transposase-mediated integration was used (development of producer cell lines), packaging
601 cell lines v1.0/2.0 were seeded at 1.11 $\times 10^6$ viable cells/mL in 90 % of the final intended volume of
602 antibiotic-free cell culture media. LVV transfer plasmids and a transposase-encoding plasmid were
603 combined at a mass:mass ratio of 9:1 (total plasmid mass in µg was equal to the total culture volume
604 in mL). Plasmids were combined with PEIpro (Polyplus-Sartorius, Illkirch-Graffenstaden, France) and
605 incubated according to manufacturer's recommendations before dropwise addition to the cell cultures
606 intended to be transfected. Transfected cell cultures were incubated for 72-96 hours according to the
607 conditions in the Subculture section prior to measurement of transfection efficiency by flow cytometry
608 and application of selective antibiotics according to the Subculture section.
609 **Subculture**
610 Cell viability and density were measured using Vi-Cell XR Cell Viability Analyser or Vi-Cell BLU Cell
611 Viability Analyser. Cell cultures were diluted to 0.3 $\times 10^6$ viable cells/mL in BalanCD HEK293 media
612 preheated to 37 °C and supplemented with 4 mM glutamine, also supplemented with selective
613 antibiotics depending on the cell line as follows. HEK293-Ox was cultured with no antibiotic selection.
614 Packaging cell line v1.0 was cultured with 3 µg/mL puromycin (Gibco, Fisher Scientific UK,
615 Loughborough, UK) and 2 µg/mL blasticidin (Gibco, Fisher Scientific UK, Loughborough, UK).
616 Producer cell line v1.0 was cultured with 3 µg/mL puromycin, 3 µg/mL blasticidin and 350 µg/mL
617 G418 (Roche, Basel, Switzerland). WXATUS0028 was cultured with no antibiotic selection.
618 Packaging cell line v2.0 was cultured with 3 µg/mL puromycin and 4 µg/mL blasticidin. Producer cell
619 line v2.0 was cultured with 3 µg/mL puromycin, 4 µg/mL blasticidin and 600 µg/mL G418. Stability
620 testing of the cell line from which the Producer cell line v2.0 was derived indicated that blasticidin and
621 puromycin were not required for stability. However, Producer cell line v2.0 was developed in-parallel
622 with this stability testing. Hence, it was not known at the time whether the producer cell lines would
623 require these antibiotics during routine subculture. Thus, they were included as a matter of prudence.
624 When HEK293-Ox cells and derivatives were incubated in Erlenmeyer flasks, the incubator settings
625 were as follows: 37 °C, 85 % humidity, 8 % CO_2, 125 rpm with 50-mm orbital diameter. When

WXATUS0028 cells and derivatives were incubated in Erlenmeyer flasks, the incubator settings were as follows: 37 °C, 85 % humidity, 8 % CO_2, 120 rpm with 25-mm orbital diameter. When all cell lines were incubated in 24 deep-well plates, the incubator settings were as follows: 37 °C, 85 % humidity, 8 % CO_2, 225 rpm with 50-mm orbital diameter. When all cell lines were incubated in 96-well plates, the incubator settings were as follows: 37 °C, 85 % humidity, 8 % CO_2, stationary.

LVV production – Fully transient

In an E125 flask or a 24 deep-well plate, WXATUS0028 or HEK293-Ox cells were seeded at 2 ×10^6 viable cells/mL in an entirely fresh volume of BalanCD HEK293 media supplemented with 4 mM GlutaMAX™ (Gibco, Fisher Scientific UK, Loughborough, UK). Cell cultures were then incubated for 24 hours as per the Subculture section. Cells were then counted and transfected using PEIpro transfection reagent with a total mass of DNA according to the production format: E125 flask, 0.3 µg DNA/10^6 viable cells; 24 deep-well plate, 1 µg DNA/10^6 viable cells. The mass ratio of the four LVV plasmids was as follows: *Rev* plasmid (Q6972), 2; *Gag-Pol* plasmid (Q6975), 5; *VSV-G* plasmid (Q6973), 4; transfer plasmid (Q6974), 5 (all plasmids produced by OXGENE, Oxford, UK). The transfection reagent:DNA ratio was 2 µL:1 µg. The total culture volume was 25 mL in E125 flask or 3 mL in 24 deep-well plate. Transfected cells were then incubated as per the Subculture section for 16-24 hours. Sodium butyrate was then added to the cell cultures at a final concentration of 5 mM. Cell cultures were then incubated as per the Subculture section for 48 hours before clarification by centrifugation at 300 RCF for five minutes. LVV supernatants were then titrated and finally stored at -80 °C.

The two commercial systems used for fully transient LVV production in Figure 2 were the ViraPower™ Lentiviral Expression Systems (Life Technologies™) and the MISSION® Lentiviral Packaging Mix (Sigma-Aldrich®). LVV productions were conducted according to manufacturers' protocols. LVV supernatants were then titrated and finally stored at -80 °C.

LVV production – Packaging cell lines

LVV production using packaging cell lines was performed as per the LVV Production – Fully transient section with the following deviations: 1) The cell lines were LVV packaging cell lines; 2) The entire mass of transfected DNA comprised LVV transfer plasmid only; 3) Doxycycline was added to the cell cultures to a final concentration of 1 µg/mL 24 hours after transfection.

LVV production – Producer cell lines

In an E125 flask or a 24 deep-well plate, various producer cell line variants were seeded at 2 ×10^6 viable cells/mL in an entirely fresh volume of BalanCD HEK293 media supplemented with 4 mM GlutaMAX™. The total culture volume was 25 mL in E125 flask or 3 mL in 24 deep-well plate. Cell cultures were then incubated for up to five hours before addition of doxycycline at 1 µg/mL final concentration. When production was performed in E125 flasks, cell cultures were incubated as per the Subculture section. When production was performed in 24 deep-well plates, cell cultures were incubated as follows: 37 °C, 85 % humidity, 8 % CO_2, 225 rpm shaking with 50 mm orbital diameter. Cell cultures were then incubated for 24 hours before addition of sodium butyrate at 5 mM final concentration. Cell cultures were then incubated for 48 hours as per the Subculture section before clarification by centrifugation at 300 RCF for five minutes. LVV supernatants were then titrated and finally stored at -80 °C.

LVV infectious titration by flow cytometry

In a flat-bottomed cell culture-treated 96-well plate, adherent HEK293T cells were seeded at 0.5 ×10^6 viable cells/mL in high glucose Dulbecco's modified eagle medium supplemented with 10 % (v/v) FBS (DMEM+FBS). Within two hours of seeding, HEK293T cells were transduced with LVV supernatants serially diluted in DMEM+FBS. Transduced cell cultures were then incubated as follows for 72 hours: 37 °C, 85 % humidity, 5 % CO_2, stationary. Media was aspirated by pipette. Cells were detached by TrypLE™ (Gibco, Fisher Scientific UK, Loughborough, UK) and re-suspended in phosphate buffered saline. Detached cells were analysed for EGFP expression by flow cytometry (Attune NxT Flow Cytometer). Per-cent EGFP values between 5 % and 20 % were used to calculate LVV titre using the following equation: $Infectious\ titre\ (TU/mL) = \frac{(\%GFP/100) \times number\ of\ HEK293T\ cells\ per\ well}{Neat\ LVV\ input\ volume\ (mL\ per\ well)}$ (Equation 1).

LVV infectious titration by qPCR

Adherent HEK293T cells were transduced, incubated, and detached as per the LVV infectious titration by flow cytometry section. Detached HEK293T cells were pelleted by centrifugation at 300 × g for five minutes, the supernatant was aspirated and discarded, and genomic DNA was extracted from the cell pellets using a DNeasy Blood and Tissue kit (Qiagen, Manchester, UK). Genomic DNA was used as template in a qPCR reaction using TaqMan® Fast Advanced Master Mix (Fisher Scientific UK, Loughborough, UK) and primers/probes against WPRE and albumin. A standard curve was generated by serial dilution of the genomic DNA from a known number of cells known to encode one LVV

genome per cell. LVV titre was calculated using the following equation: $Infectious\ titre\ (TU/mL) = \frac{(provirus\ copies\ per\ cell \times number\ of\ HEK293T\ cells\ per\ well)}{Neat\ LVV\ input\ volume\ (mL\ per\ well)}$ (Equation 2).

LVV physical titration by p24 ELISA

P24 ELISA was performed using a QuickTiter Lentivirus Titer kit (CellBioLabs, San Diego, California) as per the manufacturer's instructions.

LVV physical titration by RT-qPCR

Viral vector RNA was extracted from LVV supernatants using RNA QuickExtract solution (LGC Biosearch Technologies, Hoddesdon, UK) according to the manufacturer's instructions. Extracted RNA was used as template in an RT-qPCR reaction using TaqMan® Fast Virus 1-Step Master Mix (Fisher Scientific UK, Loughborough, UK) and PrimeTime Custom Probe-based qPCR Assay against LVV Psi (IDT, Coralville, Iowa). A standard curve was generated by RT-qPCR against serially diluted RNA generated by in-vitro transcription of a linearised LVV transfer plasmid.

Clonal cell line isolation by FACS

Cells were diluted in antibiotic-free cell culture media to 0.5-5.0 $\times 10^6$ viable cells/mL to a total volume of 1-10 mL. Cell suspension was filtered through 20-40-µm cell strainer to remove cell aggregates. Filtered cells were loaded into a Sony SH800 Cell Sorter. To identify live cells, all detection events were filtered according to BSC-A (back scatter area) against FSC-A (forward scatter area). To identify singlet cells, live cell events were filtered according to FSC-H (forward scatter height) against FSC-A (forward scatter area). To identify singlet cells with further stringency, singlet cell events were then filtered according to BSC-H (back scatter height) against BSC-A (back scatter area). Cells passing the selection criteria (live and twice selected for singlet status) were sorted into sterile, non-treated, flat-bottomed 96-well plates pre-filled with 200 µL per well of 37 °C BalanCD HEK293 media supplemented with 4 mM GlutaMAX™ and 1× InstiGRO™ HEK (Advanced Instruments, Norwood, Massachusetts). Sorted cells were incubated as follows until ≥ 25 % of cell cultures were measured at > 4 % confluence: 37 °C, 85 % humidity, 5 % CO_2, stationary. Cell lines identified as clonal were expanded to E125 flask format.

Clonal cell line isolation by single cell printing

Cells were diluted to approximately 1 $\times 10^6$ viable cells/mL to a total volume of 1-10 mL of cell culture media. Diluted cell suspension was then filtered through a 20-40-µm cell strainer to remove cell aggregates. Using a Cytena F.SIGHT single cell dispenser, singlet cells were dispensed according to

manufacturer's instructions into sterile, non-treated, flat-bottomed 96-well plates pre-filled with 200 µL per well of 37-°C BalanCD HEK293 media supplemented with 4 mM GlutaMAX™ and 1× InstiGRO™ HEK. Dispensed cells were incubated as follows until ≥ 25 % of cell cultures were measured at > 4 % confluence: 37 °C, 85 % humidity, 5 % CO_2, stationary. Cell lines identified as clonal were expanded to E125 flask format.

Preliminary LVV production screening in 96-well plate

When packaging cell lines were screened for LVV production, in 96-well plates, 180 µL of cell culture was transfected with 20 ng of LVV transfer plasmid (*EGFP* as the GOI) complexed with PEIpro transfection reagent to a total volume of 20 µL, as per the manufacturer's instructions. Transfected cell cultures were then incubated for three hours as per the Subculture section. Doxycycline was then added to the transfected cell cultures at a final concentration of 1 µg/mL, which were then incubated for 24 hours as per the Subculture section. Sodium butyrate was then added to a final concentration of 5 mM and cells were incubated as per the Subculture section for 48 hours. LVV supernatants were harvested by centrifugation at 300 RCF for five minutes. Per-cent EGFP expression in HEK293T cells transduced by LVV supernatants was used to rank packaging cell line clones.

When producer cell lines were screened for LVV production, 96-well plates were scanned by Solentim Cell Metric whole well imager to confirm cell confluence was 60-80 %. Cell culture media was aspirated and replaced with cell culture media supplemented with doxycycline at a final concentration of 1 µg/mL. Cells were then mixed by pipetting and incubated as per the Subculture section for 24 hours. Sodium butyrate was then added to a final concentration of 5 mM and cells were incubated as per the Subculture section for 48 hours. LVV supernatants were harvested by centrifugation at 300 RCF for five minutes. LVV supernatants were assayed by psi-directed RT-qPCR. Producer cell line clones were ranked based on RT-qPCR CT values and 96-well plate cell confluence.

Cryopreservation of cell lines

Cell lines were pelleted by centrifugation at 300 RCF for five minutes, then re-suspended to a final density of 1-2 $\times 10^7$ viable cells/mL in antibiotic-free cell culture media supplemented with 10 % dimethyl sulfoxide (Merck Life Science UK Limited, Gillingham, UK). Cell suspensions were transferred to 2-mL cryovials, which were cooled to -80 °C within a CoolCell container. After a minimum of 24 hours, frozen cryovials were transferred to liquid nitrogen-cooled cryostorage.

Overgrowth study

Producer cell line clones were seeded at 2 ×10^6 viable cells/mL in a final volume of 15 mL of BalanCD HEK293 media supplemented with 4 mM GlutaMAX™ per microbioreactor in an Ambr® 15 cell culture bioreactor system. Cell density and viability were measured by Vi-Cell BLU Cell Viability Analyser at 24-hour intervals. Media concentrations of glucose, lactate and ammonium, and pH were measured by FLEX2 automated cell culture analyser at 24-hour intervals. Outgrowth and monitoring continued for four days in total.

Screen for transposase gene integration

Cells from 1-mL aliquots of cell cultures were pelleted by centrifugation at 6000 RCF for two minutes. Supernatants were discarded and cell pellets were frozen at -80 °C for ten minutes, then thawed on a bead bath set to 42 °C (freeze-thaw cycle was to aid cell lysis). To extract genomic DNA (gDNA), 4 µL of cell pellet was mixed with 36 µL of QuickExtract DNA extraction solution (Lucigen, Middleton WI, USA) and thermocycled as follows: 8 × [65 °C for six minutes; 98 °C for two minutes]. To each thermocycled sample, 60 µL of nuclease-free water was added to reduce viscosity. Diluted gDNA samples were stored at -80 °C before further use. Transposase gene was probed by PCR using the following primers: PGB_F (5' GGT TCC TCC CTC GAT GAC G 3') and PGB_R (5' TTG ACA CAT ATC AAT GTT GTG CTC C 3'). G418 resistance gene was probed by PCR using the following primers: G418_F (5' GTA AAT TGT CCG CTA AAT TCT GG C 3') and G418_R (5' TCT GTG AGC TGA AGG TAC GC 3'). PCR reactions were prepared with the following volumes of components: 10 µL of Q5 High-Fidelity 2X Master Mix (New England Biolabs, Ipswich MA, USA), 1.25 µL primer F, 1.25 µL primer R, 2 µL diluted gDNA sample (0 µL in negative controls), 5.5 µL of nuclease-free water (7.5 µL in negative controls). Thermocycle conditions: 98 °C for 30 sec, 35 cycles of 98 °C for 10 sec followed by 66 °C for 30 sec followed by 72 °C for 1 min, then 72 °C for 2 min as a final extension. PCR products were separated by agarose gel electrophoresis (1 % agarose and 1:10000 SYBR Safe DNA gel stain (Thermo Fisher Scientific, Waltham MA, USA) in 1X TAE (Tris-acetate-ethylenediaminetetraacetic acid) buffer; 120 V for 50 min) and visualised on a Bio-Rad Molecular Imaging Gel Doc XR+ Universal Hood II system.

Droplet digital PCR for copy number variation analysis – CNV

Cell pellets were harvested by centrifugation at 300 RCF for five minutes at various passage numbers during stability testing. Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) and diluted to 20 ng/µL in nuclease-free water. Copy numbers of the packaging

genes and the gene of interest were determined by ddPCR using QX200 Auto DG Droplet Digital PCR System (Bio-Rad, Watford, UK) with the following primers and probes: HIV *Gag-Pol* primers: 5' CCT TGG TTC TCT CAT CTG GC 3', 5' ATC AAG CAG CCA TGC AAA TG', and probe 5' FAM-TG CAT CCA GTG CAT GCA GGG CC- IABkFQ 3'; *VSV-G* primers: 5' GGA CCA AAA TAC ATC ACG CAC AGC 3', 5' GCG TGA CTT GCA CGA TCA CG 3', and probe 5' FAM- CAG ACT AAG CAG GGA ACC TGG CTG AAC CC- IABkFQ 3'; *Rev* primers: 5' CTC TGG ATT GCA ACG AAG ACT 3', 5' CCG CTT TCC AAG ATG GTA GG 3', and probe 5' FAM- AAG CCC ACA GAT CCT GGT GGA ATC- IABkFQ 3'; WPRE primers: 5' TTG CTT CCC GTA TGG CTT TC 3', 5' CGG GCC ACA ACT CCT CAT AA 3', and probe 5' FAM- TCT CCT CCT-ZEN-TGT ATA AAT CCT GGT TGC TGT CTC - IABkFQ 3'; Human TaqMan™ Copy Number Reference Assay RNase P TAMRA™ Quencher (3'), VIC™ (5') (Fisher Scientific UK, Loughborough, UK). No systematic change in the copy numbers of measured genes was observed when comparing LVPack13-14 and the producer cell lines derived from it. Thus, the possibility of a change in the copy number of the reference gene (*RNase P*) during the development of the cell lines was ruled out. Thus, for all copy number calculations the *RNase P* copy number was assumed to be two – an approximation since HEK293T cells are often triploid or tetraploid. Gene copy number was calculated by dividing the number of copies of the target gene of interest by the number of *RNase P* copies detected per 22 µL of PCR reaction and multiplying by two.

Lentiviral vector production in stirred-tank bioreactor – Producer cell lines

To prepare seed train cells, ~90 hours prior to bioreactor inoculation, LVV producer v2.0 cells were seeded at 0.35 $\times 10^6$ viable cells/mL in an Erlenmeyer flask then incubated as per the Subculture section (EGFP_01 = passage 9; EGFP_06 = passage 11; CAR_02 = passage 11; CAR_05 = passage 11). Twenty-four hours after cell splitting, 5 % BalanCD HEK293 feed was added to the seed train. Bioreactors were seeded at 3.0 $\times 10^6$ viable cells/mL at a 1-L volume. Two hours after inoculation, doxycycline was added to a final concentration of 1 µg/mL, and 20-24 hours after inoculation, sodium butyrate, anticlumping agent and feed were added to final concentrations of 10 mM, 1:5000, and 5 %, respectively. Agitation was set to 400 rpm, pH was set to 7.1 +/- 0.2, controlled by CO_2 and 0.5-M NaOH, DO (dissolved oxygen) was set at 40 %, and temperature was set at 37 °C. Lentiviral vector was harvested 70 hours after addition of doxycycline.

Downstream processing – Clarification

Clarification was performed following harvest and Benzonase® (Merck Life Science UK Limited, Gillingham, UK) treatment under sterile conditions (5 U/mL; 37 °C; 400 rpm agitation; for two hours). To remove cell debris, LVV supernatants were centrifuged for 20 minutes at 1000 RCF. A set of two filters was used, a pre-filter for removing cell debris (PALL KA2J100P2S; 0.07 m^2 filter size; 10 µm pore size) and a second filter for removing smaller particulates (PALL KA02EKVP2S; 0.022 m^2 filter size; 0.6 µm/ 0.22 µm pore size) (both by Pall Corporation, Portsmouth, UK). Filters were flushed and air flushed with PBS prior to use and flushed with 10% PBS v/v after use.

Downstream processing – Chromatography

Anion exchange chromatography (AEX) was performed following clarification using either the AKTA Pure or the AKTA Avant system. The Sartobind® Q Strong Anion Exchanger was used in three sizes (1 mL, 3 mL and 7 mL; Merck Life Science UK Limited, Gillingham, UK). The procedure with the Sartobind ®Q column comprised equilibration (150 mM NaCl), wash (150 mM NaCl), elution, and strip (200 mM NaCl). Elution uses high salt (1200 mM), which can damage LVV after prolonged exposure, thus material was diluted immediately within the fractionation system of the AKTA system. The Equilibration, Wash and Elution buffers were kept at pH 7.2 with 20 mM Tris for buffering and 1 mM $MgCl_2$. The flow rate was 4 CV/min allowing for rapid processing without introducing issues due to high pressure, with the total processing time being 15 minutes (60 CV) plus the loading time. The 60 CV was split into 10 CV for the equilibration and strip each, and 20 CV for the wash and elution each.

Downstream processing – Tangential flow filtration

Tangential flow filtration (TFF) was performed following chromatography to buffer exchange LVV into storage buffer and to concentrate material that was diluted following AEX. The K2Ri system was used in conjunction with an appropriately sized TFF column and a standard shear rate (6000 s^{-1}) and trans-membrane pressure (0.55 bar) (Repligen, Waltham, Massachusetts). When processing 1 L of bioreactor material, D04-E500-05-N was used, which is a 500 kDa molecular weight cut-off column with a surface area of 40 cm^2. TFF comprised two steps, ultrafiltration which aimed to reduce the volume of material 10-20-fold, and diafiltration which buffer exchanged the virus into the TSS (20 mM tris HCl, 150 mM NaCl, 2 % sucrose w/v, pH 7.3) formulation buffer.

Isolation and cryopreservation of human primary T-cells

NHS research ethics committee approval was received for isolation and use of T cells from human donors (REC reference 21/NW/0202). Leukocyte cones from two donors were received from NHSBT.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient separation and any remaining red blood cells lysed by addition of RBC lysis buffer (Fisher Scientific UK, Loughborough, UK). PBMCs were resuspended in PBS and passed through a 30-µM filter. Viability and cell count measured by BioRad TC20. A portion was cultured for later flow cytometry analysis. Remaining resuspended in MACS buffer (10% FBS (Gibco), 2 µM EDTA (Merck Life Science UK Limited, Gillingham, UK), PBS (Gibco)) and incubated with CD3+ Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. Positive selection of microbead bound cells was carried out by magnetic separation using autoMACS Pro Separator (Miltenyi Biotec). The resulting CD3+ cell population was cultured at 1 $\times 10^6$ cells/mL in T-cell media [RPMI-1640 (Merck Life Science UK Limited, Gillingham, UK) + 10 % heat-inactivated FBS (Gibco) + 2 mM Ultraglutamine (Lonza) + 10 mM HEPES buffer (Gibco) + 0.5 mM sodium pyruvate (Gibco) + 1X MEM Non-Essential Amino Acids (Gibco) + 100 U/mL penicillin + 0.1 mg/mL streptomycin (Merck Life Science UK Limited, Gillingham, UK) + 55 µM 2-mercaptoethanol (Gibco)] supplemented with 50 U/mL IL-2 (Fisher Scientific UK, Loughborough, UK) in a humid static incubator at 37 °C, 5 % CO_2. The following day, PBMCs and CD3+ cells were stained with anti-CD3-PE (clone HIT3a, 1:100; BioLegend, San Diego, California), anti-CD4-FITC (clone RPA-T4, 1:50; BioLegend) and anti-CD8-FITC (clone HIT-8a, 1:100; BioLegend) for analysis on Attune NxT Flow Cytometer. Data analysis was performed using Attune NxT Flow Cytometer software. Cells were cryopreserved at 1 $\times 10^7$ cells/mL in Cryostor CS10 (STEMCELL Technologies, Cambridge, UK) according to manufacturer's instructions.

Revival and lentiviral transduction of human primary T cells

Cryopreserved CD3+ T cells were revived 72 hours before activation. Each 1 mL vial was rapidly thawed, resuspended to 10 mL in warm T-cell media supplemented with IL-2 at 50 U/mL, centrifuged at 400 RCF for 6 min, supernatant discarded and cells resuspended to 1 $\times 10^6$ cells/mL in media with IL-2 (50 U/mL). Growth was monitored and media refreshed as required. T cells were activated 16 hours prior to lentiviral transduction by incubation with Dynabeads Human T-Activator CD3/CD28 beads (Gibco) at 2:1 cell to bead ratio. A non-treated, flat bottom 96-well plate was coated with human Fibronectin (Fragment) (Miltenyi Biotec) by addition of 80 µL/well at 30 µg/mL and overnight incubation at 4 °C. The following day plates were blocked with PBS-2 % BSA (Gibco) and cells seeded at 1 $\times 10^5$ cells/well. For transduction, LVV were diluted in T-cell media supplemented with IL-2 (50 U/mL) for multiplicity of infection (MOI) of 0.5 and a final concentration of 1 $\times 10^6$ cells/mL once

added to wells (completed within two hours of seeding). Transduction was carried out to produce triplicate samples for multiple assays (flow cytometry, IVCN assay and RT-qPCR). Following addition of the LVV, the transduction plate was centrifuged at 1346 RCF for two hours at room temperature and then placed in humid static incubator at 37 °C, 5 % CO_2. At 72 hr post-transduction, cells for IVCN and RT-qPCR assays were frozen as cell pellets at -80 °C.

EGFP expression analysis of T cells

Dynabeads were removed from T cells by application of a magnet. Cells were washed twice in MACS buffer before final resuspension in MACS buffer and analysis on Attune NxT Flow Cytometer.

Protein L staining to detect cell surface CAR expression

Dynabeads were removed from all T-cell samples by application of a magnet. T cells were washed twice with PBS-4 % BSA, resuspended in 200 µL PBS-4 % BSA containing 1.5 µg biotinylated Protein L (Fisher Scientific UK, Loughborough, UK) per well and incubated at 4 °C for 30 min. Following incubation, two further PBS-4 % BSA washes were carried out, cells resuspended in 200 µL PBS-4 % BSA containing 2.5 µg Streptavidin-PE (Miltenyi Biotec) and incubated at 4 °C for 30 min. Following incubation, cells were washed three times in 200 µL PBS-1 % BSA before final resuspension in PBS-1 % BSA and analysed on Attune NxT Flow Cytometer.

CD25/69 T-cell activation marker staining

Dynabeads were removed from T cells by application of a magnet. Cells were washed once in MACS buffer, resuspended in 18 µL MACS buffer containing both anti-CD25-PerCP-Cy5.5 (BioLegend, clone M-A251, 1:40) and anti-CD69-PE (clone FN-50, 1:40; BioLegend) and incubated at 4 °C for 30 min. Following incubation, cells washed twice in MACS buffer before final resuspension in 200 µL MACS buffer and analysis on Attune NxT Flow Cytometer.

Flow cytometry data analysis

Flow cytometry data analysis was performed using Attune NxT Flow Cytometer software. Cell population was gated to exclude debris. Cell singlets were then gated by comparing FSC-H to FSC-A. Gates to identify cells positive for fluorophore were drawn using unstained/fluorophore-negative cell samples. Median fluorescence intensity (MFI) for gated cells was calculated by the software.

Integrated copy number assay by ddPCR from LVV-transduced cells

Genomic DNA was extracted from T cell pellets using DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's protocol. WPRE copy number was determined relative to known *ALB* copy number

(two copies in T cell) by duplex ddPCR assay. Each reaction mixture was prepared to a final volume of 22 µL with 2X ddPCR Supermix for Probes (No dUTP) (Bio-Rad), forward and reverse primers (IDT, 900 nM final concentration), PrimeTime qPCR probes (250 nM final concentration; IDT) and template gDNA (a mass that gave 0.2-1 reference gene copies per droplet, as recommended by Bio-Rad). Primer and probe sequences: WPRE Fwd 5' TTGCTTCCCGTATGGCTTTC 3', WPRE Rev 5' CGGGCCACAACTCCTCATAA 3', WPRE probe 5' FAM- TCTCCTCCT-ZEN-TGTATAAATCCTGGTTGCTGTCTC -3IABkFQ 3', *ALB* Fwd 5' GCTGTCATCTCTTGTGGGCTGT 3', *ALB* Rev 5' ACTCATGGGAGCTGCTGGTTC 3', *ALB* probe 5' 5SUN-CCTGTCATG-ZEN-CCCACACAAATCTCTCC -3IABkFQ 3'. Droplets were generated using AutoDG (Bio-Rad) and PCR carried out in C1000 Touch Thermocycler (Bio-Rad). Droplets analysed in QX200 Droplet Reader (Bio-Rad) and copy number quantified in QX Manager 2.0 (Bio-Rad). Thermocycle conditions: 95 °C for 10 min, 40 cycles of 94 °C for 30 sec following by 60 °C for 1 min, 98 °C for 10 min, 10 °C for 10 min. Ramp rate of 2 °C/sec for all steps.

Data processing

All flask and plate titre values were calculated as averages of biological replicates. Thus, error values indicate the population standard deviation between biological replicates. Stirred-tank bioreactor titre values were calculated as averages of analytical replicates. Thus, error values indicate the population standard deviation between analytical replicates. Linear regression and 95 % confidence interval were calculated using GraphPad Prism software.

Data availability statement

The authors declare that the data supporting the findings of this study are included within the article and its Supplemental Information file. The packaging cells and producer cells presented here are available for evaluation subject to the execution of a material evaluation agreement (MEA) with OXGENE, A WuXi Advanced Therapies Company.

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Author contributions

Conceptualization – MT, MM, MA, RPM, LD, TP, CB, RC, QL; Formal analysis – MT, MM, SPJ, MA, MR, MP, TLC, RB, SD, CB; Funding acquisition – RC, WV, DC; Investigation – MT, MM, SPJ, MA, MR, CF, BG, CFD, IM, HAT, MK, ZZ, EP, RPM, LD, MP, JK, LH, RA, LMG, TLC, RB, IS, MTA, DH, SD, HBG, CB, QL; Methodology – MT, MM, MA, HAT, EP, LM, RPM, LD, SD, HBG, CB, QL; Supervision – CB, RC, WV, DC, MIP, QL; Writing – original draft - MT, MM, MA, SD, MP, RA; Writing – review & editing - MT, CB, MIP, QL.

Declaration of interests

All listed authors are present or past employees of OXGENE, A WuXi Advanced Therapies Company. WV and DC are employees and hold stock or stock options within the company. RPM, LD, TP and RC are named co-inventors in a patent describing the molecular configuration of the packaging elements (US20200277629A1).

Keywords

Lentiviral vector, CAR-T cell therapy, Stable cell lines, Manufacturing, T cell, Cell therapy, Gene therapy, Bioprocess.

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List of figure captions/legends

Figure 1: Scheme of plasmid sequences. Lentiviral vector (LVV) transfer plasmid ID depends on which gene of interest (GOI) is encoded, please refer to Table 1. TetR = tetracycline resistance element repressor; CMV = cytomegalovirus promoter; CMV enh = cytomegalovirus promoter enhancer; CMVd1 = cytomegalovirus promoter d1; *VSV-G* = vesicular stomatitis virus protein G gene; Rabbit αG polyA = rabbit α globin poly-adenylation signal; EF-1α = human elongation factor-1α promoter; *PuroR* = puromycin resistance gene; SV40 polyA = simian vacuolating virus 40 poly-adenylation signal; βG ins = beta globin insulator; βG polyA = beta globin poly-adenylation signal; *Gag-Pol* = HIV-1 Gag-Pol gene; SV40 = simian vacuolating virus 40 promoter; *IAP* = *Bombyx mori* nucleopolyhedrovirus inhibitor of apoptosis 1 gene; *EBNA5* = Epstein-Barr virus-encoded nuclear antigen 5; BgH polyA = bovine growth hormone poly-adenylation signal; pUC ORI = pUC origin of replication; KanR = kanamycin resistance gene; RSV = rous sarcoma virus promoter; *Rev* = HIV-1 *Rev* gene; Ub = ubiquitin promoter; BlastR = blasticidin resistance gene; pMB1 ORI = pMB1 origin of replication; PGB 5' IR = piggyBac 5' inverted terminal repeat; G418R = G418 resistance gene; 3' LTR = lentiviral 3' long terminal repeat; WPRE = woodchuck hepatitis virus posttranscriptional regulatory element; SFFV = spleen focus-forming virus promoter; U6 = U6 promoter; cPPT = central polypurine tract; RRE = Rev response element; ψ= HIV-1 packaging signal; 5' LTR = lentiviral 5' long terminal repeat.

Figure 2: First iteration of stable lentiviral vector production cell line development. Comparison of (**A**) infectious titres and (**B**) physical/infectious titre ratios (LVV particles/TU) of in-house LVV plasmids in a fully transient production format compared to two equivalent commercially available plasmid sets. Physical titration by ELISA. Infectious titration by flow cytometry. N = two biological replicates, error bars indicate standard deviation. (**C**) Lentiviral packaging cell line v1.0 top four clonal cell lines

stability testing. Transfection efficiencies and LVV infectious titres at passage numbers seven and fifteen. Infectious titration by flow cytometry. N = two biological replicates. Error bars indicate standard deviation. (**D**) Long-term stability testing of top two LVV producer v1.0 clonal cell lines. 'Px' = Passage number x. Infectious titration by flow cytometry. N = three production replicates. Error bars indicate standard deviation. No AB = no antibiotics present.

Figure 3: Second iteration of packaging cell line development. (**A**) Long-term stability testing in the presence and absence of antibiotic selection of top two clonal LVV packaging cell lines derived from WXATUS0028, LVPack13-5 and LVPack13-14. Infectious titration by flow cytometry. N = two biological replicates. Error bars indicate standard deviation. (**B**) Average physical to infectious (P/I) titre ratios of top two LVPack clonal cell lines throughout long-term stability testing. N = four biological replicates. Error bars indicate standard deviation.

Figure 4: Stability testing of lentiviral vector clonal producer cell lines. (**A**) *EGFP*-encoding (EGFP_01 and EGFP_06). (**B**) Anti-CD19 CAR-encoding (CAR_02 and CAR_05). Production testing in E125 flask format. Infectious titration by flow cytometry, and qPCR, respectively. N = three biological replicates. Error bars indicate standard deviation.

Figure 5: Lentiviral vector production by *EGFP*/anti-CD19 CAR-encoding producer cell line clones in stirred-tank bioreactor. (**A**) Infectious titre post-clarification. (**B**) Total infectious titre post- purification by anion exchange chromatography and tangential flow filtration. N = one bioreactor production replicate, three titration technical replicates. Error bars indicate standard deviation of titration technical replicates.

Figure 6: T cell transduction. (**A**) Microscope images of primary T cells and HEK293T cells transduced with lentiviral vector supernatants prepared with WXATUS0028 cells, LVPack13-14 cells and EGFP_01 producer cells. Scale bars indicate 750 µm. Gene-of-interest (**B** *EGFP*; **C** Anti-CD19 CAR) expression level (median fluorescence intensity; MFI) per integrated vector copy (IVC) in Donor 8 or Donor 10 primary T cells. (**D**) Linear regression analysis between integrated *EGFP* copy number and EGFP expression level. (**E**) Linear regression analysis between integrated anti-CD19 CAR copy number and anti-CD19 CAR expression level. N = one production replicate. Error bars indicate standard deviation.

Figure 7: Lentiviral vector titre and composition when encoding several therapeutically relevant genes of interest. (**A**) Infectious titre of lentiviral vector encoding a panel of genes of interest, produced by

1101 WXATUS0028, LVPack13-14, or producer cell line pools derived from LVPack13-14. Infectious
1102 titration by qPCR. N = four production replicates, three transfection replicates per production. Error
1103 bars indicate standard deviation between production replicates. (**B**) Physical-to-infectious titre ratio of
1104 lentiviral vector preparations encoding various genes of interest. Ratio calculated by comparison of
1105 qPCR infectious titre and virus-associated p24 ELISA. (**C**) Linear regression analysis of LVPack13-14
1106 LVV titre against producer cell line LVV titre. Solid line indicates linear regression. Dotted lines and
1107 shaded area indicate 95 % confidence interval. Each data point indicates the average LVV titre when
1108 the cell lines encode a given GOI. LVPack13-14, n = four production replicates. Producer cell lines, n
1109 = six production replicates. Error bars indicate standard deviation between production replicates.

1110 **Tables**

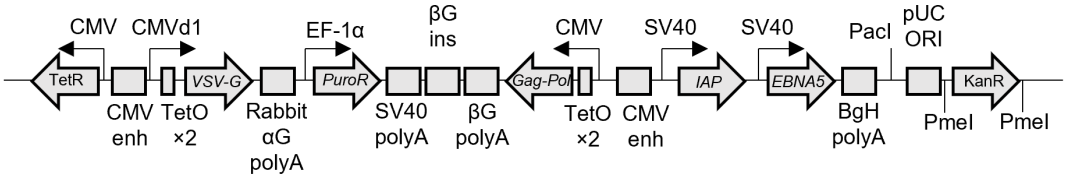
1111 **Table 1:** Summary of lentiviral vector transfer plasmids encoding several cargo genes flanked by
1112 piggyBac inverted terminal repeats.

Plasmid ID	Gene ID	Gene	Gene size (kbp)	Relevant disease
R2435	*EGFP*	Enhanced Green fluorescent protein	0.7	No disease. Control.
R3939	shRNA	Anti-α-synuclein shRNA	0.056	Parkinson's disease
R3124	Anti-CD19 CAR	Anti-CD19 chimeric antigen receptor (CD8 leader, scFV (anti-CD19), CD8 hinge, CD8 transmembrane domain, 4-1BB signalling domain, CD3ζ)	1.45	B-cell lymphoma
R3941	Anti-BCMA CAR	Anti-B-cell maturation antigen chimeric antigen receptor (domains as per anti-CD19 CAR but anti-BCMA scFV domain in place of anti-CD19)	1.5	Multiple myeloma
R3943	*PKLR*	Pyruvate kinase	1.7	Pyruvate kinase deficiency
R3949	*Cas9*	Clustered regularly interspaced short palindromic repeats-associated protein 9	4.1	No disease. This was chosen to demonstrate the compatibility of our systems with this frequently used

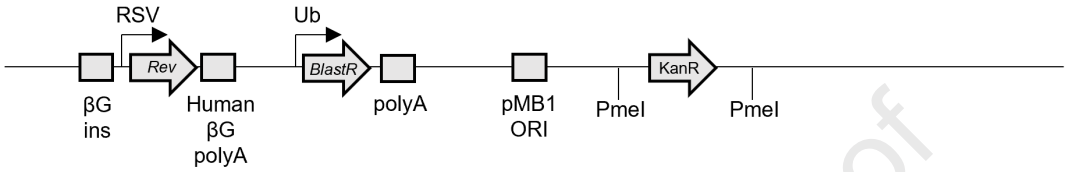
				gene editing tool for research purposes.
R4132	*STAG2Cas9*	Cas9 with gRNA against stromal antigen 2	4.1	No disease. This was chosen to demonstrate the compatibility of our systems with this frequently used gene editing tool for research purposes.
R3947	*FANCA*	Fanconi anaemia, complementation group A	4.36	Fanconi anaemia
R3945	Factor VIII	Coagulation factor VIII	4.37	Haemophilia A

1113

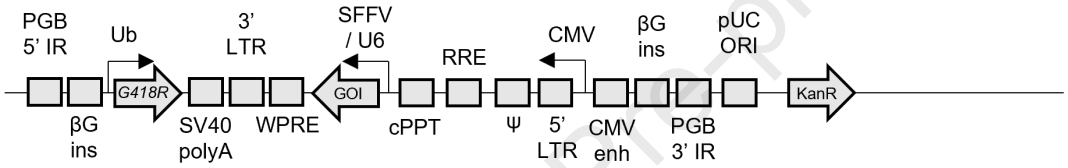
VSV-G / Gag-Pol plasmid (Q1850)

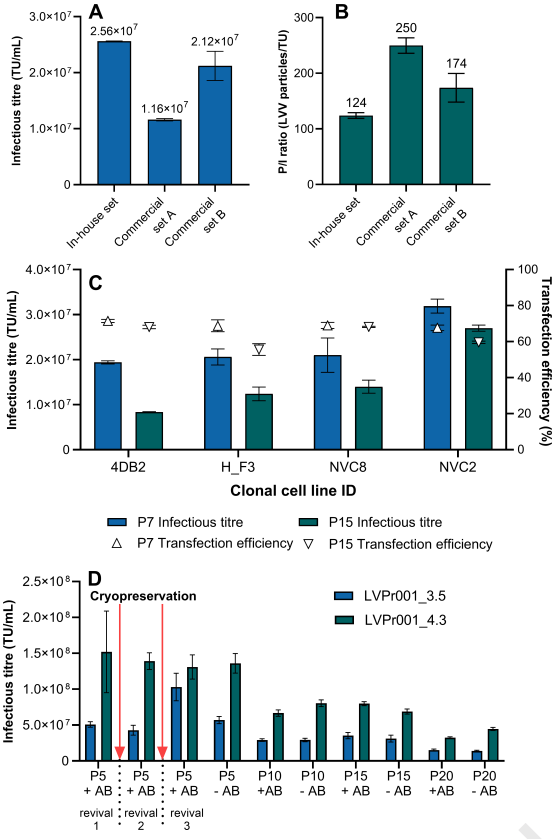


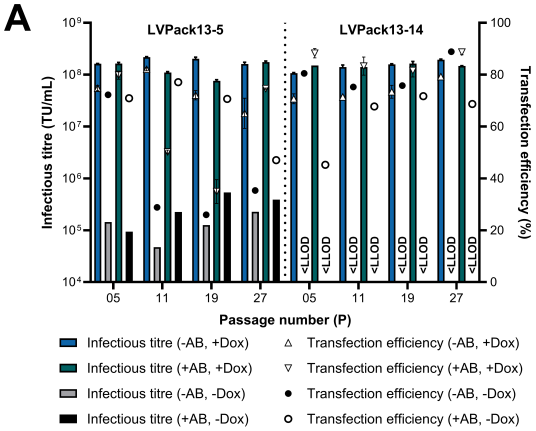
Rev plasmid (Q8890)

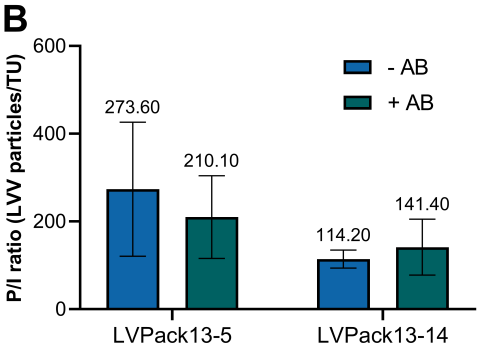


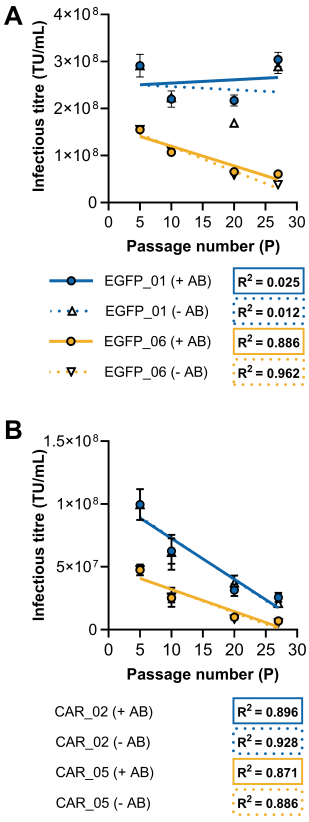
LVV transfer plasmid (see plasmid table)

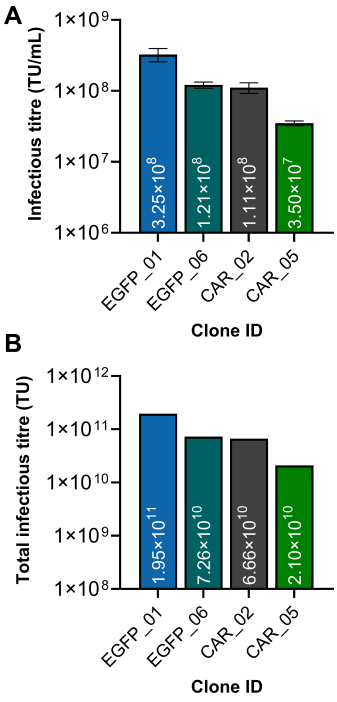


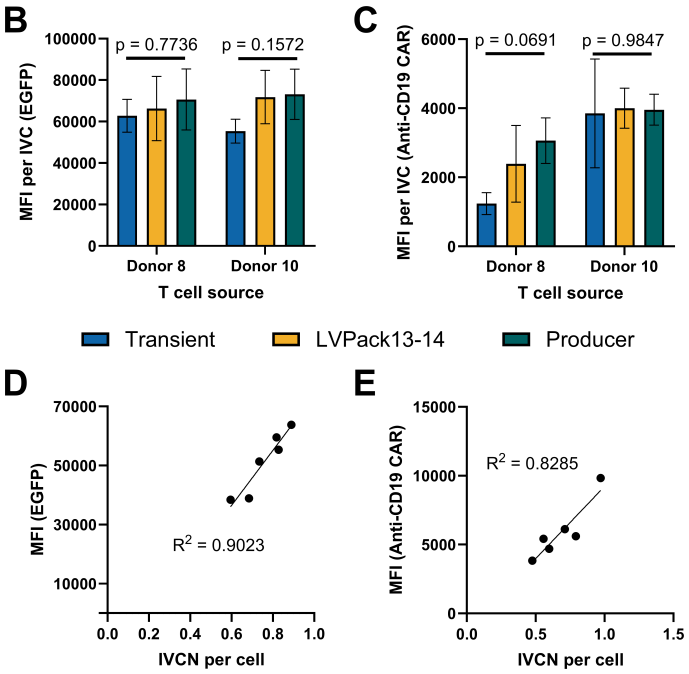


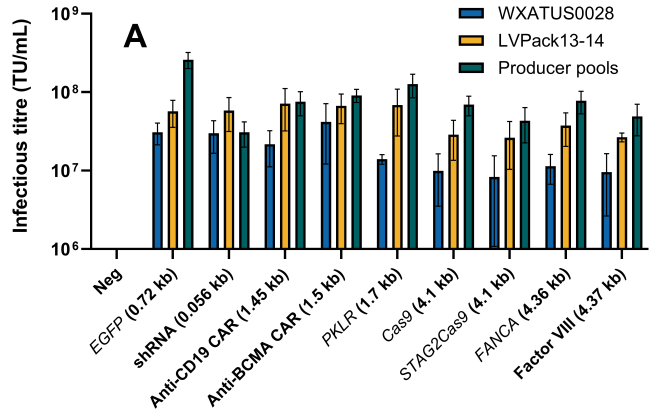


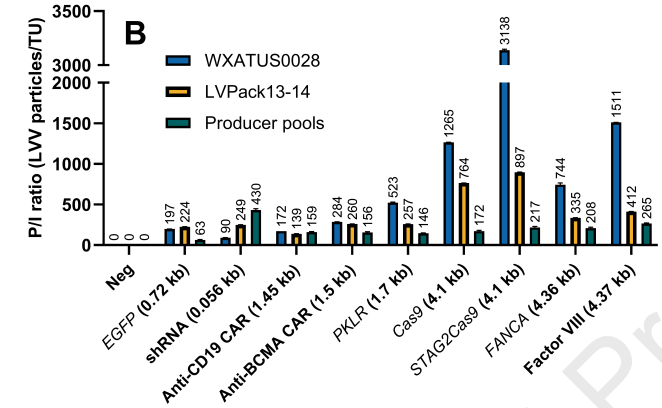


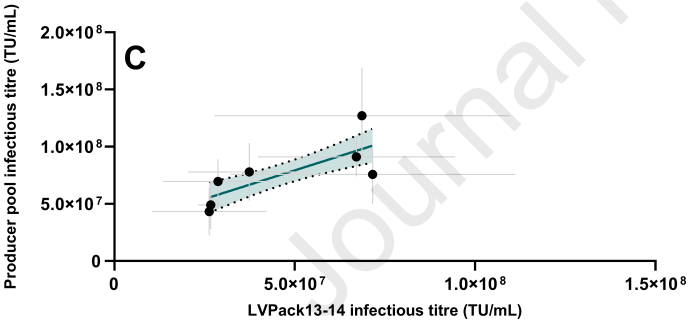












Tridgett and colleagues describe the development of packaging and producer cell lines for lentiviral vector manufacture for cell and gene therapies. These reduce or eliminate the requirement for plasmid transfection, while remaining as productive as the industry-standard procedure. By bringing costs down, patient's access to life-saving therapies can be improved.